Safety Assessment: Outcome of assessment of the of use of steviol glycosides produced by fermentation (Rebaudioside M) using Saccharomyces cerevisiae

Reference number RP1112

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

Regulated Product Dossier Assessment

Assessment finalised: 12/03/2024

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Summary

An application was submitted to the Food Standards Agency (FSA) and Food Standards Scotland (FSS) in May 2021 from Amyris Inc ("the Applicant") for changes of the existing production method of steviol glycosides (formerly E 960) to include fermentation by *Saccharomyces cerevisiae*.

The production method uses a genetically modified strain of *S. cerevisiae* to convert sugar into rebaudioside M (reb M) at a final purity of no less than 95% reb M (anhydrous).

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 from fermentation by *Saccharomyces cerevisiae* was safe under the proposed conditions of use. The proposed uses and use levels for rebaudioside M, produced via fermentation with *Saccharomyces cerevisiae* remain the same as the already authorised food additive steviol glycosides (E 960a and E960c).

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.

1. Introduction

The FSA and FSS have undertaken a safety assessment for the production of steviol glycosides from a genetically modified production strain of *Saccharomyces cerevisiae* under the common authorisation procedure for food additives, food enzymes and food flavourings legislation, assimilated eu Regulation 1331/2008. To support the safety assessment by FSA and FSS, the AEJEG provided risk assessment advice to the FSA and FSS outlined in this opinion.

Steviol glycosides are a class of compounds which function as low-calorie sweeteners that are currently authorised for use in a range of food groups which includes but is not limited to edible ices, fruit and vegetables in vinegar, oil or brine, fruit and vegetable preparations excluding compote, and table-top sweeteners in powder form, with a varying range of restrictions.

The dossier was evaluated in line with Article 3 of assimilated Regulation 1331/2008, and 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). The assessment has considered the aspects of the food additive and its production.

With thanks to the members of the AEJEG who provided advice 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.

Information regarding the identity of the substance including existing and proposed specifications were provided. In addition, information on the manufacturing process, presence of impurities, stability of the substance, fate in food, existing authorisations and risk assessments and biological and toxicological data were provided. This information was considered satisfactory. A dietary exposure assessment for steviol glycosides has not proposed any extensions of use of the product and therefore

exposure levels are expected to remain the same from the authorisation of this Application.

It was concluded that sufficient information had been provided to allow for an evaluation of the proposal for modification of the manufacturing specifications of steviol glycosides to include production by fermentation by a genetically modified production strain of *S. cerevisiae*.

Following the final review by the AEJEG In March 2024, the AEJEG advised that the new method for the production of steviol glycosides from a genetically modified production strain of *Saccharomyces cerevisiae* 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, this document received review and clearance by the Committee on Toxicity (COT) in March 2024.

2. Assessment

2.1 Identity and Characterisation of the additive

2.1.1 Identity of the substance

The Applicant stated that steviol glycosides are already authorised within the UK when originating from the leaves of the *Stevia rebaudiana* plant. Current specifications state that steviol glycosides must originate from extraction from the leaves of *S. rebaudiana*. The final product must not contain less than 95% of the 11 steviol glycosides A, B, C, D, E, F and M, steviolbioside, rubusoside, stevioside and ducloside.

The Applicant provided a brief comparison to the additive produced via fermentation. Rebaudioside M (Reb M) is proposed to be manufactured by mixing food-grade cane

sugar with a *S. cerevisiae* production strain, genetically modified to produce steviol glycosides. The Applicant stated that the production strain is absent from the final Reb M product. They requested the modification of the specifications for steviol glycosides to allow the production of highly purified Reb M from fermentation with a *S. cerevisiae* production strain.

2.1.2 Identity of the components

The Applicant provided the chemical formulae $C_{56}H_{90}O_{33}$, CAS number (CAS No. 1220616-44-3) and molecular mass of Reb M (1,291.3 g/mol) and the chemical structure of Reb M (presented below as Figure 1).

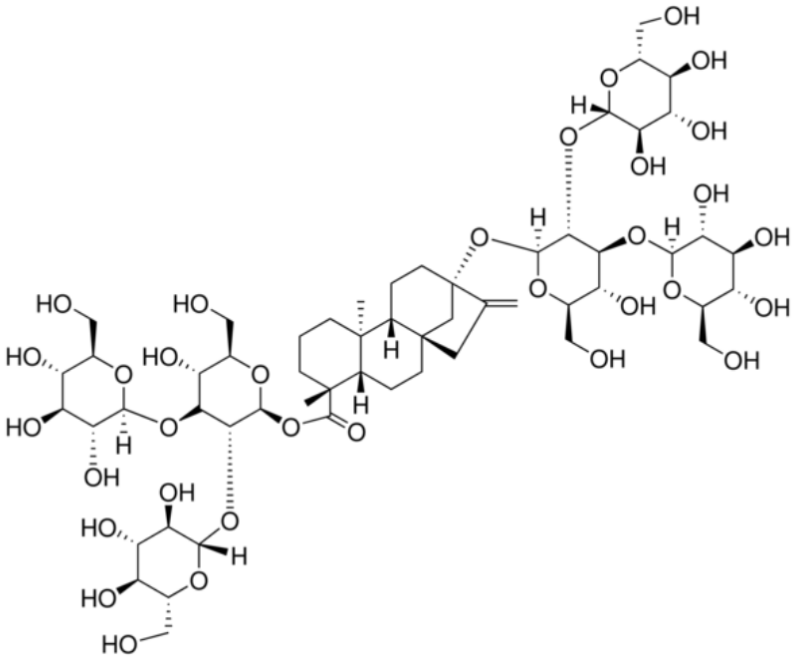

Figure 1: Chemical structure of Reb M as presented by the Applicant.

The Applicant reiterated the method of production as being via fermentation by a production strain of *S. cerevisiae* and stated the purity of the compound will not drop below 95% (as required under the specification for steviol glycosides).

2.1.3 Spectroscopic and chromatographic data

The Applicant provided a description of the analysis techniques performed on Reb M, produced by the Applicant's production strain of *S. cerevisiae*. This consisted of reverse-phase high-performance liquid chromatography with diode-array detection at 210 nm using a gradient mobile phase as the method to detect and quantify rebaudioside M in steviol glycoside samples. The Applicant provided an example of a readout for purified rebaudioside M and the readouts for five representative batches. The AEJEG was satisfied with the information provided.

The Applicant described how they used reversed phase high performance liquid chromatography with ultraviolet detection (RP-HPLC-UV) in order to assess the chemical equivalence of rebaudioside M extracted from the leaves of *S. rebaudiana* to that produced by fermentation by the Applicant's production strain of *S. cerevisiae*. This method is older than the reverse-phase high-performance liquid chromatography coupled to a diode array detector (RP-HPLC-DAD) method used to commercially measure steviols. The Applicant provided the operational procedure for RP-HPLC-UV. The AEJEG raised issue with a lack of data demonstrating the stereochemical equivalence of Reb M produced by fermentation and the Reb M extracted from *S. rebaudiana*. The AEJEG therefore requested further information to assess this, which the Applicant provided.

The Applicant conducted further analysis on the same samples that were tested with HPLC, this time, using tandem mass spectrometry (MS) methodology. Analysis was conducted using a Thermo Scientific Q Exactive Orbitrap LC-MS/MS with a Vanquish UPLC. To make the analytical method for total steviol glycosides (RP-HPLC-UV Method SOP00717 from the original submission) MS-compatible, phosphate buffer additives were replaced with 0.1% formic acid while maintaining the same percentages of water and acetonitrile using the same gradient elution program. Separation was achieved using a C18 column, electrospray ionisation was performed in negative mode (isolating

for *m/z* 1,289), and fragmentation was performed using a normalised collision energy of 35 (No units were provided).

The readouts of this analysis were presented to the AEJEG. This additional analysis was accepted by the AEJEG as evidence that the product produced by fermentation and the product produced by extraction from *S. rebaudiana* were chemical equivalents.

2.1.4 Description of physical and chemical properties

The Applicant described Reb M produced by fermentation as 'a white to light yellow powder with a pH between 4.5 and 7.0 (1% solution). The steviol glycosides from fermentation (Reb M) ingredient has an appearance similar to that of steviol glycosides derived from *S. rebaudiana* Bertoni and its physical and chemical properties conform to the existing UK specifications for steviol glycosides (E 960)'.

2.1.5 Additional information specific to additives derived from botanical sources

The Applicant stated no additional information was required due to the fact Reb M is manufactured with an *S. cerevisiae* production strain. The AEJEG accepted this.

2.1.6 Specifications

The Applicant supplied the current UK specifications for steviol glycosides. This only considers production by extraction from *S. rebaudiana* Bertoni presented below as Table 1. The Applicant also provided the current JECFA specifications for steviol glycosides from fermentation presented below as Table 2.

Table 1: Current UK specifications for steviol glycosides (at the time E960)

Parameter	**Specification**
Definition	The manufacturing process comprises two 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. The additive may contain residues of ion-exchange resins used in the manufacturing process. Several other related steviol glycosides that may be generated as a result of the production process, but do not occur naturally in the Stevia rebaudiana plant have been identified in small amounts (0,10 to 0,37% w/w).
Chemical name	Steviolbioside: 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid. Rubusoside: 13-β-D-glucopyranosyloxykaur-16-en-18-oic acid, β-D-glucopyranosyl ester. Dulcoside A: 13-[(2-O-α–L-rhamnopyranosyl-β–D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester. Stevioside: 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester. Rebaudioside A: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D- glucopyranosyl ester. Rebaudioside B: 13-[(2-O-β–D-glucopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid. Rebaudioside C: 13-[(2-O-α–L-rhamnopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester. Rebaudioside D: 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.

Parameter	Specification
	Rebaudioside E: 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester. Rebaudioside F: 13[(2-O-β-D-xylofurananosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester. Rebaudioside M: 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.
Trivial name, molecular formula and conversion factors	Steviol, C20 H30 O3, conversion factor 1.00. Steviolbioside, C32 H50 O13, conversion factor 0.50. Rubusoside, C32 H50 O13, conversion factor 0.50. Dulcoside A, C38 H60 O17, conversion factor 0.40. Stevioside, C38 H60 O18, conversion factor 0.40. Rebaudioside A, C44 H70 O23, conversion factor 0.33. Rebaudioside B, C38 H60 O18, conversion factor 0.40. Rebaudioside C, C44 H70 O22, conversion factor 0.34. Rebaudioside D, C50 H80 O28, conversion factor 0.29. Rebaudioside E, C44 H70 O23, conversion factor 0.33. Rebaudioside F, C43 H68 O22, conversion factor 0.34. Rebaudioside M, C56 H90 O33, conversion factor 0.25.
Trivial name, CAS number and molecular weight (g/mol)	Steviol, 471-80-7, 318.46. Steviolbioside, 41093-60-1, 642.73. Rubusoside, 64849-39-4, 642.73. Dulcoside A, 64432-06-0, 788.87.

Parameter	Specification
	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, 1129.15. Rebaudioside E, 63279-14-1, 967.0. Rebaudioside F, 438045-89-7, 936.99. Rebaudioside M, 1220616-44-3, 1291.30
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.
Identification (pH)	Between 4.5 and 7.0 (1 in 100 solution).
Purity (Total ash)	No more than 1%.
Purity (Loss on drying)	No more than 6% (105°C, 2 hours).
Purity (Residual solvent)	No more than 200 mg/kg methanol.
Purity (Residual solvent)	No more than 5,000 mg/kg ethanol.
Purity (Arsenic)	No more than 1 mg/kg.
Purity (Lead)	No more than 1 mg/kg.

CAS=Chemical Abstracts Service; h = hours; UK = United Kingdom

Table 2: Current JECFA specifications for steviol glycosides from fermentation (INS No. 960b)

Specification parameter	**Specification**
Synonyms	INS No. 960b
Definition	Steviol glycosides from fermentation consist of a mixture of compounds containing a steviol backbone conjugated to various sugar moieties (e.g. glucose or sucrose) depending on the specific production organism and fermentation conditions used. Steviol glycosides from fermentation are obtained from the fermentation of non-toxigenic non-pathogenic strains of Yarrowia lipolytica and Saccharomyces cerevisiae that have been genetically modified with heterologous genes from multiple donor organisms to overexpress steviol glycosides. After removal of the biomass by solid-liquid separation and heat treatment, the process involves concentration of the steviol glycosides (e.g. by resin adsorption), followed by purification of the desired steviol glycosides by crystallization and drying. Ion exchange resins may be used in the purification process. The final product may be spray-dried. Commercial products are primarily composed of either rebaudioside A, rebaudioside M, or a combination of rebaudioside M and rebaudioside D; additional minor steviol glycosides may be present.
Chemical names	See Appendix A to Steviol Glycosides Specifications Framework (JECFA, 2020)
CAS number	See Appendix A to Steviol Glycosides Specifications Framework (JECFA, 2020)
Chemical formula	See Appendix A to Steviol Glycosides Specifications Framework (JECFA, 2020)
Assay	Not less than 95% of total of steviol glycosides, on the dried basis.
Description	White to light yellow powder, odourless or having a slight characteristic odour. About 200 - 300 times sweeter than sucrose.
Functional uses	Sweetener
Characteristics	Description of characteristic
Identification (Solubility)	Freely soluble in a mixture of ethanol and water 50:50, sparingly soluble in water and sparingly soluble in ethanol.

