Safety Assessment: Outcome of Assessment of the Modification of use of Steviol Glycosides (E 960) from Stevia Leaf Extract Produced by Enzymatic Bioconversion

Reference number RP1084

Food Standards Agency (FSA) and Food Standards Scotland (FSS)

Regulated Product Dossier Assessment

Assessment finalised: 20/06/2023

Summary

An application was submitted to the Food Standards Agency (FSA) and Food Standards Scotland (FSS) in April 2021 from Purecircle/Ingredion ("the Applicant") for changes of the existing production method of steviol glycosides (E 960) to include an enzymatic conversion process to yield high purity steviol glycosides.

The production method uses the enzymatic conversion of highly purified steviol glycosides rebaudioside A (reb A) and/or stevioside extracted from Stevia leaves (*S. rebaudiana*) to rebaudioside M (reb M) and rebaudioside D (reb D) and/or to rebaudioside AM (reb AM; an isomer of reb D), respectively, using UDP-glucosyltransferases (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13) enzymes, derived from genetically modified strains of *Escherichia coli*.

To support the FSA and FSS in evaluating the dossier the Joint Expert Group on Additives, Enzymes and other regulated products (AEJEG) were asked to review the dossier and the supplementary information from the applicant. The AEJEG concluded that the new method for the production of steviol glycosides (E960) using enzymatic bioconversion was safe under the proposed conditions of use. The proposed uses and use levels for rebaudioside AM, D and M, produced via enzymatic bioconversion of purified stevia leaf extract remain the same as the already authorised food additive steviol glycosides (E 960).

The views of the AEJEG have been taken into account in this safety assessment which represents the opinion of the FSA and FSS on the modification of the production for steviol glycosides using enzymatic bioconversion. The Committee on Toxicity (COT) also reviewed the AEJEG safety assessment agreeing with the conclusions of the AEJEG.

1. Introduction

The FSA and FSS have undertaken a partial risk assessment for the production of high purity steviol glycosides using enzymatic bioconversion under the common authorisation procedure for food additives, food enzymes and food flavourings legislation, retained EU Regulation (EU) 1331/2008. To support the risk assessment

by FSA and FSS, the AEJEG provided advice to the FSA and FSS outlined in this assessment.

The Applicant has presented the technical dossier detailing the modification in the specifications of steviol glycosides (E 960) to include a new manufacturing process as an alternative method using a novel enzymatic conversion process to yield high purity steviol glycosides.

The dossier was evaluated on behalf of the FSA and the FSS by the AEJEG. In line with Article 3 of retained EU regulation 1331/2008 (REUL 1331/2008), the assessment has considered the aspects of the food additive and its modification of use. This, and the guidance put in place by EFSA for food additive applications, has formed the basis and structure for the assessment (EFSA, 2012).

With thanks to the members of the AEJEG during the course of the assessment who were: Dr Allain Bueno, Dr Claude Lambré, Dr Martin Rose, Dr Olwenn Martin, Professor Qasim Chaudhry and Dr Claire Stephenson.

Following the review by the AEJEG at their meeting in November 2021, the AEJEG concluded that the new method for the production of steviol glycosides (E960) using enzymatic bioconversion was safe under the proposed conditions of use.

This document outlines the conclusions of the AEJEG assessment on the safety of the new manufacturing process to produce high purity steviol glycosides.

2. Assessment

2.1 Identity and Characterisation of the additive

The Applicant has proposed an enzymatic conversion of highly purified steviol glycosides rebaudioside A (reb A) and/or stevioside extracted from Stevia leaves (*S. rebaudiana*) to rebaudioside M (reb M) and rebaudioside D (reb D) and/or to rebaudioside AM (reb AM; an isomer of reb D), respectively, using UDP-glucosyltransferases (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13) enzymes, derived from genetically modified strains of *Escherichia coli* K12. A Steviol glycosides generic structure is provided in Figure 1 and a summary of individual

steviol glycosides that may be present after enzymatic conversion is provided in Table 1.

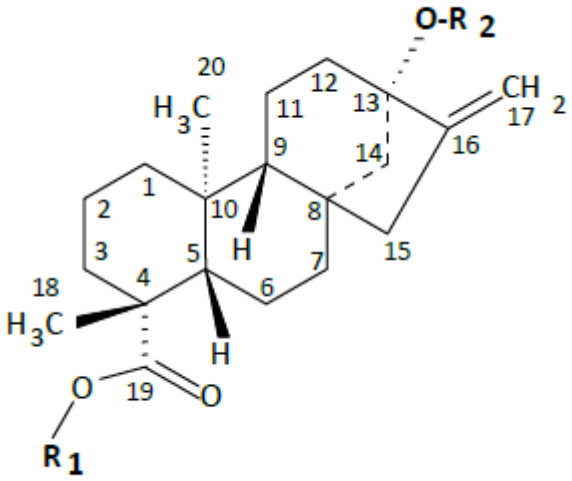


Figure 1. Backbone structure for steviol glycosides

Table 1. Individual steviol glycosides that may be present in steviol glycosides produced by enzymatic conversion

Common Name	Trivial Formula	Mol. Wt.	R_1	R_2
Starting material: Rebaudioside A (>95%) from stevia leaf extract				
Rebaudioside A	SvG4	967	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside D	SvG5	1,129	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside M	SvG6	1,291	Glcβ(1-2)[Glcβ (1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Starting material: Stevioside (>95%) from stevia leaf extract				
Stevioside	SvG3	805	Glcβ1-	Glcβ(1-2)Glcβ1-
Rebaudioside E	SvG4	967	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-
Rebaudioside AM	SvG5	1,129	Glcβ(1-2)[Glcβ (1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-

Glc = glucose; Mol. Wt. = molecular weight.

The Applicant is seeking to amend the existing specifications for E 960 Steviol Glycosides to allow for the inclusion of this enzymatic conversion manufacturing process as an alternate method to produce high purity steviol glycoside preparations.

The Applicant has submitted information on the proposed specifications (Table 2) for steviol glycosides to be produced via the enzymatic process (Figure 2). Information to support this characterisation was provided by the Applicant. The composition of

the additive was verified by high-performance liquid chromatography (HPLC) on 3 batches of the additive. The Applicant also provided the existing specification for steviol glycosides in the United Kingdom (Table 3)

The current application describes blends of selected steviol glycosides consisting primarily of rebaudiosides M and D and/or rebaudiosides AM and E. The Applicant has submitted information on the proposed specifications for steviol glycosides to be produced via the enzymatic process.

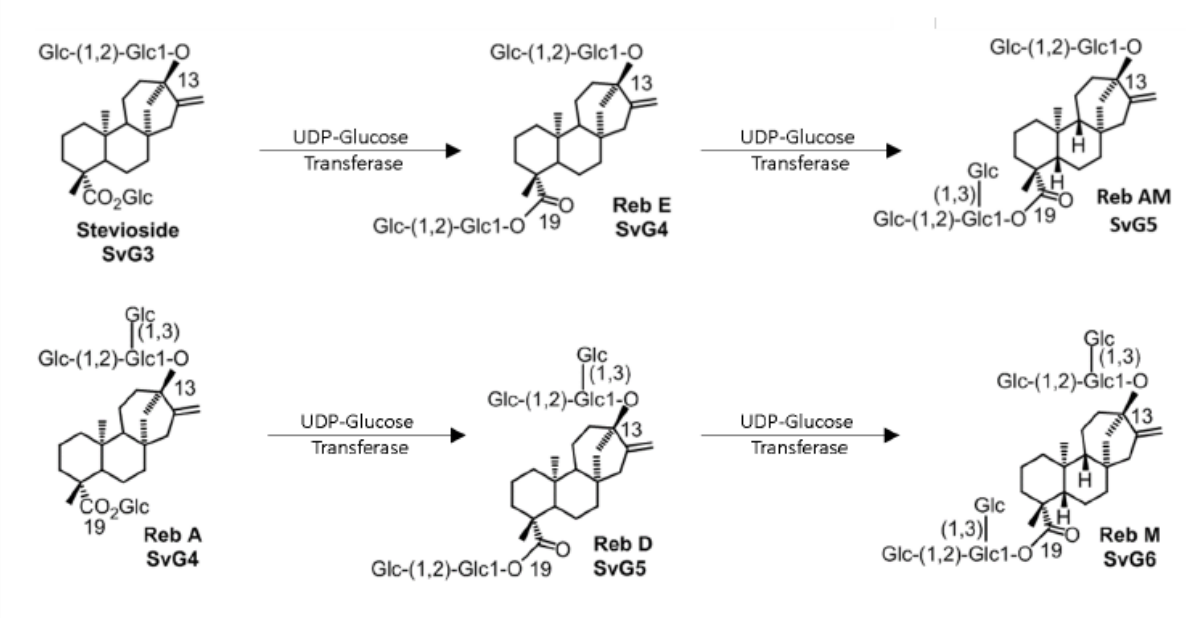


Figure 2. Schematic pathways of enzymatic conversion of highly purified stevioside and Reb A from stevia leaf extract to selected steviol glycosides, as provided by the Applicant.

Table 2. Proposed Specifications for "E 960c(ii) Steviol Glycosides Produced via Enzymatic Bioconversion of Steviol Glycosides from Stevia Plant" in the United Kingdom as Provided by the Applicant.

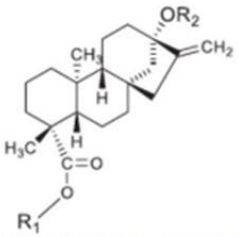
E 960c(ii) STEVIOL GLYCOSIDES PRODUCED VIA ENZYMATIC BIOCONVERSION OF STEVIOL GLYCOSIDES FROM STEVIA PLANT	
Synonyms	
Definition	Steviol glycosides produced via enzymatic bioconversion of steviol glycosides from stevia are composed predominantly of rebaudioside M, rebaudioside D, and/or rebaudioside AM. Steviol glycosides are obtained via enzymatic bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides) using UDP-glucosyltransferase and sucrose synthase enzymes produced by genetically modified strains of *E. coli* (pPM294, pFAH170, and pSK041) that facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds. After removal of the enzymes by solid-liquid separation and heat treatment, the purification involves concentration of the steviol glycosides by resin adsorption, followed by recrystallisation of the steviol glycosides resulting in a final product containing not less than 95 % of total steviol glycosides, including one or more of rebaudiosides D, M and/or AM. Viable cells of *E. coli* (pPM294, pFAH170, and pSK041) or their DNA shall not be detected in the food additive.
Chemical name	 Where R_1 and R_2 are as follows (Glc = glucose): **Trivial name** / **R_1** / **R_2** Rebaudioside M / Glcβ(1-2)[Glcβ(1-3)]Glcβ1- / Glcβ(1-2)[Glcβ(1-3)]Glcβ1- Rebaudioside D / Glcβ(1-2)Glcβ1- / Glcβ(1-2)[Glcβ(1-3)]Glcβ1- Rebaudioside AM / Glcβ(1-2)[Glcβ(1-3)]Glcβ1- / Glcβ(1-2)Glcβ1-
Molecular formula	**Trivial name** / **Formula** / **Conversion factor** Rebaudioside M / $C_{56}H_{90}O_{33}$ / 0,25 Rebaudioside D / $C_{50}H_{80}O_{28}$ / 0,29 Rebaudioside AM / $C_{50}H_{80}O_{28}$ / 0,29
Molecular weight and CAS No	**Trivial name** / **CAS Number** / **Molecular weight** Rebaudioside M / 1220616-44-3 / 1 291,30 Rebaudioside D / 63279-13-0 / 1 129,15 Rebaudioside AM / 2222580-26-7 / 1 129,15
Assay	Not less than 95 % of total steviol glycosides on the dried basis, including one or more of rebaudiosides D, M and/or AM.
Description	White to light yellow powder, approximately between 150 and 350 times sweeter than sucrose (at 5 % sucrose equivalency).
Identification	
Solubility	Freely soluble to slightly soluble in water
pH	Between 4,5 and 7,0 (1 in 100 solution)
Purity	
Total ash	Not more than 1 %
Loss on drying	Not more than 6 % (105 °C, 2h)
Residual solvents	Not more than 5 000 mg/kg ethanol
Arsenic	Not more than 0.015 mg/kg
Lead	Not more than 0.2 mg/kg
Cadmium	Not more than 0.015 mg/kg
Mercury	Not more than 0.07 mg/kg
Residual protein	Not more than 5 mg/kg