Specification parameter	Specification
Identification (HPLC Chromatic profile)	The main peaks in a chromatogram obtained by analysing a sample following the procedure in METHOD OF ASSAY correspond to steviol glycosides.
Identification (pH)	Between 4.5 and 7.0 (1 in 100 solution)
Purity (Total ash)	Not more than 1%
Purity (Loss on drying)	Not more than 6% (105°, 2 h)
Purity (Residual solvents)	Not more than 200 mg/kg methanol and not more than 5000 mg/kg ethanol (Method I, General Methods, Organic Components, Residual Solvents)
Purity (Arsenic)	Not more than 1 mg/kg. Determine using a method appropriate to the specified level (Use Method II to prepare sample solution). The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General Methods, Metallic Impurities”).
Purity (Lead)	Not more than 1 mg/kg. Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, under “General Methods, Metallic Impurities”.
Purity (Microbiological criteria)	Total (aerobic) plate count: Not more than 1,000 CFU/g Yeasts and moulds: Not more than 200 CFU/g Escherichia coli: Negative in 1 g Salmonella: Negative in 25 g.

CAS= Chemical Abstracts Service; CFU = colony forming units; HPLC = high-performance liquid chromatography; JECFA = Joint FAO/WHO Expert Committee on Food Additives

The Applicant provided proposed modifications to the specifications for steviol glycosides derived from fermentation. These modifications to the specification have been drafted following the format of UK specifications and have been proposed to align to the JECFA definition of steviol glycosides from fermentation. The Applicant proposed the addition of a purity parameter for kaurenoic acid with a limit of no more the 300 mg/kg. These specifications are presented below as Table 3 with new proposed text presented in bold writing.

Table 3: Proposed modifications to the definition and purity parameters in the UK specification for steviol glycosides.

Parameter	Specification
Definition	Extraction: The manufacturing process comprises two 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. The additive may contain residues of ion-exchange resins used in the manufacturing process. Several other related steviol glycosides that may be generated as a result of the production process, but do not occur naturally in the Stevia rebaudiana plant have been identified in small amounts (0,10 to 0,37% w/w). Fermentation: The manufacturing process comprises two main phases: the first involving fermentation of a non-toxigenic non-pathogenic strain of Saccharomyces cerevisiae that has been genetically modified with heterologous genes from multiple donor organisms to overexpress steviol glycosides. After removal of the biomass by solid-liquid separation and heat treatment, the second phase involves purification and concentration of rebaudioside M by ultra-, nano-, and press-filtration, and optional recrystallisation of rebaudioside M from aqueous ethanol and carbon treatment resulting in a final product containing not less than 95% rebaudioside M.
Purity (Kaurenoic acid)	Not more than 300 mg/kg

UK = United Kingdom

2.1.7 Analytical data for steviol glycosides

The Applicant provided purity data from 5 commercial batches of steviol glycosides from fermentation. The Applicant presented that the percentage of Reb M within the 5 batches ranged from 95-99%.

Following this, data from batch analyses were provided to demonstrate compliance of the product with the current purity specifications for steviol glycosides within the UK presented below as Table 4.

Table 4: Results of batch analyses for 5 representative commercial lots of steviol glycosides from fermentation (Reb M)

Current UK specifications for steviol glycosides parameter[a]	**Current UK specifications for steviol glycosides limit**	**Analytical method**	**Manufacturing lot P0145**	**Manufacturing lot P0146**	**Manufacturing lot P0164**	**Manufacturing lot P0211**	**Manufacturing lot P1012**
Assay	Not less than 95% steviol glycosides[b]	IT_CORP_LQA_076	99	95	98	99	99
Description	White to light yellow powder	IT_CORP_LQA_077	White	White	White	White	White
Description	Between 200 and 350 times sweeter than sucrose (at 5% sucrose equivalency)	N.A	N.A	N.A	N.A	N.A	N.A
Identification (Solubility)	Freely to slightly soluble in water	IT_CORP_LQA_122	Freely soluble	Freely soluble	Freely soluble	Freely soluble	Freely soluble
Identification (pH)	Between 4.5 and 7.0 (1 in 100 solution)	IT_CORP_LQA_074	6.1	5.7	5.9	6.1	6.7
Purity (Total ash)	No more than 1%	IT_CORP_LQA_079	0.1	0.1	0.1	0	0.1
Purity (Loss on drying)	No more than 6% (105°C, 2 hours)	IT_CORP_LQA_075	4	4	3	3	4

Current UK specifications for steviol glycosides parameter[a]	Current UK specifications for steviol glycosides limit	Analytical method	Manufacturing lot P0145	Manufacturing lot P0146	Manufacturing lot P0164	Manufacturing lot P0211	Manufacturing lot P1012
Purity (Residual solvent)	No more than 200 mg/kg methanol	USP 467	<100	<100	<100	<100	<100
Purity (Residual solvent)	No more than 5,000 mg/kg ethanol	USP 467	1900	1600	920	1500	910
Purity (Arsenic)	No more than 1 mg/kg	AOAC 993.14	<0.01	<0.01	<0.01	<0.01	<0.01
Purity (Lead)	No more than 1 mg/kg	AOAC 993.14	<0.005	<0.005	<0.005	<0.005	<0.005

AOAC = Association of Official Analytical Chemists; h = hours; NMT = not more than; Reb M = rebaudioside M; UK = United Kingdom; USP = United States Pharmacopeia

[a] Current specifications for steviol glycosides in the UK as per: EUR-Lex, 2012. Assimilated Regulation No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to assimilated Regulation No 1333/2008. Available online: https://www.legislation.gov.uk/eur/2012/231/contents

[b] Reported values for the analysed batches are based on rebaudioside M content only

The Applicant provided microbiological analyses for the same batches comparing CFU/g for total aerobic plate count, yeast, moulds, *Escherichia coli* and *Salmonella* within the batches to the JECFA specification (Table 5).

Table 5: Results of microbiological analysis for 5 representative commercial lots of steviol glycosides from fermentation (Reb M).

Parameter	**JECFA specification[a]**	**Analytical Method**	**P0145**	**P0146**	**P0164**	**P0211**	**P1012**
Total (aerobic) plate count (CFU/g)	Not more than 1,000	AOAC 990.12	30	<10	90	<10	<10
Yeast (CFU/g)	Not more than 200	AOAC 997.02	<10	<10	<10	10	<10
Moulds (CFU/g)	Not more than 200	AOAC 997.02	<10	<10	<10	<10	<10
Escherichia coli	Negative in 1g	AOAC 991.14	Negative	Negative	Negative	Negative	Negative
Salmonella	Negative in 25g	AOAC 2016.01	Negative	Negative	Negative	Negative	Negative

AOAC = Association of Analytical Communities; CFU = colony forming units; JECFA = Joint FAO/WHO Expert Committee on Food Additives.

[a]JECFA specification for Steviol Glycosides from Fermentation (JECFA, 2020).

The Applicant provided information on the sweetness potency and sensory characteristics, stating that Reb M is approximately 200 times the sweetness of sugar. They reported the results of a sensory analysis and sweetness equivalence test. This concluded that Reb M from fermentation is 200-300 times the sweetness of sucrose. The Applicant stated this is in line with the JECFA specifications which describe steviol

glycosides as ‘approximately 100-300 times sweeter than sucrose’. They claimed Reb M produced by fermentation left less of a lingering bitter taste and was less bitter and astringent relative to sucrose.

The Applicant stated that information on particle size, shape and distribution was not required. They stated the current UK specification for steviol glycosides (at the time E 960) does not include information on particle size, shape, and distribution and therefore, data pertaining to these properties were not included in this application.

2.2 Production Process

The Applicant provided detailed information on the production process of Kaurenoic acid which the AEJEG reviewed. In summary, the production process can broadly be divided into two stages, upstream processing and downstream processing. In upstream processing, food-grade cane sugar is mixed with *S. cerevisiae* production strain and fermented to produce rebaudioside M and other steviol glycosides. Following this, the production organism is deactivated and removed from the fermentation mixture. In downstream processing, a series of purification and concentration steps are implemented, producing the final product, ≥95% rebaudioside M powder.

The Applicant stated that their steviol glycosides, produced from fermentation were manufactured in compliance with current Good Manufacturing Practice (cGMP) and a food safety plan which included Hazard Analysis Critical Control Point (HACCP) plan. They continued that all additives, processing aids and food contact articles used within the production process complied with food grade requirements.

The AEJEG were satisfied with the information provided by the Applicant regarding the manufacturing process.

2.3 Presence of impurities

The Applicant stated Reb M is a high purity ingredient containing no less than 95% steviol rebaudioside M. The Applicant also stated the compound is in compliance with UK specifications evidencing the batch reports presented in Table 4. The Applicant continued that the final product contained no production strain, evidenced by an absence of DNA and viable cells. The Applicant noted the presence of an additional impurity in the production of steviol glycosides from fermentation. This is kaurenoic acid. This is a metabolite present within the biosynthetic pathway for steviol glycosides and acts as a precursor to steviol. The conversion of kaurenoic acid to steviol is displayed below in Figure 2.

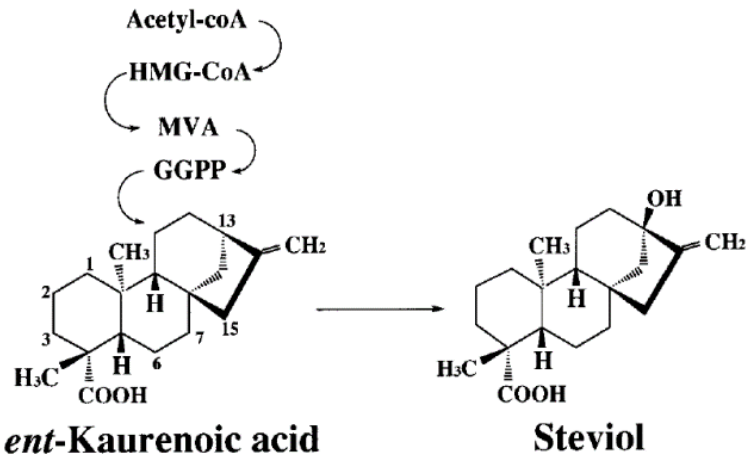

Figure *2*: Biosynthetic pathway and structures of ent-kaurenoic acid and steviol (Kim et al., 1996). GGPP = geranylgeranyl pyrophosphate, HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A, MVA = mevalonic acid. ent = enantiomer

For measuring kaurenoic acid, the Applicant stated that ‘a high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS; Waters HClass UPLC with QDa Detector) [method] was developed and validated.’ The method standard operating procedure and method validation report were provided by the Applicant and deemed satisfactory by the AEJEG.

A primary source of kaurenoic acid with 94.26% purity manufactured by the Applicant was used to generate a standard curve and was confirmed with a 100% purity kaurenoic acid supplied by Sigma-Aldrich. The 5 representative lots of product used in previous testing were analysed for kaurenoic acid with an average concentration of 150.2 mg/kg within the Reb M. Based on this data, the Applicant proposed a limit of 300 mg/kg to be included within the UK specifications for steviol glycosides produced by fermentation. The table displaying the concentrations of kaurenoic acid from the 5 individual lots is presented below as Table 6.

Table 6: Results of kaurenoic acid analysis (mg/kg) for 5 representative commercial lots of steviol glycosides from fermentation (Reb M)

Parameter	**Proposed specification**	**Analytical Method**	**P0145**	**P0146**	**P0164**	**P0211**	**P1012**
Kaurenoic acid	≤ 300mg/kg	O-TC-MET36016	75	119	219	145	193

The AEJEG requested the Applicant provide further information on the origin of kaurenoic acid within the product.

The Applicant stated that kaurenoic acid is a precursor in the biosynthetic pathway of the steviol aglycone, in which it is present as a precursor to steviol. The Applicant stated that the presence of kaurenoic acid within steviol glycosides produced by water extraction of *S. rebaudiana* has been considered as plausible by EFSA despite its absence from current specifications (Smeraldi, 2018).

The AEJEG were satisfied with the information provided by the Applicant.

The AEJEG also requested justification for the selection of a 300 mg/kg limit for kaurenoic acid within the proposed specifications.

The Applicant stated that kaurenoic acid had been infrequently addressed in steviol glycoside Generally Recognised As Safe (GRAS) notices to the US Food and Drug Administration (FDA) and steviol glycoside safety evaluations submitted to EFSA. The Applicant stated that the impurity is present in some steviol products (and is a precursor intermediate in the biosynthetic pathway to produce steviol glycosides).

The Applicant stated that in 2018 an *ad hoc* meeting between EFSA and industry representatives on steviol glycosides addressed the topic of kaurenoic acid and in particular the level of kaurenoic acid within their steviol glycoside-based sweeteners (Smeraldi, 2018). The Applicant stated that EFSA noted kaurenoic acid had not been reported by any previous Applicants who had submitted Application dossiers for evaluation. The Applicant stated EFSA had not recommended adding limits to kaurenoic acid to the specifications for E 960. As such, currently there are no limits for kaurenoic acid within the specifications for steviol glycosides by EFSA or the FDA. The Applicant stated that they have proposed these limits in order to ensure any potential worst case exposure scenario to kaurenoic acid from products containing Reb M is safe.