Table 3. Existing specifications for “E 960 Steviol Glycosides” in the United Kingdom as provided by the Applicant

E 960 STEVIOL GLYCOSIDES			
Molecular formula	Trivial name	Formula	Conversion factor
	Steviol	$C_{20}H_{30}O_3$	1,00
	Steviolbioside	$C_{32}H_{50}O_{13}$	0,50
	Rubusoside	$C_{32}H_{50}O_{13}$	0,50
	Dulcoside A	$C_{38}H_{60}O_{17}$	0,40
	Stevioside	$C_{38}H_{60}O_{18}$	0,40
	Rebaudioside A	$C_{44}H_{70}O_{23}$	0,33
	Rebaudioside B	$C_{38}H_{60}O_{18}$	0,40
	Rebaudioside C	$C_{44}H_{70}O_{20}$	0,34
	Rebaudioside D	$C_{50}H_{80}O_{28}$	0,29
	Rebaudioside E	$C_{44}H_{70}O_{23}$	0,33
	Rebaudioside F	$C_{43}H_{68}O_{22}$	0,34
	Rebaudioside M	$C_{56}H_{90}O_{33}$	0,25
Molecular weight and CAS No	Trivial name	CAS Number	Molecular weight
	Steviol		318,46
	Steviolbioside	41093-60-1	642,73
	Rubusoside	64849-39-4	642,73
	Dulcoside A	64432-06-0	788,87
	Stevioside	57817-89-7	804,88
	Rebaudioside A	58543-16-1	967,01
	Rebaudioside B	58543-17-2	804,88
	Rebaudioside C	63550-99-2	951,02
	Rebaudioside D	63279-13-0	1 129,15
	Rebaudioside E	63279-14-1	967,01
	Rebaudioside F	438045-89-7	936,99
	Rebaudioside M	1220616-44-3	1 291,30
Assay	Not less than 95 % steviolbioside, rubusoside, dulcoside A, stevioside, rebaudiosides A, B, C, D, E, F, and M, on the dried basis, in any combination and ratio.		
Description	White to light yellow powder, approximately between 200 and 350 times sweeter than sucrose (at 5 % sucrose equivalency).		
Identification			
Solubility	Freely soluble to slightly soluble in water		
pH	Between 4,5 and 7,0 (1 in 100 solution)		
Purity			
Total ash	Not more than 1 %		
Loss on drying	Not more than 6 % (105 °C, 2h)		
Residual solvents	Not more than 200 mg/kg methanol		
	Not more than 5 000 mg/kg ethanol		
Arsenic	Not more than 1 mg/kg		
Lead	Not more than 1 mg/kg		

2.2 Production Process

The manufacturing process includes 2 main phases: the first involving water extraction of the leaves of the *Stevia rebaudiana* Bertoni plant and preliminary purification of the extract by employing ion exchange chromatography to yield a steviol glycoside primary extract, and the second involving recrystallisation of the steviol glycosides from methanol or aqueous ethanol resulting in a final product containing not less than 95 % of the below identified 11 related steviol glycosides, in

any combination and ratio. These include: stevioside; rebaudiosides A, B, C, D, E, F and M; steviolbioside; rubusoside; and dulcoside. Purified stevia leaf extracts may further be converted to minor glycosides that are present in the leaf (e.g., rebaudiosides M, D, E, and AM) via enzymatic conversion using enzymes (e.g., UDP-glucosyltransferases, sucrose synthase) derived from genetically modified strains of *Escherichia coli* K12.

The Applicant states that the steviol glycoside purification steps prior to and following enzymatic conversion are in accordance with those described in the EFSA Panel's Scientific Opinion on the safety of steviol glycosides (EFSA ANS Panel, 2010), in the Chemical and Technical Assessment (CTA) for steviol glycosides from *S. rebaudiana Bertoni* published by FAO/JECFA (FAO, 2016), and in the JECFA specification monograph for Enzyme Modified Steviol Glycosides (JECFA, 2020). The Applicant proposes a manufacturing process comprising 4 steps:

- Extraction of *S. rebaudiana* leaves to yield a stevia extract, which is then purified;
- Inoculation of the *E. coli* production strains (LE1B109) carrying the expression vectors for the corresponding enzymes, followed by fermentation, cell lysis and purification of the UDP-glucosyltransferase (UGT-Sr and UGT-Sl) and sucrose synthase (SuSy-At) enzymes;
- Enzymatic conversion process including the products of steps 1 and 2;
- Flocculation: the removal of the mechanical particles, proteins, polysaccharides, and other impurities.

The AEJEG noted that the production process had recently been evaluated by the European Food Safety Authority (EFSA, 2021).