The Applicant therefore supplied as a justification for the ≤ 300 mg/kg limit for kaurenoic acid in their steviol glycosides from fermentation (reb M) 2 key principles:

- “To ensure that the maximum potential kaurenoic acid exposure from consumption of Amyris’ steviol glycosides from fermentation (Reb M) would not exceed established TTCs for substances of any Cramer Class designation

- To establish an upper limit for kaurenoic acid that would allow for the inherent batch-to-batch variability that can be reasonably expected during the manufacture of food ingredients.”

The AEJEG were satisfied with the justification supplied by the Applicant.

The Applicant's discussion of kaurenoic acid exposure, used to justify the ≤ 300 mg/kg limit is later discussed in the section of this advice document on 'Proposed Uses and Exposure Assessment / Dietary Exposure Assessment and Data on Dietary Sources'. Further discussion on the choice of Cramer class used within this assessment and TTC values is presented in the 'Establishing a safe human exposure level' within this advice document.

2.4 Stability of the substance and reaction and fate in foods

The Applicant stated that information on the stability of steviol glycosides and fate in foods was submitted by Applicants to various global scientific and regulatory bodies including JECFA, EFSA and Food Standards Australia/ New Zealand (FSANZ). These regulatory bodies reviewed and evaluated the stability of the compound and its fate in food based on the work submitted by Applicants and the work of several published studies (Chang and Cook, 1983; Kroyer, 1999).

The Applicant discussed the 68th Committee meeting of JECFA where the stability of steviol glycosides in conditions mimicking their usage in food was investigated (JECFA 2007). The Applicant stated that JECFA considered steviol glycosides reasonably stable under elevated temperatures that are commonly used in food processing as they 'do not undergo browning or caramelization when heated'. The Applicant stated steviol glycosides are stable for at least 180 days when stored under acidic conditions (pH 2 – 4) at 24^{o}C. It was noted that when held at an elevated temperature of 80^{o}C, 4% and 8% degradation were seen at pH 4 and 3, respectively. The Applicant stated that this suggested steviol glycoside stability is pH and temperature dependent. They stated that at 100^{o}C, decomposition occurred at 10% and 40% at pH 4 and 3, respectively. The Applicant continued that JECFA concluded steviol glycosides are thermally and hydrolytically stable for use in food and acidic beverages under normal processing and storage conditions.

The Applicant then discussed a 2017 study investigating the effect of manufacturing process on steviol glycosides from 3 separate batches. They stated the testing of batches was performed on samples of untreated stevia leaves, the first water extract and a high purity end product (≥ 95% steviol glycosides (Oehme *et al*., 2017). Changes in steviol glycoside composition were analysed by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) and high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). The authors reported they had detected all 9 JECFA-defined steviol glycosides (rebaudiosides A, B, C, D, F, stevioside, steviolbioside, rubusoside, and dulcoside A) and demonstrated that stevia extract processing does not chemically alter or modify the individual steviol glycoside content.

The Applicant then proceeded to discuss the stability of steviol glycosides produced via fermentation with the genetically modified production organism specified within this Application. The Applicant conducted an accelerated storage stability study on 4 lots of Reb M (lots P0115, P0135, P0146 and P0151). This entailed storing the batches at 40^{o}C at a relative humidity of 75%. All 4 lots were stored in foiled packing with 2 lots also being stored in double high density polyethylene (HDPE) packaging. On average the preparations maintained a Reb M content of no less than 95%. The Applicant states this indicates that the Reb M produced by fermentation is stable for 6 months under accelerated conditions which the Applicant states is representative of 24 months in real time. The tables displaying the results of this study have been presented below as Tables 7 and 8.

Table 7: Rebaudioside M content (%) of 4 production lots of steviol glycosides from fermentation (Reb M) under accelerated stability test conditions stored in foil packaging

Lot number	0 weeks	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	8 weeks	13 weeks	26 weeks
P0115	96.5	97.8	95.2	96.0	95.4	97.5	96.4	98.6	96.1	96.1
P0135	96.1	95.4	95.2	94.8	96.9	95.8	97.5	98.3	95.5	96.3
P0146	95.3	96.4	96.2	96.2	95.5	97.9	94.5	96.2	94.6	95.2
P0151	97.0	97.2	97.6	96.3	97.5	97.7	95.1	95.4	96.3	95.6
Average	96.3	96.7	96.1	95.8	96.3	97.2	95.9	97.12	95.6	95.8

Table 8: Rebaudioside M content (%) of 4 production lots of steviol glycosides from fermentation (Reb M) under accelerated stability test conditions stored in double HDPE packaging.

Lot number	0 weeks	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	8 weeks	13 weeks	26 weeks
P0146	95.3	97.5	94.8	94.8	94.8	95.7	98.3	95.0	96.6	95.2
P0151	97.0	94.5	95.1	95.1	96.2	96.0	99.1	95.2	98.1	96.3
Average	96.2	96.0	95.0	95.5	95.9	98.7	98.7	95.1	97.3	95.8

2.5 Methods of analysis in food

Within the risk management section of the technical dossier, the Applicant stated that in 2010 EFSA recognised the use of validated in-house analytical methods using HPLC to identify stevioside, rebaudioside A and related minor steviol glycosides, degradation products or constituents in matrices of food and beverage products (EFSA ANS Panel, 2010). The Applicant stated that 2 further analytical methods employing HPLC to determine steviol glycosides in food have been published (Geuns, 2008; Gardana *et al.,* 2010)

The Applicant stated that their steviol glycoside from fermentation (Reb M) is specifically analysed by reverse-phase high performance liquid chromatography with diode-array detection at 210nm (RP-HPLC DAD) using a gradient mobile phase. This method has been validated to detect and quantify rebaudioside M in steviol glycoside samples. The Applicant provided further detail on the standard operating procedure and the validation report for this method.

The AEJEG were satisfied by the information provided regarding methods of analysis in food.

2.6 Existing Authorisations and Risk Assessments

2.6.1 Existing authorisations

The Applicant summarised the existing authorisations for the European Union and the United Kingdom, the United States of America, Australia and New Zealand Canada, Asia and a number of other jurisdictions. Brief summaries are provided below.

In the EU/UK, steviol products must comply with the specifications for steviol glycosides last updated in 2016. They must be produced via extraction from the leaves of the *S. rebaudiana* Bertoni plant and contain no less 95% of the named 11 steviol glycosides dulcoside, rebaudiosides A, B, C, D, E, F, and M, rubusoside, steviolbioside, and stevioside. In 2020 the EFSA Food and Flavourings Panel published an opinion on an amendment, which saw the European Commission expand the list of acceptable steviol glycosides to include all those that naturally occur in the leaves of the *S. rebaudiana* plant. At the time the Applicant's dossier was received by the FSA this was still under review by the European Commission. The same EFSA panel evaluated the safety of rebaudioside M produced via enzyme-catalysed bioconversion of purified stevia leaf extract in 2019, issuing an opinion that the additive did not pose a safety concern.

In the United States, Amyris' steviol glycosides from fermentation (Reb M) produced by an engineered daughter strain of CEN.PK113-7D *S. cerevisiae* has Generally Recognized As Safe (GRAS) status for food and beverage uses with no objection from the U.S. FDA (GRN 812 – U.S. FDA, 2018). The U.S. FDA has no objections to the GRAS status for multiple methods of manufacture for steviol glycosides.

Regarding the regulatory position of steviol glycosides in Australia and New Zealand, the Applicant stated 'FSANZ [Food Standards Australia and New Zealand] has included steviol glycosides in the Australia New Zealand Food Standards Code as an intense sweetener under the food additive code number 960. Foods in Australia and New Zealand are permitted to contain intense sweeteners as specified in Standard 1.3.1(Food Additives) either as a flavour enhancer or as a replacement for sweetness normally provided by sugars. Steviol glycosides are permitted in a range of products (e.g., biscuits, cakes and pastries, formulated meal replacements and formulated supplementary foods, and sugar confectionery) at levels up to 1,100 mg/kg, measured as steviol equivalents, as described in Schedule 15 (Substances that may be used as food additives) of the Australia New Zealand Food Standards Code. Furthermore, specifications for steviol glycoside preparations described in Schedule 3 (Identity and Purity) include rebaudioside M" (S3-31), "steviol glycoside mixtures containing rebaudioside M" (S3-32), steviol glycosides from Stevia rebaudiana Bertoni" (S3-35), and "steviol glycosides from fermentation" (S3-39) (FSANZ, 2020). Each of these specifications indicate that these preparations must contain no less than 95% steviol glycosides on a dry weight basis. Steviol glycosides from fermentation are currently permitted in Australia/New Zealand when prepared by a S. cerevisiae production strain CD15407 as defined in S3-39.'

In Canada, the Applicant stated that steviol glycosides are approved for uses as sweeteners in a variety of food and beverage products. As of March 2021, Health Canada has limited the sources of steviol glycosides to: *Stevia rebaudiana* Bertoni; *Saccharomyces cerevisiae* CD15380; *Saccharomyces cerevisiae* CD15407; *Saccharomyces cerevisiae* Y63348 (Health Canada, 2020a). Steviol glycosides are

permitted in products at a maximum level of 0.35% in finished products as described in the *List of permitted Sweeteners* (Health Canada, 2020b).

The Applicant stated that stevia extracts are permitted for use in several countries in the Northern, Southern and Pacific regions of Asia. These include China, Hong Kong, Indonesia, Malaysia, Myanmar, Pakistan, Philippines, Singapore, Taiwan, Thailand and Vietnam (PureCircle Stevia Institute, 2021). They note 3 forms of stevia extract are authorised in Japan by the Ministry of Health and Welfare. They also stated other general use sweeteners such as purified stevioside (crude extract, 50% pure extract or ≥90% pure extract) and *S. rebaudiana* leaf extract are also permitted in a variety of Japanese food and beverage products (Marie, 1991; Das *et al*., 1992; Ferlow, 2005). The Applicant also stated that the use of steviol glycosides in food and beverages has been authorised in India by the Food Safety and Standards Authority (FSSAI, 2015; MOHFW, 2016).

The Applicant has stated that steviol glycosides are also authorised in several countries as a low-calorie sweetener in Central and South America, Africa and the Middle East (PureCircle Stevia Institute, 2021). The Applicant noted steviol glycosides from fermentation have been authorised in some Central and South American countries including Brazil, Colombia, Costa Rica, Peru, Bolivia, Panama, Ecuador and Mexico.

2.6.2 Risk Assessment / Safety Opinions

The Applicant collated the conclusions of safety opinions from JECFA, EFSA, FSANZ and Health Canada.

The Applicant stated that JECFA had reviewed the safety of steviol glycosides in 1998, 2004, 2007, 2008 and 2016 at their 51st, 63rd, 68th, 69th and 82nd meetings, respectively (JECFA, 1999, 2006, 2007, 2009, 2017). An acceptable daily intake (ADI) of 0 to 2 mg/kg bw was established, however on reviewing additional data in 2008 the ADI was increased to 0 to 4 mg/kg bw. JECFA upheld the ADI of 0 – 4 mg/kg bw/day in 2016. It

was noted that the Applicant had not provided descriptions of the uncertainties, uncertainty factors or the key studies used to establish this health-based guidance value.

The Applicant discussed how in their 87th meeting JECFA established a framework to develop specifications for steviol glycosides produced by 4 production methodologies. This included extraction, fermentation, enzymatic modification and enzymatic glucosylation (JECFA, 2019). The Applicant stated the latest Compendium of Food Additive Specifications had recently been published listing the specifications. The Applicant provided the full definition of steviol glycosides by fermentation defined by the JECFA framework, provided below:

> *"Steviol glycosides from fermentation consist of a mixture of compounds containing a steviol backbone conjugated to various sugar moieties (e.g. glucose or sucrose) depending on the specific production organism and fermentation conditions used. Steviol glycosides from fermentation are obtained from the fermentation of non-toxigenic non-pathogenic strains of Yarrowia lipolytica and Saccharomyces cerevisiae that have been genetically modified with heterologous genes from multiple donor organisms to overexpress steviol glycosides. Commercial products are primarily composed of either rebaudioside A, rebaudioside M, or a combination of rebaudioside M and rebaudioside D; additional minor steviol glycosides may be present"* (JECFA, 2020).

The Applicant stated that their steviol glycosides abide by the specifications laid out by JECFA for steviol glycosides manufactured by fermentation, complying with purity standards.

The Applicant provided an overview of EFSA's safety opinion. The Applicant stated the safety of steviol glycosides had been evaluated by the EFSA panel on Food Additives and Nutrient Sources Added to Food (ANS panel). Within this safety assessment an ADI of 4 mg/kg bw was established (EFSA ANS Panel 2010). In 2015 the ANS Panel

extended the specification of steviol glycosides to include rebaudiosides D and M as alternatives to rebaudioside A as the predominant components of steviol glycosides, stating this would not be a safety concern. The Panel stated that the ADI of 4 mg/kg bw could also be applied when the total steviol glycosides comprise more than 95% of the material. No uncertainties, uncertainty factors or key studies regarding this authorisation were provided by the Applicant (EFSA ANS Panel 2015a).

The Applicant then described how in 2019 EFSA published an opinion stating that enzymatic bioconversion of purified stevia is not of concern to health. The Applicant also discussed EFSA's evaluation of a proposed amendment to the specification of steviol glycosides, which aimed to expand the list of permissible steviol glycosides to include all steviols that are present on the leaves of *S. rebaudiana*. The Applicant stated that EFSA considered that all 60 steviol glycosides had been observed to share the same metabolic fate and considered the proposed amendment safe due to the vast toxicological database previously evaluated for these compounds. EFSA therefore considered the 4 mg/kg bw ADI was applicable to all 60 identified steviol glycosides (EFSA FAF Panel, 2020).