The AEJEG queried if introducing additional sugar molecules to the glycoside molecule would result in a change in structure and conformation of the molecule. It was noted that the three-sugar structure was already existing within the glycoside form. The AEJEG considered the information provided to be satisfactory.

2.2.1 Enzymes used in the Manufacturing Process

Glucose moieties added to steviol are through the action of UDP-glucosyltransferases that transfer glucose from an activated donor molecule (e.g., UDP-glucose) to the acceptor molecule steviol (Richman *et al*., 2005). Following the synthesis of steviolmonoside, successive glucose moieties are added, leading to the formation for steviolbioside, and then stevioside etc. *In vitro*, the availability of UDP-glucose for this reaction can be re-generated with the enzyme sucrose synthase that catalyses the conversion of UDP and sucrose to fructose and UDP-glucose (Wang *et al.*, 2015).

The enzymes used to convert reb A to reb D and reb M or stevioside to reb E and reb AM include 2 UDP-glucosyltransferases (UGT-Sr, UGT-Sl; EC 2.4.1.17) and 1 sucrose synthase (SuSy-At; EC 2.4.1.13), which are produced by microbial fermentation of the *E. coli* production strains (LE1B109) carrying the expression vector for the corresponding enzyme gene. The enzymes in question are produced by microbial fermentation of the *E. coli* production strains (LE1B109) carrying the expression vector for the corresponding enzyme gene.

2.2.2 Carbohydrate Source

Commonly used carbohydrate sources sucrose and UDP disodium salt (5'-UDP-Na_2) are added to the reaction mixture to form the intermediate glycosides reb D or reb E and the final glycosides reb M or reb AM.

2.2.3 Presence of impurities

The Applicant has provided detailed information to support their claim regarding the absence of impurities in the final product. The Applicant noted that no traces of *E. coli* (MPN/g) were detected in 9 production batches tested, confirming the absence of the production strain in the final product. The Applicant also noted the possible presence of residual ethanol, which is used in the desorption and crystallisation processes but also commented that the maximum residual level of ethanol detected in a final product was 1,340 mg/kg, which is below the acceptable limit for ethanol residues of 5,000 mg/kg consistent with the existing purity criteria for E 960 Steviol

Glycosides. Therefore, the evaporation steps used to remove the ethanol from the final ingredient are sufficient.

The Applicant has tested batches of the product for residual proteins using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The Applicant describes the method as follows: “Test samples were dissolved to a concentration of 1,000 ppm, and about 10 µL from each dissolved sample was stained with 3X protein loading dye and loaded onto a precast polyacrylamide gel (10% Mini-PROTEAN® TGX™ Precast Protein Gels, BIORAD). Electrophoresis was conducted at 60 minutes at 130 V and the gel was stained with 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% water for 1 hour. Gels were destained by soaking for 4 hours in a mixture of 10% acetic acid, 50% methanol, and 40% water. If protein was present in the sample, it would be visually detected on the gel (limit of detection = 0.1 µg protein).”

The Applicant noted: “No visible protein bands have been detected above the limit of detection (limit of detection = 0.1 µg protein) in any batches of final product, including 4 of the same tested batches of steviol glycosides with a high reb M and/or reb D content (Reb M Lot Nos. BM050517, SK-B-U2D1, SK-B-U3D1; Reb D Lot No. PTBRD 150218).”

The Applicant also tested the final products for residual protein using the bicinchoninic acid (BCA) assay based on the Thermo Scientific Pierce BCA Protein Assay Kit protocol and applying some minor deviations. Samples were prepared at 1,000 ppm steviol glycosides (1 mg/mL) and the protein content in the solution was measured against the known protein concentration standard (Bovine Serum Albumin 250 µg to 1 mg) provided by the Assay Kit. The assay protocol required having 1:8 ratio of the sample and working reagents in the final volume (e.g., 125 µL sample: 875 µL working reagent); steviol glycoside concentration in the assay solution was 125 µg/ml. An incubation for 30 minutes at 37°C was applied to develop the colour. The limit of detection for the assay was 5 ppm. To note, this limit of detection applies to the original undiluted samples, not the diluted samples. The test samples and the standard curve Bovine Serum Albumin samples were all diluted 8-fold with the working reagent. The Applicant submitted samples to an external lab for analysis according to the above-described BCA assay protocol. Certificates of analysis for

protein analysis were provided by the Applicant demonstrating that no protein was detected above the limit of detection in 6 non-consecutive lots of steviol glycosides with a high reb M content (Lot Nos. BRM 005-001, BRM 005-002, BRM 005-003, BRM0919049, BRM0120054, BRM1219053), 4 non-consecutive lots of steviol glycosides with a high reb D content (Lot Nos. PTBRD20082018, PTBRD22082018, PTBRD070818, PTBRD080818), and 3 non-consecutive lots of steviol glycosides with a high reb AM content (Lot Nos. AM10122018, AM11122018, AM12122018).

The Applicant noted: “These data confirm the absence of any residual protein in the final product. Furthermore, the starting stevia leaf extract raw material has also been tested for protein using the same assay as the final product: the BCA assay. A certificate of analysis for protein analysis 1 lot of reb A starting stevia leaf extract was provided demonstrating that there is no protein in the starting raw material above the limit of detection of 5 ppm for the assay.”