The Applicant described how the FSANZ has established an ADI of 0 – 4 mg/kg bw/day in 2008 (FSANZ, 2008). The definition of steviol glycosides has been expanded twice, in 2015 and 2017 to include all steviol glycosides present in the leaves of the *S. rebaudiana* plant (FSANZ 2015, 2017). This recognised at least 40 steviol glycosides, with the regulator stating that steviol glycoside extracts must contain no less than 95% steviol glycosides on a dry weight basis (FSANZ, 2017). In 2020 FSANZ expanded the specifications for steviol glycosides to include production from fermentation by *S. cerevisiae* in addition to enzymatic conversion of stevia leaf extract. The Applicant also states 'Moreover, in October of 2020, FSANZ evaluated the safety of Amyris' rebaudioside M as a steviol glycoside from *S. cerevisiae* (i.e., the same product as is the subject for this application) for use as an intense sweetener food additive and concluded that *"no potential public health and safety concerns have been identified with*

Amyris's Reb M produced from S. cerevisiae expressing steviol glycoside biosynthesis pathway genes".' (FSANZ, 2020).

The Applicant described Health Canada's authorisation. Health Canada first evaluated steviol glycosides (defined as stevioside rebaudiosides A, B, C, D, F, M, dulcoside A, rubusoside, and steviolbioside) in 2012, establishing an ADI of 4 mg/kg bw/day (Health Canada, 2012a,b). The definition of steviol glycosides was expanded to include rebaudioside M in 2016 (Health Canada, 2016), and all steviol glycosides within the *S. rebaudiana* plant in 2017. The Applicant stated that in Canada the specifications set for steviol glycosides stipulate that the specifications in the Food Chemicals Code by JECFA must be met where steviol glycoside preparations must contain no less than 95% steviol glycosides on a dried weight basis.

No uncertainties, uncertainty factors or key studies were provided regarding this authorisation. The Applicant stated 'In several recent premarket safety assessments, Health Canada did not identify any safety concerns for the use of steviol glycosides produced by fermentation with various *S. cerevisiae* production strains in foods and has therefore included these strains as acceptable alternative methods of steviol glycoside production in the List of Permitted Sweeteners (Health Canada, 2020a,b). Steviol glycosides produced from *S. rebaudiana* Bertoni, *S. cerevisiae* CD15380, *S. cerevisiae* CD15407, and *S. cerevisiae* Y63348 are all permitted for use in the same food products and at the same use levels in Canada.'

2.7 Proposed Uses and Exposure Assessment / Dietary Exposure Assessment and Data on Dietary Sources

This Application concerns an amendment to the specifications of steviol glycosides to introduce a new method of manufacture, therefore the Applicant claimed there will be no change to exposure to steviol glycosides from approval of this Application. The Applicant provided the currently authorised uses for steviol glycosides within the UK pursuant of assimilated Regulation No. 1131/2011.

The Applicant reiterated the claim that there will be no change to exposure from a change in specifications regarding manufacturing process. The Applicant stated ‘It is also important to note that since steviol glycoside use levels are expressed as steviol equivalents, and not for any one particular steviol glycoside, specific use levels are not required for each individual glycoside. Steviol glycoside use levels are instead based on the total steviol content of the final food product resulting from the addition of any steviol glycoside preparations that fall within the standard specifications.’.

The AEJEG requested that the Applicant perform an exposure assessment for the minor impurity kaurenoic acid.

The Applicant stated that exposure to kaurenoic acid from steviol glycosides manufactured by fermentation (Reb M), can be effectively estimated using recent estimates of anticipated steviol glycoside exposure conducted by EFSA. A worst case maximum estimated daily exposure of up to 4.3 mg steviol glycosides/kg body weight per day was reported for toddlers (12 to 35 months), the population sub-group with the greatest anticipated exposure relative to body weight (EFSA ANS Panel, 2015a). A summary of the anticipated exposure estimates for steviol glycosides across a range of population sub-groups, as reported by EFSA, was provided by the Applicant and is presented below as Table 9.

Table 9: Summary of anticipated exposure to steviol glycosides (mg/kg bw per day) utilising the EFSA comprehensive database and the maximum permissible levels (reproduced from EFSA, 2015)

Exposure level	**Toddlers (12-35 months) mg/kg bw/day**	**Children (3-9 years) mg/kg bw/day**	**Adolescents (10-17 years) mg/kg bw/day**	**Adults (18-64 years) mg/kg bw/day**	**Elderly (≥ 65 years) mg/kg bw/day**
Mean	0.6 to 2.4	0.5 to 1.8	0.2 to 0.8	0.1 to 1.1	0.1 to 0.5
High level	2.0 to 4.3	1.4 to 3.9	0.6 to 1.9	0.5 to 2.3	0.4 to 1.4

The Applicant stated that the upper limit for kaurenoic acid within the proposed specifications for steviol glycosides was identified as ≤ 300 mg/kg. Utilising this level and the highest estimated intakes (upper bound estimates) above in Table 9, converted into µg/kg bw per day, the Applicant predicted the maximum anticipated exposure to kaurenoic acid from the use of steviol glycosides produced by fermentation at mean and high levels of consumption. These have been presented below as Table 10. The Applicant considered this to be a highly conservative assessment.

Table 10: Summary of maximum anticipated exposure to kaurenoic acid (µg/kg bw/day) from all steviol glycoside preparations.

Exposure level	**Toddlers (12 to 35 months)**	**Children (3 to 9 years)**	**Adolescents (10 to 17 years**	**Adults (18 to 64 years)**	**Elderly (≥ 65 years)**
Mean (µg/kg bw/day)[a]	0.72	0.54	0.24	0.33	0.15
High level (95th Percentile) (µg/kg bw/day)	1.29	1.17	0.57	0.69	0.42

Bw = bodyweight

[a]Calculation: (highest estimated intake of steviol glycoside [from Table 9 (mg//kg bw/day)] * 0.0003) * 1,000.

The Applicant considered that assuming a maximum concentration of 300 mg/kg for kaurenoic acid in the Applicant's steviol glycosides from fermentation and the worst case scenario of 4.3 mg/kg bw per day exposure to steviol glycosides in the sub-population with the highest relative exposure (toddlers aged 12 – 35 months), the highest worst case maximum daily exposure to kaurenoic acid as an impurity from steviol glycosides manufactured by fermentation is 1.29 µg/kg bw per day (toddlers).

The Applicant discussed the conservative nature of this assessment stating that the EFSA comprehensive database contained data from 17 different countries and that conservatively the estimates from the country with the highest intakes were included in the current exposure assessment, which they claimed largely overestimates exposure

by the majority of the population groups. In addition, they stated this exposure assessment assumed that all commercial batches of steviols including those already approved would contain kaurenoic acid. They also stated that this assessment assumed all batches of steviols contained the 300 mg/kg upper limit for kaurenoic acid which they considered unrealistic. They stated that the realistic exposure to kaurenoic acid from consumption of Reb M from *S. cerevisiae* could be reasonably expected to be far lower than the level within this assessment.

The Applicant discussed applying estimated absorption factors for kaurenoic acid (4.7 – 12.5%) derived from in the work of Choi *et al*. (2018). They applied these values to the exposure estimates. This reduced the worst-case exposure estimate from 1.29 µg/kg bw per day to 0.16 µg/kg bw per day from applying the 12.5% absorption factor. The Applicant continued that applying the C_{max} value instead (4.7%) a systemic exposure of 0.06 µg/kg bw per day can be derived for the 300 mg/kg limit for kaurenoic acid. The Applicant stated these values were well below the threshold for toxicological concern for a Cramer class 1 compound (30 µg/kg bw per day where there is no concern for genotoxicity) (Kroes *et al*., 2004; EFSA FAF Panel, 2019).

The AEJEG noted that Choi et al. had used pure kaurenoic acid to determine the concentration of kaurenoic acid within a plant extract, and that this plant extract had been administered to subjects, not pure kaurenoic acid. Study details are presented within the ‘Metabolic fate’ section, under the ‘Safety of Kaurenoic Acid’.

Since the dietary exposure estimates for kaurenoic acid were below the TTC threshold without the need for further refinement, the approaches suggested by the applicant were not reviewed by the AEJEG.

The COT reviewed the Applicant’s use of the C_{max} value to calculate systemic exposure and were unsupportive of this approach, as Members did not consider that the C_{max} value would adequately reflect the bioavailability of the compound.

The Applicant's discussion on the selection of a TTC value based on the relevant Cramer class and the AEJEG's critique on the Applicant's selection is discussed later within the 'Safety assessment of kaurenoic acid' under the subsection 'Establishing a safe human exposure level'.

The AEJEG requested further work to be performed regarding the exposure assessment of kaurenoic acid (discussed in 'Safety Assessment of Kaurenoic Acid' later in this document). This was provided by the Applicant and the AEJEG was overall satisfied with the exposure assessment for kaurenoic acid.

The COT noted that there were other possible sources of exposure to kaurenoic acid, and this uncertainty was acknowledged by the AEJEG within 'Establishing a safe human exposure level' section.

2.8 Biological and toxicological data

The Applicant stated that the safety of steviol glycosides has been thoroughly investigated by multiple regulatory and advisory scientific bodies as a food additive, and these investigations are detailed in 'existing authorisations and risk assessments'. They stated a large safety database has been generated for steviol glycosides, including extensive metabolic and pharmacokinetic investigations in humans and animals alongside a large amount of toxicity testing including genotoxicity, carcinogenicity, subchronic, chronic and reproductive and developmental studies. The Applicant provided referenced studies for these claims (Curry and Roberts, 2008; Curry *et al*., 2008; Nikiforov and Eapen, 2008; Williams and Burdock, 2009).

The Applicant stated that these studies were previously reviewed by the EFSA ANS Panel within their 2010 initial assessment. The Applicant then described EFSA's later 2015 review on the safety of Reb M extracted from *S. rebaudiana* leaves, Reb M produced by enzyme-catalysed bioconversion of purified stevia leaf extract in 2019 and

all steviol glycosides present within the leaves of *S. rebaudiana* in 2020 (EFSA ANS Panel 2015b, EFSA FAF Panel, 2019, 2020). The Applicant then summarised that EFSA considers all 60 steviol glycosides safe and that an ADI of 4 mg/kg was established and applicable to all 60 steviol glycosides present within the leaves of *S. rebaudiana* Bertoni.

The Applicant noted that as their steviol glycoside produced from fermentation (Reb M) is the chemical equivalent extracted from *S. rebaudiana* Bertoni leaves and due to the shared metabolic fate of steviol glycosides that the safety of their Reb M product can be established by applying the current safety database through a read-across approach.

The Applicant also stated that they had identified studies which had been released since EFSA's latest opinion (studies published from January 1st, 2018 – February 24th, 2021).

The Applicant reiterated the absence of any *S. cerevisiae* production strain or DNA from their product. The Applicant presented DNA analysis demonstrating a lack of DNA within their product. They stated that they had evaluated the Reb M production strain in line with the retained EFSA Guidance on the Risk Assessment of Genetically Modified Microorganisms and their Products for Food and Feed Use (EFSA GMO Panel, 2011) as a category 1 product and the more recent EFSA (2018) 'Guidance on the characterisation of microorganisms used as feed additives or as production organisms' with Qualified Presumption of Safety (QPS) status. They supported this claim with a safety assessment. This was accepted by the AEJEG.

The Applicant also discussed the presence of the impurity kaurenoic acid within their product, which is present at an approximate mean level of 150 mg /kg across 5 representative lots. The Applicant has performed a literature review and the studies were collated in the Application.

The AEJEG noted that in the various studies collated from the scientific literature by the Applicant within their literature review, some studies featured routes of administration other than oral. The AEJEG considered that alternate routes of administration may affect metabolism of steviol glycosides and kaurenoic acid. The AEJEG have considered the toxicological studies on a case-by-case basis, integrating the variations in administration routes into their considerations to increase confidence in toxicity predictions as part of their assessment. The AEJEG has assessed the studies below on the strengths and weaknesses of each exposure experimental protocol and their relevance to the proposed uses in food, as described in this Application.

2.8.1 Toxicokinetics

The Applicant stated that due to similarities between already approved steviol glycosides, and their product produced by fermentation, supported by chromatographic data, the metabolic pathway is expected to be similar. The Applicant stated as follows 'Rebaudioside M is expected to undergo complete *in vivo* hydrolysis to steviol following cleavage of the triglycoside moieties at R2 and R1 of the structure of rebaudioside M by gastrointestinal microflora. Thus, with steviol being the only compound systemically available following consumption of steviol glycosides from fermentation (Reb M), the toxicology database and associated ADI previously derived for steviol (EFSA ANS Panel, 2010) are considered relevant and applicable to Amyris' steviol glycosides from fermentation (Reb M).'

The Applicant outlined one new study in an updated literature search, by Purkayastha and Kwok (2020), which investigated the metabolic fate of 5 steviol glycoside samples using *in vitro* colonic microbiota samples. The authors concluded that "Given a common metabolite structure and a shared metabolic fate in all ages, safety data for individual steviol glycosides can be used to support safety of all steviol glycosides produced by extraction and enzymatic conversion of stevia leaf extract".

The AEJEG was satisfied with the toxicokinetic data provided by the Applicant.

2.8.2 Subchronic toxicity

The Applicant has identified five new studies regarding subchronic toxicity. These included:

The Applicant discussed a 20-day *in vivo* feed intake and digestibility study in goats by Han *et al*., (2019). There were 3 male Xiangdong goats per group and goats were dosed with either 0, 400 or 800 mg stevioside/kg forage consisting of dry rice straw. Forage was supplemented with feed concentrate at 0.5% body weight. The stevioside isolates (97% purity) were dissolved in water before being added to the forage. A replicated 3 x 3 latin square design was used for animal treatment group selection. The Applicant discussed the results as follows: 'The authors reported significant, linear increases in dry intake of forage and total diet consumed that correlated with stevioside dose, presumably through increased palatability. Concurrently, a significant quadratic decrease in volatile fatty acid and significant quadratic increase in rumen pH was measured. Animals in the control and low-dose group exhibited higher isobutyrate and isovalerate; animals in the high-dose group had lower concentrations of isobutyrate and isovalerate, both in a significant quadratic relationship. Neutral and acidic detergent fibre digestibility was also increased in a statistically significant linear and quadratic relationship with increased digestibility in control and low-dose animals, which was opposite for animals in the high-dose group. The hematologic metrics were not significantly affected by administration of stevioside. Thus, the authors concluded that consumption of food containing 400 or 800 mg stevioside/kg from supplemented forage increased the intake of dry matter and the digestibility of neutral and acidic detergent fibre in goats.'

The Applicant identified an *in vivo* study on potential effects of steviol glycosides on the microbiota of male Sprague-Dawley rats, by Nettleton *et al*., (2019) where groups of 8 rats were treated for 9 weeks with (Group 1) Control (water); (Group 2) Rebaudioside A, 2 to 3 mg/kg body weight/day via drinking water; (Group 3) Oligofructose-enriched inulin

(prebiotic), 10% wt/v in the diet; and (Group 4) Rebaudioside A + prebiotic. Regarding the results the Applicant stated ‘Animals in Group 2 consuming rebaudioside A did not exhibit any significant effects on body weight, fat and lean mass, total weight, percent body fat, liver weight, and cecum weight in response as compared to those in Group 1 (control). However, the ratio of liver weight to body weight was significantly decreased (p=0.01). Intake of food and fluids were documented for 5 consecutive days every second week, and no significant differences were observed between Group 1 and Group 2. In the insulin tolerance test, the blood glucose area under the curve (AUC) for the oral glucose tolerance test (OGTT) was unchanged between groups. Blood glucose was significantly elevated 30 minutes into the OGTT (p=0.017) but returned to control levels for the rest of the test. The bar plot of microbiota abundance at the family level was similar between groups 1 and Group 2, and between Group 3 and Group 4. The relative abundance of Clostridiales family XIII and Ruminococcaceae UCG 005 were decreased in Group 2 compared to the control, and further decreased in Group 4. Animals in Group 2 exhibited increased relative abundance of Akkermansia muciniphila, Akkermansiaceae, Bacteroides goldsteinii, and Bacteroides thetaiotaomicro as compared to control. In the prebiotic and prebiotic + rebaudioside A groups, Akkermansia muciniphila and Akkermansiaceae were increased further. These findings are of unknown relevance to human consumption of rebaudioside A.’

A study by Sánchez-Tapia *et al*. (2019) on the effects of a range of natural and artificial sweeteners combined with a high fat diet on adipocyte function in rats was reported. Within the study, groups of 6 Wistar rats of 5 weeks of age were dosed with steviol glycoside sweetener via drinking water at 2.5% (w/v) for 4 months and fed either a regular, high fat or ad libitum diet. The Applicant stated that ‘the authors concluded that steviol glycoside intake did not impact the functionality of adipocytes in rats consuming 2.5% steviol glycosides in water..’

The Applicant identified a study by Schiano *et al*. (2019) investigating the effects of low-calorie sweeteners (administered by drinking water) on inflammatory response, behavioural changes and endothelial progenitor cells (EPCs) in mice. Regarding the

study the Applicant stated ‘Animals (male C57BL/6 mice; n=6/group) were administered one of the following for 8 weeks: sucrose (0.75 g/kg body weight/day); D-(+)-glucose (0.80 g/kg body weight/day); D-fructose (0.80 g/kg body weight/day); aspartame (3.75 mg/kg body weight/day); sodium cyclamate (7.00 mg/kg body weight/day); rebaudioside A (2.80 mg/kg body weight/day) in drinking water; or a double concentration of rebaudioside A (5.6 mg rebaudioside A/kg body weight/day). The results will focus on the rebaudioside A group compared to the control group, unless otherwise stated. Both doses of rebaudioside A decreased EPC significantly (single dose, $p<0.05$; double dose, $p<0.01$), and increased body weight in both groups ($p<0.001$). Blood biochemistry analysis revealed a significant increase in blood glucose (single and double dose, $p<0.05$), decrease in total cholesterol (single dose, $p<0.05$), decrease in high-density lipoprotein cholesterol (HDL-C) (single dose, $p<0.05$; double dose, $p<0.01$), increase in low-density lipoprotein cholesterol (double dose, $p<0.01$) and decrease in alanine aminotransferase (ALT) (single dose, $p<0.05$; double dose, $p<0.01$). Blood triglycerides, aspartate aminotransferase, total bilirubin, blood urea nitrogen, and alkaline phosphatase (ALP) were unchanged throughout the course of the experiment. The rebaudioside A-treated animals exhibited significantly increased volume of carrageenan-induced paw oedema ($p<0.001$) and significantly decreased the ipsilateral paw withdrawal threshold following carrageenan injection ($p<0.001$). The consumption of rebaudioside A over 8 weeks did not significantly affect or induce depressive-like behaviours, spatial memory, or obsessive-compulsive behaviour. Schiano *et al*. (2019) concluded that “administration of both natural and artificial sweeteners affects EPC number according to the presence or absence of inflammation but does not induce adverse effects on behavioural changes”.

The Applicant also identified a week-long study by Mbambo *et al*. (2020) investigating the effects of non-nutritive sweeteners (NNS) on diabetes-related parameters on rats. The Applicant stated the group size and sex of the animals was not reported by the authors. Sweeteners were dissolved in drinking water at sweetness dilutions equivalent to 10% sucrose, provided *ad libitum*. Regarding the study the Applicant stated ‘Food and fluid consumption were monitored daily. Body weight changes and 3-hour “tail-tip”

fasting blood glucose were measured weekly. OGTTs were performed in the final week to measure blood glucose and following the sacrifice of the animals at the end of Week 5, blood was collected to perform serum biochemical analyses. For the purposes of this dossier, only results concerning the steviol glycoside group compared to the control are reported, unless otherwise stated. The body weight of animals in the stevia group was significantly reduced ($p<0.05$). No significant changes in non-fasting blood glucose were observed following the administration of the non-nutritive sweeteners, including stevia. The results of the OGTT revealed significantly increases serum insulin levels ($p<0.05$) in the stevia group. All non-nutritive sweeteners, except aspartame, significantly decreased serum triglycerides ($p<0.05$) when compared to control group. No change in serum total cholesterol was seen in the stevia group, whereas serum HDL-C significantly increased in the stevia group ($p<0.05$). The administration of the non-nutritive sweeteners, including stevia, did not significantly affect the remaining blood chemical parameters (i.e., serum aspartate transaminase, ALT, ALP, urea, uric acid, creatinine, lactate dehydrogenase, and albumin). The authors of the study concluded that “stevia-based commercially available NNS showed the most favourable effects on some diabetes related parameters in non-diabetic rats, and thus may be useful in glycaemic and body weight management”.

The Applicant provided further details for these studies, of which the AEJEG was satisfied.

2.8.3 Genotoxicity

The Applicant presented two new genotoxicity studies.

The first study, conducted by Pasqualli *et al*. (2020), investigated venous-blood derived lymphocyte cultures obtained from a healthy human donor. A dose-range finding study was conducted dosing cells to a concentration up to 1 mg/mL to determine the LC_{50} of 178.8µg/mL, then an alkaline comet assay, chromosomal aberration test and mitotic index determination after both 24 and 48 hours at concentrations 1, 10 and 50 µg

steviol/mL were performed. The major lymphocyte populations identified were $CD3^+$, $CD4^+$ and $CD8^+$. The Applicant stated that cell viability was maintained at 50 µg/mL however cell proliferation was reduced at this concentration. The CD4+ population was reported as significantly smaller than control when treated at 10 and 50 µg/mL steviol and there was a statistically significant decrease of the double lymphocyte population following exposure to all steviol concentrations compared to controls. The CD3+ population was unchanged when lymphocytes were treated with all concentrations of steviol. A significant increase in the DNA damage index was noted at 10 and 50 µg/mL groups in the alkaline comet assay compared to controls. In the chromosomal aberration test 300 metaphase cells were counted and aberrations were evident in the 10 and 50 µg/mL groups. The Applicant claimed that whilst the mitotic index values of cells were unchanged, treatment conditions used in this study were 129 times greater than normal steviol exposures in the human diet and therefore the results have limited relevance to human consumption.

The AEJEG queried the Applicant's choice of wording that mitotic index values for cells were unchanged. The Applicant responded stating that 'the mitotic index reported across each of the test concentrations does appear to indeed 'remain unchanged' following exposure to steviol (CAS No. 471-80-7), as the perceived reduction in mitotic index was statistically insignificant as test concentrations were increased.' The Applicant then discussed the work of Pasqualli et al., (2020) as follows: 'the presentation of these data in Table 1 of the publication is ambiguous and the authors of this publication do not discuss the observed mitotic index results. Moreover, the authors did not consider that steviol is a product of the microbial hydrolysis of steviol glycosides and that it is not intended for direct consumption. It is unclear how the concentrations of steviol evaluated in this study can be related to dietary exposure from consumption of rebaudioside ingredients. In addition, the conclusions drawn by Pasqualli et al. (2020) are not consistent with prior studies (Uçar et al., 2018, Silva et al., 2018), nor are they consistent with the most recent Scientific Opinion reported by the European Food Safety Authority (EFSA) Panel on Food Additives and Flavourings (2023), which states that the results published by Pasqualli et al. (2020) "… were considered as not reliable

and of low relevance due to inconsistent reporting and methodological shortcomings. Therefore, they were not further considered in the genotoxicity assessment of the food additive." An extensive safety database exists for steviol glycoside ingredients, which includes two product-specific studies of genotoxicity conducted with Amyris' steviol glycosides from fermentation (Reb M), which support the conclusion that this ingredient is non-mutagenic, non-clastogenic, and non-aneugenic. Additionally, as described in Section 2.2.8 of the confidential technical dossier, the intended conditions of use for Amyris' ingredient are fully substitutional for other steviol glycoside preparations that are currently available in the UK marketplace, at levels that are not to exceed currently permitted levels. As such, the intended conditions of use for Amyris' steviol glycoside from fermentation (Reb M) are not expected to increase dietary exposure to steviol glycosides, or the metabolite steviol, to levels greater than those which have already been established as safe in the UK and the EU.'

The AEJEG reviewed the response by the Applicant and noted the Applicant's comments on the ambiguity of the data presented within table 1 of the study and the methodology of the study, as well as EFSA's conclusions on this paper. On review, the AEJEG considered that the study was unreliable and not relevant to this assessment.

The second study, by Yilmaz *et al*. (2020) investigated the impact of steviol glycosides (rebaudioside A, 98.65% purity) on oxidative and genotoxic measures in BALB/c mice. The duration of the study was 28 days. There were 4 mice per sex per group, with doses of 0, 470, 620, 940 and 1,880 mg/kg bw/day administered in a distilled water vehicle by gavage. Mice were inspected for clinical observations, food consumption and body weights weekly with blood and bone marrow obtained at the end of the study for haematological, biochemistry and mitotic index measurements with chromosome aberration analysis. Colchicine was administered intraperitoneally (5 mg/kg bw) 2 hours prior to termination to arrest mitosis. All but the lowest dose group at 470 mg/kg exhibited a significant ($p<0.05$) increase in chromosomal aberrations, most commonly sister union and polyploidy. The Applicant stated 'The authors tabulated results indicating an increase in mitotic index in all treatment groups, despite description of

insignificant differences in mitotic index between control and treatment groups in the text of the article; rendering the authors' overall interpretation of the data unclear. Cholesterol, of both high- and low-density varieties, were unchanged in all treatment groups as compared to control. Observations of tabulated data were used to determine no change in antioxidant status, oxidant status, or oxidative stress index in all study groups; 1 statistically significant decrease was reported in the 620 mg/kg group for paraoxonase-1 enzyme ($p<0.01$); however, the relevance of these data are limited as the doses of rebaudioside A administered were in excess of the human ADI of 4 mg/kg.'

The COT noted that paraoxonase-1 enzyme was also decreased significantly at 1880 mg/kg within the above study. However, in their review, EFSA (2023) concluded that the study was unreliable and of low relevance.

The AEJEG were satisfied with the genotoxicity data provided by the Applicant.

2.8.3.1 Studies with steviol glycosides from fermentation

The Applicant provided information on studies conducted with the Applicant's steviol glycosides derived from fermentation with a measured quantity of kaurenoic acid (approximately 422.13 mg/kg) and an isolated high concentration sample of kaurenoic acid in order to confirm a lack of genotoxicity for this contaminant.