The steviol glycoside final product was tested by polymerase chain reaction (PCR) method for the absence of any residual recombinant DNA using three primer pairs targeting one of the three genes coding for the three enzymes (two different UDP-glucosyltransferases genes and one sucrose synthase gene). Primers were designed to amplify the genes of interest that were inserted into the *E. coli* production organisms. The limit of the detection for the method was 0.002 ng DNA. Genomic DNA was extracted using a DNA extraction kit according to manufacturer’s protocol. An aliquot of powder (100 mg) was transferred into a tube containing bead and 750 µL of homogenization buffer. The sample was homogenised on a disruptor at speed of 2,840 rpm for 10 minutes. The supernatant was filtered. The filtrate was mixed with lysis buffer and transferred into a spin column. The column was washed twice with buffers. Finally, the purified DNA was eluted in 50 µL of buffer. The genomic DNA was quantified using a spectrophotometer and the extracted genomic DNA was evaluated for the presence of the gene of interest. A 25 µL reaction contained 1 µL of DNA, 12.5 µL of 2x PCR master mix, 0.2 µM forward primer and 0.2 µM reverse primer. The thermal profile used was 2 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 30 seconds at 57°C, and 30 seconds at 72°C. This PCR method was used to test the same 9 lots of steviol glycosides produced by enzymatic conversion of highly purified reb A or stevioside from stevia leaf extract

(Reb M Lot No. BM050517, SK-B-U2D1, SK-B-U3D1; Reb D Lot Nos. PTBRD 150218, PTBRD 070818, PTBRD 080818; Reb AM Lot Nos. AM10122018, AM11122018, AM12122018).

No PCR products were detected in any batches of final product (limit of detection = 0.002 ng DNA).

The Applicant has analysed three separate batches of each SuSy, UGT-Sr, and UGT-Sl enzymes respectively for enzyme activity, protein, total organic solids, water, diluents, and ash. The analytical results of the batches are shown in Tables 4 - 6:

Table 4. Product Specifications for Sucrose Synthase SuSy-At

Specification Parameter	**Specification**	**Manufacturing Lot**		
		PM2-34-001	**PM-39-001**	**PM-40-001**
Activity	≥400 U/mL	413	547	512
Total viable count	<50,000 CFU/g	<100	<100	<100
Salmonella spp.	Absent in 25 g	Conforms	Conforms	Conforms
Escherichia coli	Absent in 25 g	Conforms	Conforms	Conforms
Total coliforms	≤30 CFU/g	<10	<10	<10
Antimicrobial activity	Negative	Negative	Negative	Negative
Lead[a]	≤5 mg/kg	0.11	0.14	0.11
TOS (%)	NS	9.48	10.49	9.62

CFU = colony forming units; NS = not specified; TOS = total organic solids; U = units (1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0).

[a] Limit of detection for lead (Pb) = 0.02 mg/kg

Table 5. Product Specifications for UDP-Glucosyltransferase UGT-Sr

Specification Parameter	**Specification**	**Manufacturing Lot**		
		FAH-a-U3D1	**FAH-a-U4D1**	**FAH3-002**
Activity	≥1 U/mL	1.22	1.66	2.00
Total viable count	<50,000 CFU/g	<100	<100	<100
Salmonella spp.	Absent in 25 g	Conforms	Conforms	Conforms
Escherichia coli	Absent in 25 g	Conforms	Conforms	Conforms
Total coliforms	≤30 CFU/g	<10	<10	<10
Antimicrobial activity	Negative	Negative	Negative	Negative
Lead[a]	≤5 mg/kg	0.08	0.07	0.08
TOS (%)	NS	10.53	13.61	14.17

CFU = colony forming units; NS = not specified; TOS = total organic solids; U = units (1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0).

[a] Limit of detection for lead (Pb) = 0.02 mg/kg

Table 6. Product Specifications for UDP-Glucosyltransferase UGT-SI

Specification Parameter	**Specification**	**Manufacturing Lot**		
		SK4-14-001	**SK4-18-001**	**SK4-19-001**
Activity	≥7 U/mL	9.6	12.0	9.2
Total viable count	<50,000 CFU/g	<100	<100	<100
Salmonella spp.	Absent in 25 g	Conforms	Conforms	Conforms
Escherichia coli	Absent in 25 g	Conforms	Conforms	Conforms
Total coliforms	≤30 CFU/g	<10	<10	<10
Antimicrobial activity	Negative	Negative	Negative	Negative
Lead[a]	≤5 mg/kg	0.12	0.06	0.09
TOS (%)	NS	10.47	13.47	11.41

CFU = colony forming units; NS = not specified; TOS = total organic solids; U = units (1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0).

[a] Limit of detection for lead (Pb) = 0.02 mg/kg

Overall, the AEJEG was satisfied with the information provided and considered that there would be no viable cells of the production organism, residual protein nor any recombinant DNA present in the final product. The rest of the impurities were within their respective regulatory limits.

2.3 Specification

The Applicant has submitted information on the proposed specifications for steviol glycosides to be produced via the enzymatic process. A schematic of the proposed enzymatic reaction as a stepwise conversion of the starting steviol glycosides extracts, is detailed in Figure 2 above.

The proposed modification of the EU specifications for steviol glycosides is presented below, with changes to the E 960 Steviol Glycosides specification captured in **bold text**.

Rebaudioside M:

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

Chemical formula: $C_{56}H_{90}O_{33}$

Molecular weight: 1291.30

CAS number: 1220616-44-3

Rebaudioside D:

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-β-D-glucopyranosyl ester

Chemical formula: $C_{50}H_{80}O_{28}$

Molecular weight: 1129.15

CAS number: 63279-13-0

Rebaudioside AM:

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-β-D-glucopyranosyl ester

Chemical formula: $C_{50}H_{80}O_{28}$

Molecular weight: 1129.15

CAS number: **2222580-26-7**

The Applicant has stated that steviol glycosides produced by enzymatic conversion may contain remnants of the starting material reb A (comprising 4 glucose units conjugated to the steviol backbone), the intermediate glycoside reb D (comprising 5 glucose units), and the final glycoside reb M (comprising 6 glucose units).

2.4 History of Use

The history of use data supplied does not indicate any further areas for evaluation.