The Applicant performed a bacterial reverse mutation assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* tester strain WP2 uvrA. The Applicant stated that the study was performed in line with OECD Test Guideline TG471 (OECD, 2020) and following the OECD Principles of Good Laboratory Practice (GLP) (ENV/MC/CHEM(98)17) (OECD, 1998).

The assay was performed using both Ames plate incorporation and pre-incubation methods at up to 8 dose levels in triplicate both in the presence and absence of phenobarbitone/β-naphthoflavone-induced rat liver S9. The initial toxicity mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay

and provide a preliminary mutagenicity evaluation. The Applicant then performed a confirmatory mutagenicity assay to evaluate and confirm the mutagenic potential of the test article.

The dose range for the initial experiment (plate incorporation) ranged between 1.5 – 5000 µg/plate. The experiment was performed on a separate day (pre-incubation method) using fresh cultures of the bacterial strains and fresh test item formulations. The Applicant amended the doses following experiment 1 to 15 – 5000 µg/plate. Six test concentrations were then selected for experiment 2 to ensure at least 4 non-toxic doses were achieved in line with OECD TG471 guidelines. These doses were informed by the lack of cytotoxicity in the results of experiment 1 and potential for a change in cytotoxicity following the change from the plate incorporation to pre-incubation method. DMSO was utilised as the vehicle and the control. The Applicant reported that revertant colony counts within the control plates were within normal ranges. They noted all positive controls induced marked increases in revertant colony frequency validating the sensitivity and efficacy of the S9 mix.

The Applicant reported no test item precipitate in the presence or absence of metabolic activation in both experiments 1 and 2. The Applicant reported no increases of revertant colonies at any dose level in experiments 1 and 2 in both the presence or absence of metabolic activation.

Regarding the *in vitro* bacterial reverse mutation assay the Applicant concluded: ‘In summary, in this bacterial reverse mutation assay “Ames test” (plate incorporation method and pre incubation method) using *S. typhimurium* strains TA1535, TA1537, TA98, and TA100 and *E. coli* strain WP2uvrA (OECD TG 471) at the maximum recommended dose level of 5,000 µg/plate the test item did not induce an increase in the frequency of revertant colonies that met the criteria for a positive result, either with or without metabolic activation (S9-mix). Therefore, under the conditions of this test, steviol glycosides from fermentation (Reb M) was considered to be non-mutagenic’.

The Applicant also performed an *in vitro* mammalian cell micronucleus test using human peripheral blood lymphocytes in both the absence and presence of an phenobarbitone/β-naphthoflavone-induced liver S9 activation system. The Applicant stated this study was performed according to OECD TG 487 following the OECD principles of GLP (ENV/MC/CHEM (98)17) (OECD, 1998, 2016). A preliminary toxicity test was performed and from these results a dose range of 62.5 – 2000 µg/mL was selected for the micronucleus assay, with DMSO as the vehicle.

The Applicant reported that all DMSO vehicle controls featured frequencies of cells with micronuclei within normal ranges for normal human lymphocytes and considered acceptable for addition to the laboratory's historical negative control data. Positive control items induced statistically significant increases in the frequency of cells with micronuclei in line with historical positive controls. The Applicant stated this validated the sensitivity of the assay and the efficacy of the S-9-mix.

The Applicant reported the test item was non-toxic and did not induce any statistically significant increases in the frequency of binucleate cells with micronuclei at any of the 3 exposure levels including the maximum recommended dose of 2000 µg/mL. They also stated there were no significant concentration related increases when the results were evaluated with a trend test. The Applicant stated these results fulfilled the criteria of a clearly negative outcome.

Regarding the *in vitro* micronucleus test the Applicant concluded 'In summary, based on the findings of this *in vitro* mammalian cell micronucleus test performed at the maximum recommended dose level of 2,000 µg/mL, the test item was considered to be non-clastogenic and non-aneugenic to human peripheral blood lymphocytes *in vitro*'.

The Applicant concluded from these two studies that the Applicant's Reb M does not exhibit mutagenic, aneugenic or clastogenic potential. The presence of kaurenoic acid within the samples corroborate later results of testing undertaken solely for kaurenoic acid.

The AEJEG were satisfied with the studies produced by the Applicant regarding steviol glycosides produced by the Applicant via fermentation.

2.8.4 Chronic toxicity and carcinogenicity

The Applicant could not find any updated studies investigating chronic toxicity and carcinogenicity since EFSA's 2020 evaluation.

The AEJEG were satisfied with the search undertaken by the Applicant regarding chronic toxicity and carcinogenicity.

2.8.5 Reproductive and developmental toxicity

The Applicant found two new studies regarding reproductive and developmental toxicity for steviol glycosides.

This included a study using a diabetic rat model induced by a single intraperitoneal injection of streptozotocin (60 mg/kg) and nicotinamide (120 mg/kg) to evaluate the serum hormone levels, key steroidogenesis enzymes, and testicular damage in male Wistar rats following consumption of aqueous stevia extract (Gholizadeh *et al*., 2019).This study featured 12 animals treated with either 1 mL water (diabetic control), 400 mg/kg bw/day aqueous stevia extract or 500 mg/kg bw/day metformin treatment administered orally for 2 weeks. 12 nondiabetic control animals receiving 1 mL of water were also used. The Applicant discussed the study as follows 'Upon study termination, body weight was measured and testicles were collected along with blood samples, which were then analysed for a number of haematological and histochemical metrics. A significant decrease in fasting glucose ($p<0.05$) levels of stevia-treated animals compared to diabetic controls was observed. Serum luteinising hormone and testosterone were significantly decreased in the diabetic rats as compared to diabetic control ($p=0.026$ and $p<0.001$, respectively). Treatment with stevia returned serum

luteinising hormone levels to that of non-diabetic controls, but also significantly decreased serum testosterone (p=0.004) in the same regard. Testis weight and volume were decreased in diabetic rats as compared to non-diabetic control (p=0.016 and p=0.014, respectively); attenuation in both groups was achieved by stevia administration. Seminiferous tubules and germinal epithelium volume, in addition to the number of spermatogonia, spermatocytes, round spermatids, long spermatids, Sertoli cells, and Leydig cells were statistically decreased in diabetic rats ($p \leq 0.016$ in all cases); these were attenuated by administration of stevia. Round spermatids and Sertoli cells were significantly decreased compared to the non-diabetic control (p=0.012 and p=0.001, respectively), results were exacerbated in diabetic animals. Similarly, stevia treatment ameliorated the increase in non-progressive sperms ($p<0.05$). A significant reduction in sperm count and significant increases in sperms with abnormal morphology and percentage of non-viable sperms were observed in diabetic animals ($p<0.05$) as compared to control. Thus, the authors concluded that oral stevia administration in a diabetic rat model had "*positive effects on testicular steroidogenesis, spermatogenesis, and function*".'

The Applicant also found a study by Li *et al*. (2020) on the effects of non-nutritive sweeteners on taste receptor type 1 subunit 3 (T1R3) and taste receptor type 1 subunit 2 (T1R2) expression in the uterine and ovarian tissues of guinea pigs. 30 female Harley-white guinea pigs were split into 5 groups of 6 and were administered either water (control), 1.5mM or 7.5mM saccharin solution or 0.5mM (approximately 40 mg/kg bw/day) or 2.5 mM (approximately 174 mg/kg bw/day) rebaudioside A solution *ad libitum* for 28 days. Regarding the study the Applicant stated: 'Daily food consumption measurements and weekly body weight measurements were taken throughout the study; puberty onset was recorded as the day of vaginal opening, and daily vaginal smears were used to track the oestrous cycle. The following description of post-mortem analyses of treated animals will be referring to the rebaudioside A-treated animals as compared to control unless otherwise stated. Food consumption was significantly elevated in Week 1 of the study ($p<0.05$) but returned to control levels by Week 2. A significant decrease in water intake was observed in the high-dose rebaudioside A

group from Weeks 2 to 4 ($p<0.05$). At Week 2, the average body weight of both rebaudioside A groups was significantly increased ($p<0.05$) but was no different from control at any other time point. Water intake was decreased in high-dose rebaudioside A animals throughout Weeks 1 and 2, accompanied by rebaudioside A-related weight gain, as compared to control during Week 2 through Week 4 ($p<0.05$). Observed ovary weight, oestradiol levels, and day of puberty onset were not significantly affected by rebaudioside A administration throughout this study. Low-dose rebaudioside A animals exhibited increased serum progesterone and uterine T1R2 expression, as compared to control ($p<0.05$); however, no significant corresponding changes were observed in the expression of T1R3 in the ovary and uterus. Ovary follicle distribution in rebaudioside A-treated animals were regular and atretic follicles were qualitatively increased; however, the number of antral follicles was not statistically increased as compared to control. The number of corpus luteum in the ovaries of animals receiving high-dose rebaudioside A were significantly increased ($p<0.05$). No histological or morphological changes in uterine tissue were observed. Staining of T1R2 and T1R3 in ovarian follicles revealed no significant changes, although, corpus luteum T1R3 staining was increased in the lutein cells in animals treated with all doses of rebaudioside A. Uterine expression of T1R2 in high-dose animals was significantly increased, particularly in the epithelial and stromal cells. The authors reported increases in the uterine expression of T1R2 and an increased number of corpus luteum in the ovaries of animals treated with the high-dose rebaudioside A (approximately 174 mg steviol/kg body weight/day). Given that the doses of rebaudioside A administered in this study were in significant excess of the human ADI for steviol glycosides, the relevance of this study to human consumption is limited.'..

The AEJEG were satisfied with the studies provided by the Applicant regarding reproductive and developmental toxicity.

2.8.6 Human studies

The Applicant presented five studies conducted since EFSA's 2020 evaluation. These are discussed below.

A double-blind randomised control trial of sweetener consumption in children (male and female aged 6 – 9 years) by Cocco *et al*. (2019) to assess sweetener consumption against various indicators of dental health. Children were provided with snacks sweetened with the test items. The Applicant concluded form this study that 'Authors reported no adverse effects in response to treatment with stevia and concluded that substitution of stevia sweetened snacks for sugar sweetened snacks for 42 days would "positively modify some important caries-related variables, i.e., mutans streptococci, lactobacilli and plaque pH, reducing the probability of developing new caries in the future" in children at risk for dental caries (Cocco *et al*., 2019).'

A 3-arm single-blinded randomised crossover trial investigated the relationship between energy intake and postprandial glucose response satiety by Farhat *et al*. (2019). The Applicant discussed the study as follows: 'Adult human subjects [10 males, 20 females; mean age 26.1 ± 10.56 years; body mass index (BMI) 23.44 ± 3.42 kg/m^2] fasted 8 hours prior to study start, where a breakfast meal was provided. 3 hours later, subjects received a 300 mL preload of water with: citric acid (trace); sugar (60 g); or stevia (1 g; purity not reported). 30 minutes post-preload, lunch was provided to the study participants. A different preload was provided to each participant on 3 different days, 4 to 5 days apart. Blood glucose was measured before both the preload and lunch, and at 30-minute intervals until 120 minutes after lunch. AUC for glucose was significantly increased by intervention with sugar as compared to water or stevia ($p=0.001$, $p=0.007$; respectively); water and stevia were not significantly different from each other. Following sugar preload, postprandial glucose levels were significantly increased ($p\leq0.05$) although further blood glucose adjustment resulted in no significant difference. The

postprandial glucose levels following stevia consumption were lower than those measured after sugar consumption. The authors concluded that stevia consumption did not cause subjects to seek to energy compensation, nor did it alter energy intake; moreover, it was observed to lower subjective feelings of hunger as compared to water.'

The Applicant identified a study by Higgins and Mattes (2019). This was an investigation of rebaudioside A among other sweeteners and their effects on induced changes in bodyweight, food and fluid intake behaviours and glucose tolerance in overweight or obese adults. The Applicant stated the authors 'employed a parallel-arm study design in which participants were randomly assigned to 1 of 5 study groups who received: sucrose, rebaudioside A, saccharin, aspartame, or sucralose. Focusing only on the sucrose (control, n=39, mean age 28.2 ± 9.5 years; BMI 30.4 ± 4.1 kg/m2) and rebaudioside A (n=28, mean age 27.1 ± 9.6 years; BMI 29.9 ± 3.8 kg/m2) groups, subjects were instructed to consume between 1.25 to 1.75 L/day of a coloured fruit-flavoured beverage for 12 weeks that was sweetened with either sucrose or rebaudioside A. The steviol glycoside beverage was sweetened with 0.66 g rebaudioside A to match the sweetness of an 8% w/v sucrose beverage. Intake of rebaudioside A was approximately 7.8 mg rebaudioside A/kg body weight (2.6 mg/kg steviol equivalents)/day. Participants were monitored for changes in body weight, body composition, dietary intake, energy expenditure, appetite, and glycemia throughout the test period. Secondary outcomes observed were serum lipids, sweetness perception, and an overall enjoyment score of the beverage. Compliance was monitored via the urinary biomarker para-aminobenzoic acid. The body weight, total fat mass, total fat-free mass, and android and gynoid fat mass remained unchanged in rebaudioside A-treated subjects over the 12-week administration period, whereas all parameters increased significantly in sucrose-treated subjects ($p=0.001$ for fat-free mass; $p<0.001$ for others). Total body water was not significantly different between groups. No differences in OGTT responses, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), glycated haemoglobin (HbA1c), and serum triacylglycerol were reported between groups after 12 weeks of beverage consumption. The authors therefore

concluded that daily rebaudioside A consumption for 12 weeks at approximately 2.6 mg steviol equivalents/kg body weight/day raises no safety concerns.'