2.5 Proposed Use and Anticipated Intake

The current uses and levels of steviol glycosides manufactured by the traditional method were provided. The current application is to include a new specification for the production method of steviol glycosides to include the new manufacturing method, therefore the Applicant is proposing steviol glycosides produced by enzymatic conversion is used as a high-intensity sweetener in food and beverages under the same conditions as those already approved for steviol glycosides in the UK.

As the proposed uses and use levels for rebaudioside AM produced via enzymatic bioconversion of purified stevia leaf extract remain the same as the already authorised food additive steviol glycosides (E 960), the Applicant did not provide an exposure estimate. The Applicant has however, referred to EFSAs recent exposure estimates for steviol glycosides (EFSA ANS Panel, 2015b).

Following consideration of the provided information, the AEJEG concluded that there would be no significant change in exposure to steviosides as a result of this Application, due to the fact that the steviosides produced using the process in the current Application will be subject to the existing conditions of use for steviol glycosides.

The information provided is sufficient and does not raise any further areas for evaluation.

2.6 Biological and Toxicological Data

2.6.1 Absorption, Distribution, Metabolism and Excretion (ADME)

As part of their dossier, the Applicant has submitted literature studies relevant to the safety of steviol glycosides as well as data from their own *in vitro* metabolic studies. The Applicant noted that the safety of steviol glycosides have previously been evaluated by several advisory scientific bodies including the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS Panel) (EFSA ANS Panel, 2010, 2015a) and it has been concluded that steviol glycosides, as a group of substances, share a similar structure (i.e., glycosylated derivatives of the aglycone steviol with various glucose, xylose, or rhamnose units attached at the R1 and R2 positions), and that these substances undergo a common metabolic pathway following ingestion (i.e., hydrolysation of the glycoside units from the steviol backbone to generate steviol). As such, safety studies conducted with an individual steviol glycoside can extend to other steviol glycosides due to the shared metabolic fate. A detailed overview of the general metabolic fate of steviol glycosides, as provided by the EFSA ANS Panel (2015a), is also summarised and discussed below.

To demonstrate that steviol glycosides produced by enzymatic conversion of reb A or stevioside from stevia leaf undergo a similar metabolic fate as steviol glycosides extracted from *S. rebaudiana* Bertoni, the Applicant has conducted *in vitro* studies, investigating the anaerobic metabolism of rebaudioside D and rebaudioside M derived from bioconversion and steviol glycosides minor components from leaf extract in pooled human faecal homogenates from healthy male and female adult subjects.

The Applicant stated that “Overall, these data demonstrate clearly that reb D, reb M, and reb AM produced by enzymatic conversion in the presence of human faecal homogenates are metabolised completely to steviol within 12 hours and confirm that steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract undergo an almost identical rate and degree of hydrolysis as steviol glycosides from *S. rebaudiana* Bertoni. The similarity in the degree and rate of hydrolysis of the steviol glycoside materials produced via enzymatic conversion further demonstrates there will be a similar rate and level of

absorption of steviol, leading to the conclusion that the overall pharmacokinetics of the products will be identical leading to a lack of concern regarding differences in exposure and elimination. Furthermore, the overall similarity in the metabolic profile of steviol glycosides produced by enzymatic conversion and steviol glycosides extracted from *S. rebaudiana* Bertoni indicates the acceptability to be able to bridge to the steviol glycoside safety data base thereby underpinning the generalisability of the safety of steviol glycosides produced by enzymatic conversion."

The AEJEG discussed the extrapolation of the fate of steviol glycosides produced by bioconversion in the gut based on that of steviol glycosides produced in the "traditional" manner. It was concluded that this would be possible because steviol glycosides are not degraded by the gut, but by microorganisms and therefore there would be no enzymes present in faecal matter. The AEJEG also discussed that, as the hydrolysis process relied on the activity of microbial enzymes in the gut, it may differ in those with gut dysbiosis and could potentially result in differences in the pharmacokinetics and safety of steviol glycosides produced by enzymatic conversion. However, after discussion, the Group agreed that in those with gut dysbiosis, the steviol glycosides would be excreted in their original state rather than being absorbed and were not likely to pose any particular safety concern.

The AEJEG was therefore satisfied that glycosides produced by enzymatic conversion of reb A or stevioside from stevia leaf undergo a similar metabolic fate as steviol glycosides extracted from *S. rebaudiana* Bertoni.

2.6.2 Toxicological information

No toxicity studies on rebaudioside AM from stevia extract or produced via enzymatic bioconversion of purified stevia leaf extract has been submitted by the Applicant, however, the Applicant submitted a comprehensive summary of studies in support of the application. As discussed previously, the AEJEG considered that these studies alongside the information provided regarding the evaluation of steviol glycosides by other regulatory bodies could be used to assess the safety of steviol glycosides produced by the proposed manufacturing method.

2.6.2.1 Genotoxicity

The Applicant has presented studies relevant to the genotoxicity of steviol and steviol glycosides (Rumelhard *et al*., 2016; Sharif *et al*., 2017; Uçar *et al*., 2018; Zhang *et al*., 2017). The Applicant has not submitted any of their own genotoxicity study data, noting that the *in vitro* and *in vivo* genotoxicity of both steviol glycosides and steviol have previously been addressed in EFSA's 2010 Opinion (EFSA ANS Panel, 2010). In their 2010 Opinion, the Panel concluded that they were not genotoxic based on a review of the experiments conducted with stevioside and rebaudioside A.

2.6.2.2 Chronic Toxicity and Carcinogenicity

The Applicant noted that both chronic and carcinogenicity of steviol glycosides were previously evaluated by the Scientific Committee on Food (SCF) in 1999 and EFSA in 2010 and consequently presented a brief summary of the studies considered (EFSA ANS Panel 2010; SCF, 1999). They noted that the results of a chronic toxicity study in F344 rats, the combined chronic toxicity and carcinogenicity study in Wistar rats, and a carcinogenicity study in F344 rats indicated a lack of toxic effect and a lack of toxicity, although the 3 studies presented within EFSA's 2010 opinion were not without limitations (EFSA ANS Panel, 2010).

The Applicant further discussed that the EFSA Panel noted that the tumour occurrence in all 3 studies was typical for the species and strains of rats and were deemed to be negative with respect to carcinogenic potential. The NOAEL established in the 2-year carcinogenicity study conducted in F344 rats was 388mg steviol equivalents/kg body weight/day (Toyoda *et al*., 1997; EFSA ANS Panel, 2010). This NOAEL formed the basis of the ADI. The Applicant did not identify any new studies.

2.6.2.3 Reproductive and Developmental Toxicity

The Applicant identified two new studies as summarised below:

The potential effect of reb A (R107558; obtained from Aladdin Co., Ltd., China) on ovary function in rats was investigated by Jiang *et al*. (2018). Reb A was provided in drinking water to weanling Sprague-Dawley rats (n=6 females/group) for a total of 48 days at dose levels of 0, 0.5, or 2.5 mM (reported to be equivalent to 0, 210, or 1,430 mg/kg body weight/day). Food and water were provided *ad libitum*. In the high dose group, body weight was significantly decreased from Day 18 to Day 30; however, at the end of the study, no significant difference in body weight between the high dose reb A and control groups was observed. Water consumption was significantly increased in the high-dose group during the entire study period, while the low-dose group was reported to be notably different from the control group after 21 days of treatment. Furthermore, the high-dose group water consumption was reported to be higher than the low-dose group in the last 3 weeks of treatment.

Conversely, reb A was not reported to influence food intake in either group. No significant differences between the control and test-article treated groups were reported for puberty onset or body weights at puberty, nor were differences in oestrous cycles observed. No morphological changes in the ovaries of the reb A treated groups were reported. Serum levels of progesterone in the reb A groups were significantly decreased compared to the control group and decreased expression of 3β- hydroxysteroid dehydrogenase, an enzyme involved in progesterone synthesis, was measured in the ovaries via western blotting. Several other steroidogenesis-related factors were also reported to decrease based on the western blot results; however, the significance of these findings with respect to safety are limited as no effects on ovarian morphology or oestrous cyclicity were reported following reb A exposure for 48 days.

Gharheri *et al*. (2018) investigated the effects of *Stevia rebaudiana* extract (purity not reported) on reproduction function in diabetes-induced healthy adult male albino rats of Wistar strain. Diabetes mellitus was induced in rats via intraperitoneal injection of 50 mg streptozotocin/kg. The rats that reached fasting glucose levels greater than 250 mg/dL after 72 hours were selected for the study. Animals (7/group) were administered stevia extract at doses of 5, 50, or 100 mg/kg by gavage for 28 days. A diabetic and non-diabetic control group received 2 mL distilled water only. Sexual behaviours of the rats were recorded for 30 minutes every 2 weeks for 1 month,

including mount latency, intromission latency, mount frequency, intromission frequency, ejaculation latency, the mount latency post ejaculation, and ejaculation frequency. Following the study period, animals were killed, and serum concentration of testosterone was measured. Histological examination was carried out on the right testis and epididymis.

In diabetic rats, a significant increase in the frequency of intromission was observed in the low-dose group, compared to diabetic control rats. In addition, diabetic rats of the low-dose group showed a significant increase in the frequency of ejaculation, compared to the diabetic control and high-dose animals. However, a significant decrease in the latency of ejaculation was observed in the low-dose group when compared to the high-dose animals, although, the effect was not significant between the treated animals and the controls. Significant differences in other sexual behaviour parameters measured were not observed in the animals. Furthermore, a significant reduction in the number of Leydig cells in high-dose animals was noted, compared to the non-diabetic control group; however, this effect was not significantly different compared to the diabetic control rats. Organ weights and serum testosterone levels showed no significant differences among the study animals. Based on the results of the study, the authors concluded that there is no risk to reproductive parameters and consumption of stevia. The authors reported that intake of stevia may be effective in the promotion of blood glucose reduction and preventing destruction of Leydig cells.

2.6.2.4 Human Studies

The Applicant identified 3 new studies since the publication of the EFSA opinion (EFSA, 2010) in addition to the single-dose pharmacokinetics trial cited (Roberts *et al*., 2016). These included a study investigating the hypoglycaemic and hypolipidaemic effects of stevia leaf powder were studied in human volunteers with type 2 diabetes mellitus (Ritu and Nandini, 2016), a study evaluating the acute effect of stevia extract on postprandial glucose metabolism in adult subjects with metabolic syndrome (Kassi *et al*., 2016) and a study investigating the potential effects of a natural stevia preparation (Al-Dujali *et al*., 2017).

Other studies were cited, which investigated the potential effects of stevia leaf powder (prepared from dried stevia leaves; steviol glycoside content not reported) on postprandial glycemia, appetite, palatability, gastrointestinal discomfort, and anthropometric parameters (Ahmad *et al*., 2018a) and the beneficial effect of *S. rebaudiana* in male and female patients (Rizwan *et al*., 2018), however these were not considered in the area of safety of the product.

The AEJEG considered that the information presented from the literature could be sufficient to evaluate the safety of steviol glycosides produced by enzymatic bioconversion.

2.7 Allergenicity

The Applicant has employed a sequence homology search to check the potential for cross-reactivity among the inserted heterologous gene sequences in the production strain, in accordance with the FAO/WHO (FAO/WHO, 2001) and the Codex Alimentarius (2009) using the AllergenOnline Database version 18B (available at http://www.allergenonline.org; updated 23 March 2018) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2018). The Applicant aimed to determine whether the UGT-Sr, UGT-Sl, and SuSy-At enzymes contain amino acid sequences similar to other known allergens that might produce an allergenic response.