The Applicant found a study conducted by Ajami *et al*. (2020) on the effects of stevia and sucralose-sweetened teas on the glycaemic and lipid profile of type 2 diabetic subjects. The applicant stated 'Subjects (male & female; BMI 18.5 to 29.9 kg/m2) were assigned to groups that were administered 1 cup of black tea, sweetened with either 2% stevia extract (n=15; mg/kg dose and purity not reported) or 2% sucralose (n=19) daily for 8 weeks. Blood was drawn at study initiation and in Weeks 4 and 8 for complete blood cell counts, glycaemic and lipid profiles, fasting blood glucose, and post-prandial glucose levels, and serum HbA1c. No significant differences between baseline and all other treatment timepoints were observed in both groups. Thus, the authors concluded that 2% stevia extract in tea daily for 8 weeks caused no significant adverse effects or changes in glycaemic response in type 2 diabetic subjects'.

The Applicant identified a randomised, controlled, open-label, 2-parallel arm study by Stamataki *et al*. (2020) on the relationship between glucose homeostasis and consumption of stevia. The Applicant reported 'Participants (n=28, 25 ± 5 years) were administered 2 daily doses of stevia (5 drops of Sweet Leaf Stevia) or 1 teaspoon table sugar, which are of equal sweetness, for 12 weeks. Subjects fasted at Week 0 and Week 12 for measurements of glucose homeostasis markers. No significant changes were observed between the baseline and Week 12 measurements of body weight, BMI, waist circumference, oral glucose tolerance test results, and insulin response. The authors concluded that their "data provide evidence that the daily consumption of stevia in real-life doses does not affect glycaemia in healthy normal-weight individuals but could aid in weight maintenance and the moderation of energy intake".'

The AEJEG were satisfied with the additional data on human studies collected by the Applicant.

2.8.7 Immunotoxicity, hypersensitivity/ allergy and food intolerance

Three studies were identified by the Applicant.

The first study by Sánchez-Delgado *et al*. (2021) consisted of an evaluation of the effects of non-calorie sweeteners on nutrient and calorie intake, adipose mass, triglycerides, and serum proinflammatory cytokines in humans during a 7-week study split into 2 phases. The Applicant reported 'In Phase I, a food frequency questionnaire was completed, and anthropometric and body composition measurements (weight, BMI, total fat percentage, muscle mass, and waist circumference) were made at study initiation. A 1-week washout period was implemented to restrict food and drinks with added sugar and non-calorific sweeteners prior to administration initiation. In Phase II, blood samples were drawn from fasted participants to measure biochemical and immunological parameters [blood glucose, triglycerides, cholesterol, interleukin (IL)-1β, IL-6, IL-10, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ] before and after the 6-week administration period. Subjects were randomly assigned 1 of 3 administration groups: Group 1 (n=12, eight 5-g packs sucrose/day); Group 2 (n=13, four 1-g packs sucralose/day, each pack containing 0.012 g sucralose); and Group 3 (n=13, four 1-g packs steviol glycoside/day, each packet containing 0.025 g steviol glycosides). The composition of steviol glycosides were not specified. The assigned sweetener was added to drinks or food every day, and subjects were asked to restrict the use of added sugar or sweeteners in the rest of their diet during the administration phase. Intakes were monitored using 24-hour diet recalls and anthropometric and body composition parameters were measured weekly. Mean energy intake in the steviol glycoside group was reduced compared to baseline. Nutrient distribution showed a significant decrease in carbohydrate intake (p=0.002) and an increase in protein intake (p=0.0001) in the steviol glycoside group. No changes were observed in lipid intake, body weight, BMI, or muscle mass in the steviol glycoside group; however, body fat was significantly decreased (p=0.0287). Immunological parameters in the steviol glycoside group from baseline to Week 7 of study showed a significant decrease in TNF-α concentrations (p= 0.0029) and no significant change in IL-6 concentrations. Concentrations of IFN-γ and

IL-10 were below limit of detection. The Applicant noted the following observation from the study conclusion: “The data reported in the present study corroborates previously reported anti-inflammatory effects of steviol glycosides and support the notion that these compounds may have beneficial effects for human health…”.’

The second study by Rosales-Gómez *et al*. (2018) investigated effects of sweetener consumption on glycaemia, cytokines, hormones, and lymphocytes of gut associated lymphoid tissue (GALT) in CD-1 mice. The Applicant discussed the study as follows ‘CD1 mice pups were divided into 3 treatment groups: Group A (n=8, no treatment, 3 weeks old); Group B (n=32, 6 weeks treatment, 9 weeks old at end of treatment period); and Group C (n=32, 12 weeks treatment, 15 weeks old at end of treatment period). Groups B and C were each divided further into subgroups (n=8/group): Control (water), sucrose (41.66 mg/mL), sucralose (4.16 mg/mL), and stevia (4.16 mg/mL). Sweetener solution was available for 5 hours daily and water was provided for the remaining 19 hours/day. Body weight, food consumption, and blood glucose measurements (via lancet) were taken from 3 to 15 weeks of age. Blood samples at study termination were used to measure GIP [gastric inhibitory peptide], insulin, and leptin. Samples of small intestine tissue were prepared for: lamina propria and Peyer’s patches lymphocytes observation ($CD3^+$ T cells and $CD19^+$ B cells); IgA^+ plasma cells; and the cytokines (IL-4, IL-5, IFN-γ, and TNF-α) by flow cytometry. Unless otherwise stated, results presented refer to the Stevia subgroups as compared to controls. Body weight, blood glycaemia, GIP, or leptin levels were unchanged in the 9th and 15th weeks; however, food consumption was significantly decreased ($p=0.042$ and $p=0.017$, respectively), insulin secretion was significantly increased at both time points ($p<0.05$) , and the homeostatic model assessment (HOMA) index increased significantly at both weeks ($p<0.05$) according to the tabulated data, but only at Week 9 as explained in the article text. $CD3^+$ T cells, $CD19^+$ B cells, and IgA^+ plasma cell populations were significantly increased in the lymphocyte population of the Peyer’s patches at Weeks 9 and 15, but statistics compared to the control were not reported. Significant changes, relative to control, were reported in the lamina propria as follows: increased percentage of $CD3^+$ T cells at Week 9; increased percentage of $CD19^+$ B cells at Week 15; decreased percentage of IgA^+

plasma cells at Week 9 and an increase at Week 15. Significant changes, relative to control, were reported in the Peyer's patches as follows: significantly decreased IFN-γ producing T cell percentage at Week 9 (p=0.001), TNF-α producing cell percentage at Week 9 (p=0.001), IL-4 producing cell percentage at Weeks 9 and 15 (both p=0.001), IL-5 producing cell percentage at Week 15 (p=0.001), IL-10 producing cell percentage at Week 9 (p=0.001); and significantly increased TNF-α producing cell percentage at Week 15 (p=0.001),), IL-10 producing cell percentage at Week 9 (p=0.001), and IL-5 producing cell percentage at Week 9 (p=0.001). In the lamina propria, the percentage of $CD4^+$ cells secreting IFN-γ, IL-4, IL-5, and IL-10 was decreased at Weeks 9 and 15 and TNF-α was increased at both weeks but statistical significance compared to the control group was not reported. The authors concluded that: "Stevia consumption stimulates humoral immunity in Peyer's patches by increasing the percentage of B cells and IgA, with an increase in anti-inflammatory cytokines IL-4 and IL-10, although in lamina propria it triggers an inflammatory response due to increased TNF-α secretion". However, the relevance of these data with respect to human exposure levels has not yet been determined.'

The third study by 'Martínez-Carrillo *et al*. (2019) investigated the effects of sweeteners on microbiome and immunity in the mouse small intestine. Test items were administered through drinking water. The Applicant reported that: "Study animals (n=72) were divided into 3 groups: Control; Group A (6 weeks treatment); and Group B (12 weeks of treatment). Groups A and B were further divided into 4 subgroups (n=8/group): control, sucrose, Splenda [A commercial sweetener, containing 1g of carbohydrates including dextrin, maltodextrin and sucralose], and Stevia [A commercial sweetener] (sucrose, isomalt, 2.5 g steviol glycoside/100 g and 0.6 g sucralose/100 g). Solutions for sweetener administration were prepared in ultrapure water and 41.66 mg/mL sucrose or 4.1 mg/mL of Splenda or Stevia; exposures were not reported. Anthropometric measurements, the percentage of lymphocytes, IL-6, and IL-17A producing cells in Peyer's patches and lamina propria, and concentration of leptin, resistin, C-peptide, and serum TNF-α were taken at treatment cessation. Small intestine microbiota was investigated in sampled faeces. The following results are concerning Stevia groups

related to control. Body weight and BMI remained unchanged. At Weeks 6 and 12, water consumption with Stevia was increased. In Peyer's patches and lamina propria, the percentage of $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes and IL-6 and IL-17A producing cells were determined using flow cytometry and the following statistically significant changes were reported compared to the respective control:

- Increased percentage of $CD8^+$ lymphocytes in Peyer's patches after 12 weeks (p=0.001);
- Increased percentage of $CD4^+$ lymphocytes in the lamina propria after 12 weeks (p<0.001);
- Decreased percentage of $CD8^+$ lymphocytes in the lamina propria after 12 weeks (p,0.001);
- Decreased IL-6 producing cells in Peyer's patches at 6 weeks and increased at 12 weeks (p=0.001); increased IL-17A producing cells at 6 and 12 weeks (p=0.001) in Peyer's patches; and
- Increased IL-6 producing cells (p=0.001) and IL-17A producing cells (p=0.001) in lamina propria at both timepoints.

Resistin and C-peptide were increased significantly (p<0.001) after 6 weeks; however, this change was not significant after Week 12. Fourteen genera and 36 different species were identified in faecal samples from all groups during microbiome analyses; *Streptococcus saliviloxodontae* and the genus *Bacillus* (*B. aerius, B. circulans, B. licheniformis, and B. safensis*) were identified in the Stevia group at 6 weeks, and *Bacillus safensis, Oceanobacillus sojae*, and *Staphylococcus lugdunensis* were identified at 12 weeks. Martínez-Carrillo *et al*. (2019) concluded that: "The consumption of sweeteners increases the percentage of $CD3^+$ $CD8^+$ lymphocytes in Peyer's patches and $CD3^+$ $CD4^+$ in the lamina propria, in addition to modifying the composition of the intestinal microbiota… . However, the relevance of these data with respect to human exposure levels to steviol glycosides has not yet been determined."'

The AEJEG were satisfied by the additional studies provided by the Applicant regarding immunotoxicity, hypersensitivity, allergy and food intolerance.

2.8.8 Safety assessment of kaurenoic acid

2.8.8.1 Metabolic fate

The Applicant provided three studies from the literature discussing the metabolic fate and pharmacokinetics of kaurenoic acid within rats and humans.

The initial study was performed by Jiang *et al*. (2019). The authors developed and validated a high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method to allow quantification of kaurenoic acid in rat plasma. They then determined its application to a pharmacokinetic study. The Applicant discussed the study as follows: 'The lower limit of quantification was 5 ng/mL and the relative standard deviation for intra- and inter-day precision ranged from 3.0 to 11.4%. This validated UPLC-MS/MS method was applied to the pharmacokinetic analysis of kaurenoic acid after oral administration of 10, 20, and 40 mg/kg in male Sprague-Dawley rats (250 g ± 15 g). For the low dose, C_{max} (peak concentration) was 12.6 ± 4.0 µg/L, T_{max} (time of peak concentration) was 0.70 ± 0.30 hours and $t_{1/2z}$ (terminal elimination half-life) was 16.6 ± 9.8 hours [low dose area under the curve (AUC_{0-t}) of 95.7±45.7 µg/L*h]. For the medium dose, C_{max} was 44.7 ± 24.6 µg/L, T_{max} was 0.68 ± 0.28 hours, and $t_{1/2z}$ was 10.7 ± 5.2 hours [medium dose AUC_{0-t} of 282.8 ± 105.4 µg/L*h]. For the high dose, C_{max} was 48.0 ± 20.2 µg/L, T_{max} was 0.68 ± 0.23 hours, and $t_{1/2z}$ was 12.6 ± 12.5 hours [high dose AUC_{0-t} of 364.1 ± 200.4 µg/L*h]. AUC and C_{max} had a dose-dependent increase from 10 mg/kg to 20 mg/kg, but non-linear pharmacokinetics from 20 mg/kg to 40 mg/kg. The AUC and C_{max} values of the high-dose were 1.29 and 1.07 times higher than that of the medium-dose group, which was speculated to result from the saturation of the enzyme [assumed to be P450(s)]. The dose amount (i.e., 10 to 40 mg/kg) did not affect the elimination process of kaurenoic acid *in vivo*. The high apparent volume of distribution ($V_{z/F}$) value (1,667.9 ± 1,254.4, 1,539.4 ± 422.2, and 1,356.3 ± 1,138.1 L/kg for the low-, medium, and high-dose, respectively) indicated that kaurenoic acid was widely distributed in body tissues and fluids, which may be related to the liposolubility of this

molecule. The study results indicated that kaurenoic acid was mainly distributed in the tissue and experiences a slow elimination *in vivo* after oral administration (Jiang *et al*., 2019).