The Applicant has also carried out a FASTA search, using default settings (E-value/score cut-off = 1 and maximum alignments of 20), yielding several matches to known allergen sequences for all 3 enzyme sequences. The Applicant noted “E values/scores for these matches were between 0.13 and 0.76, indicating that these alignments are not significant as E-values/scores larger than 1×10^{-7} are unlikely to identify proteins that may share immunologic or allergic cross-reactivity to known allergens (Hileman *et al*., 2002). Additionally, none of these sequences shared greater than 50% identity with the identified allergens, indicating the unlikely potential for cross-reactivity to these allergens (Aalberse, 2000).

Additionally, in accordance with the Codex Alimentarius criterion for use in flagging proteins that might be of some concern of cross-reactivity for genetically engineered plants, an 80-amino acid sliding window (segments 1–80, 2–81, 3–82, etc.) was used to scan the amino acid sequence of each heterologous gene inserted in the production strain against the allergen database using FASTA to search for matches of >35% identity (Codex Alimentarius, 2003, 2009). No matches greater than 35% were identified among the 80-mer sliding windows in comparison to known allergens."

The Applicant concludes that there are no safety concerns regarding allergenicity associated with exposure to steviol glycosides produced by enzymatic bioconversion.

3. Discussion

The Applicant concluded:" The determination of the safety of steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract was based on a detailed *in vitro* metabolism study in comparison to the positive control reb A, a component of the current UK specification for steviol glycosides. The study involved the use of human faecal homogenate samples from 6 healthy male and 6 healthy female volunteers. A steviol glycoside equal mixture of reb M and reb D both produced by enzymatic conversion of reb A from stevia leaf extract, a mixture of minor glycosides containing reb AM produced by enzymatic conversion of stevioside from stevia leaf extract, and the positive control reb A, were incubated in pooled faecal homogenate samples at concentrations of 0.20 and/or 0.40 mg/mL under anaerobic conditions at 37°C for between 4 to 72 hours in triplicate.

The data generated demonstrates clearly that reb M, reb D, and reb AM produced by enzymatic conversion are metabolised to steviol at a similar rate as reb A extracted from *S. rebaudiana* Bertoni by the human faecal homogenates under anaerobic conditions. *In vivo*, the generated steviol is subsequently absorbed and conjugated with glucuronic acid to form steviol glucuronide, which is excreted primarily via the urine in humans. The fact that the rates of hydrolysis are remarkably similar indicates

that there will be no difference in the overall pharmacokinetics of the aglycone steviol.

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, rebaudioside A, and rebaudioside 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.

Overall, the fact that steviol glycosides produced via enzymatic conversion and steviol glycosides extracted from *S. rebaudiana* Bertoni undergo a common metabolic pathway following ingestion provides the pivotal confirmatory data supporting the read across approach to the extensive steviol glycoside safety database for supporting the safety of steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract."

The AEJEG agreed with the Applicants conclusions.

4. Conclusions

The FSA and FSS agreed on the assessment undertaken by the AEJEG who agreed with the proposed change in the steviol glycoside specification to include a production method using enzymatic conversion of highly purified rebaudiosides A and/or stevioside extracted from the leaves of *Stevia rebaudiana* Bertoni to give rebaudiosides M and D and/or rebaudioside AM and concluded that the proposed production method is safe under the proposed conditions of use and at the anticipated levels of intake.

The AEJEG concluded that sufficient information had been provided to allow for an evaluation of the proposal for modification of the manufacturing specifications of steviol glycosides (E 960) to include the enzymatic bioconversion process and that as the product is comparable to steviol glycosides extracted via conventional methods, there were no concerns over safety of the proposed process.

The FSA and FSS therefore conclude that the modification of the manufacturing specifications of steviol glycosides (E 960) to include the enzymatic bioconversion process as described within this application would not pose a risk to health. The FSA and FSS also conclude that the product is comparable to steviol glycosides extracted via conventional methods and that therefore there were no concerns over safety of the proposed process.

These conclusions were based on the information in the food additive dossier plus the supplementary information and could not have been reached without the data claimed as proprietary by the Applicant.

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6. Abbreviations

ADI – Acceptable daily intake

AEJEG – Joint Expert Group on Additives, Enzymes and other Regulated products

ANS - EFSA Panel on Food Additives and Nutrient Sources added to Food

BCA - Bicinchoninic Acid Assay

CoA - Certificate of Analysis

cGMP - Current Good Manufacturing Practices

DNA - Deoxyribonucleic acid

EC - European Commission

E. coli - *Escherichia coli*

EFSA - The European Food Safety Authority

EU - European Union

FAF - The EFSA Panel on Food Additives and Flavourings

FAO - The Food and Agriculture Organization

FCC - The Food Chemicals Codex

FDA - The United States Food and Drug Administration

FSANZ - Food Standards Australia New Zealand

GRAS - Generally Recognized as Safe

HACCP - Hazard Analysis of Critical Control Points

IPTG - Isopropyl β-D-1-thiogalactopyranoside

JECFA - The Joint FAO/WHO Expert Committee on Food Additives

mg/kg – Milligram per kilogram

mg/kg bw/day - mg per kg body weight per day

ML – Maximum permitted level

MPN/g - Most probable number per gram

ng/kg bw/day - nanograms per kilogram of body weight per day

NOAEL - No observed adverse effect level

PCR - Polymerase chain reaction

REB A – Rebaudioside A

REB AM – Rebaudioside AM

REB D – Rebaudioside D

REB M – Rebaudioside M

SCF - Scientific Committee on Food

SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SG - steviol glycoside

SuSy - Sucrose synthase

UDP - uridine diphosphate

UGT - UDP glucosyltransferase

WHO - World Health Organization

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