The Applicant continued that 'Based on the reported mean body weight of the test animals (250 g), estimated doses of 2.5, 5, and 10 mg kaurenoic acid can be calculated for the 10, 20, and 40 mg kaurenoic acid/kg test groups, respectively. Assuming a blood volume of 16 mL for a 250-g rat (Lee and Blaufox, 1985), the reported C_{max} values for each test group correspond to approximately 0.0002, 0.0007, and 0.0008 mg kaurenoic acid (i.e., <0.01% of dose), circulating at C_{max}, and the reported $AUC_{(0-t)}$ values (95.7 ± 45.7, 282.8 ± 105.4, and 364.1 ± 200.4 µg/L*h) correspond to approximately 0.0015, 0.0045, and 0.0058 mg kaurenoic acid (i.e., 0.06, 0.09, and 0.06% of dose), respectively, during 0 to 36 hours after dose administration.'

The Applicant identified a second study by de Matos *et al*. (2018) where the pharmacokinetic profile and bioavailability of kaurenoic acid (which they also name KA in this instance) was investigated in Wistar rats, which had been submitted to jugular vein canulation. Fifty mg/kg kaurenoic acid was administered either intravenously or orally. The Applicant discussed the study as follows: 'The cannula allowed serial blood collection over 10 hours. Analytical quantification by reverse phase HPLC-UV and mobile phase composed of acetonitrile acidified water was reliable and validated between 0.75 and 100 µg/mL. Oral kaurenoic acid treatment did not provide detectable KA plasma levels; therefore, the bioavailability of kaurenoic acid was not determined. The authors suggested that poor absorption or an extensive pre-systemic elimination of kaurenoic acid via the oral route was possible (de Matos *et al*., 2018). However, as noted by Choi *et al*. (2018), this study may have had a methodological shortcoming in that the bioavailability of kaurenoic acid could not be evaluated because of insufficient lower limit of quantification (LLOQ, 5 ng/mL). Results following intravenous administration of KA were not considered relevant to the current safety assessment of kaurenoic acid and therefore are not discussed herein.'

The third study identified by the Applicant was work by Choi *et al*. 2018 where the human pharmacokinetic profile was characterised after administration of an oral dose of *Araliae Contintentalis Radix* extract powder (approximately 1.15 mg kaurenoic acid per 1g powder) to Korean study volunteers and evaluated the mechanism of its absorption. The Applicant stated 'The purchased *Araliae Continentalis Radix* extract powder used in the study was prepared from the dried root of *Aralia continentalis Kitagawa*. An analytical method was developed and validated for the detection of kaurenoic acid in human plasma, and concentrations were quantified by UPLC-MS/MS. This pharmacokinetic model of kaurenoic acid was described by a 2-compartment model with first-order absorption. A plasma C_{max} value of 18.02 µg/L was reported for kaurenoic acid following the oral dose of 1.15 mg kaurenoic acid (15.69 µg/kg for mean bodyweight of 73.3 kg for 10 subjects; delivered in *Araliae continentalis Radix* extract powder) (Choi *et al*., 2018). Assuming that the approximate amount of plasma in a human is 3 L (Hurley, 1975; Sharma and Sharma, 2018), this amount corresponds to approximately 0.054 mg kaurenoic acid (4.7% of the administered dose at T_{max}, 0.6 hours), circulating in the plasma at C_{max}. Plasma concentrations after T_{max} gradually decreased as kaurenoic acid was cleared from the circulation ($t_{1/2}$ 4.97 hours). Alternatively, using the plasma AUC value of 47.89 µg x hr/L, this amount during 0 to 12 hours after dose administration corresponds to an integrated amount of approximately 0.14 mg kaurenoic acid (12.5% of the administered dose). A Caco-2 monolayer model was used to identify efflux transporters involved in the absorption of kaurenoic acid. Kaurenoic acid was considered to be well distributed throughout the body based on model estimated pharmacokinetic parameters (i.e., 88.49 L total volume distribution compared to 3 L, the volume of human plasma). Also, it was determined that kaurenoic acid has high lipophilicity, which indicates that the hydrophobic interaction between biological membrane and kaurenoic acid can play an important role in the absorption of kaurenoic acid. It was suggested that kaurenoic acid is highly permeable through the intestinal membrane compared to permeability of propranolol. Results from the Caco-2 bidirectional transport study suggested that kaurenoic acid was a potential substrate of efflux transporters (Choi *et al*., 2018), indicating that kaurenoic acid is expected to be actively transported across the human intestinal epithelium. Considering the basic

environment of the small intestine, it is postulated that if kaurenoic acid does survive passage through the stomach it would likely then be neutralised in the small intestine to a K salt, water and heat.'

The AEJEG noted that the study authors had used pure kaurenoic acid to determine the concentration of kaurenoic acid within a plant extract, and that there may be possible interference of the pharmacokinetics of kaurenoic acid, from components within the extract. It was considered that it was not possible for the AEJEG to predict the nature of the interactions between kaurenoic acid and the other components of the plant extract, and how those potential interactions may modulate kaurenoic acid pharmacokinetics, without additional supporting evidence.

Regarding the work of Choi et al., the COT noted that the calculation of the dose of kaurenoic acid present in the body based on the C_{max} and volume of plasma assumes that all of the compound is present in the plasma. The COT stated this is unlikely to be correct and would result in an underestimation of the amount of compound in the body. The COT also commented that the use of Caco-2 cells was intended to measure active influx or efflux. No specific transporters were studied and that the final conclusion of this study was that there was equivocal efflux transport. The COT also clarified that the study authors had compared the permeability of kaurenoic acid to that of propranolol, concluding that as the values were similar and propranolol is a high permeability compound, kaurenoic acid was also a high permeability compound.

The Applicant summarised 'kaurenoic acid was detected in plasma following oral administration in 2 of the above identified studies, with higher extrapolated % of dose values calculated for humans compared to rats. Therefore, these human % of dose values were considered appropriate for use in calculating the potential systemic exposure to kaurenoic acid from the consumption of Amyris' steviol glycosides from fermentation (Reb M) (….) Kaurenoic acid had a shorter half-life in human plasma (t1/2 4.97 hours) compared to rats (t1/2 up to 16.6 ± 9.8 hours for low-dose group) demonstrating efficient clearance from the circulation.'

Regarding the metabolic fate of kaurenoic acid, overall the AEJEG were satisfied with the information provided by the Applicant.

2.8.8.2 Genotoxicity

The Applicant commissioned two studies to investigate the genotoxic profile of kaurenoic acid with an *in vitro* bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus test.

The Applicant tested kaurenoic acid in a bacterial reverse mutation assay using *S. typhimurium* tester strains TA98, TA100, TA1535, and TA1537 and *E. coli* tester strain WP2 uvrA. The Applicant stated that the assay was performed according to OECD TG 471 (OECD, 2020) and followed the OECD Principles of GLP (ENV/MC/CHEM(98)17) (OECD, 1998). The assay was performed using the Ames plate incorporation method in experiment 1 and the pre-incubation method in experiment 2 at up to 8 dose levels, in triplicate both in the presence and absence of phenobarbitone / / β-Naphthoflavone-induced rat liver S9 mix.

The Applicant stated that the dose range for experiment 1 was based on OECD TG 471 ranging from 1.5 – 5000 µg/plate. The experiment was then repeated on a second day (experiment 2) using fresh bacterial strains and fresh test item formulations. Following experiment 1 the dose range was amended to 15 – 5000 µg/plate. Six test concentrations were then selected for experiment 2 to ensure at least 4 non-toxic doses were achieved in line with OECD TG471 guidelines. These doses were informed by the lack of cytotoxicity in the results of experiment 1 and potential for a change in cytotoxicity following the change from the plate incorporation to pre-incubation method. DMSO was utilised as the vehicle and the control. The Applicant reported that revertant colony counts within the control plates were within normal ranges. They noted all positive controls induced marked increases in revertant colony frequency validating the sensitivity and efficacy of the S9 mix.

The Applicant reported that there were no significant increases in the frequency of revertant colonies for any bacterial strain with any dose of test item either in the presence or absence of metabolic activation in experiment 1. The Applicant noted there were no biologically relevant increases in revertant colony frequency in experiment 2 at any dose level both in the absence and presence of metabolic activation however there was one statistically significant increase noted for the strain TA100 at 50 µg/ plate. The Applicant argued that as this represented only a maximum of a 1.2 fold increase from the concurrent vehicle control and the mean colony count was within the in-house vehicle/ control range for the strain this finding was not of biological significance and did not meet the criteria for a positive response.

The Applicant summarised as follows: 'In summary, in this Bacterial Reverse Mutation Assay "Ames Test" (plate incorporation method and pre incubation method) using *S. typhimurium* strains TA1535, TA1537, TA98, and TA100 and *E. coli* strain WP2uvrA (OECD TG 471) at the maximum recommended dose level of 5000 µg/plate, the test item kaurenoic acid did not induce an increase in the frequency of revertant colonies that met the criteria for a positive result, either with or without metabolic activation (S9-mix). Under the conditions of this test kaurenoic acid was considered to be non-mutagenic'

The Applicant also performed an *in vitro* mammalian cell micronucleus test using human peripheral blood lymphocytes in both the absence and presence of an phenobarbitone/β-naphthoflavone-induced rat liver S9 activation system. The Applicant stated this study was performed according to OECD TG 487 following the OECD principles of GLP P (ENV/MC/CHEM(98)17) (OECD, 1998, 2016). A preliminary toxicity test was performed and due to an absence of cytotoxicity was repeated to attempt to achieve the required cytotoxicity. From these results the final dose levels in each condition for the micronucleus assay ranged from 16 to 128 µg/mL for the 24-hour exposure without S9, 32 to 96 µg/mL for the 4-hour exposure without S9, and 32 to 128 µg/mL for the 4-hour exposure with S9. DMSO was selected as the vehicle.

The Applicant stated that duplicate cultures of human peripheral blood lymphocytes treated with the test item were evaluated for micronuclei in binucleate cells at three dose levels in addition to the vehicle (negative control quadruplicate cultures) and positive controls (duplicate cultures). The Applicant stated three exposure conditions were used for the 4-hour exposure in the presence and absence of S9 metabolic activation at a final concentration of 2% and a 24-hour period in the absence of metabolic activation. After the exposure period cell cultures were washed and incubated for a further 24 hours with cytochalasin B.

The Applicant reported that all DMSO vehicle controls featured frequencies of cells with micronuclei within normal ranges for normal human lymphocytes and were considered acceptable for addition to the laboratory's historical negative control data. Positive control items induced statistically significant increases in the frequency of cells with micronuclei in line with historical positive controls. The Applicant stated this validated the sensitivity of the assay and the efficacy of the S9-mix.

The Applicant stated the test item demonstrated cytotoxicity in all three exposure groups. The 24-hour exposure group achieved optimum cytotoxicity in the initial experiment at 64 µg/ml with 54% cytotoxicity achieved with no statistically significant increases in frequency of micronucleated cells. They stated the 4 hour exposure groups in both the absence and presence of S9 were repeated twice in an attempt to achieve the necessary level of cytotoxicity with no significant increases in the frequency of binucleate cells in a dose range which included the dose considered to achieve optimal cytotoxicity.

The Applicant summarised the *in vitro* micronucleus test as follows: 'In summary, based on the findings of this *in vitro* mammalian cell micronucleus test performed at the maximum feasible concentrations limited by test item-induced cytotoxicity, the test item, kaurenoic acid, did not induce any genetic toxicologically significant increases in the frequency of binucleate cells with micronuclei in either the absence or presence of a

metabolising system. Therefore, kaurenoic acid was considered to be non-clastogenic and non-aneugenic to human peripheral blood lymphocytes in vitro'.

The Applicant also referenced several published studies which have investigated the genotoxic profile of kaurenoic acid within this section. This included *in vitro* studies (*in vitro* bacterial mutation, micronucleus, chromosomal aberration and/or comet assays) (Pezzuto *et al*., 1985, 1986; Cavalcanti *et al*., 2006, 2010; Cano *et al*., 2017; Cardoso *et al*., 2017; Damasceno *et al*., 2019; Rocha *et al*., 2019); references for *in vivo* comet assays were also provided (Cavalcanti *et al*., 2010; Dalenogare *et al*., 2019).The Applicant stated 5 of the above studies concluded kaurenoic acid non-genotoxic in their test systems (Pezzuto *et al*., 1985, 1986; Cano *et al*., 2017; Damasceno *et al*., 2019; Dalenogare *et al*., 2019) whilst 4 articles considered kaurenoic acid positive for genotoxicity (Cavalcanti *et al*., 2006, 2010; Cardoso *et al*., 2017; Rocha *et al*., 2019). The Applicant argued that in the 4 articles that concluded kaurenoic acid had mutagenic and genotoxic potential, cell lines had displayed cytotoxicity and that kaurenoic acid was negative at test concentrations that were not cytotoxic. They concluded that 'Prevailing guidance (OECD, 2015) for interpretation of genotoxicity assays states that proper study design requires determination of whether the compound is cytotoxic in the test system because biologically irrelevant false positive results can occur at cytotoxic concentrations. Further, a positive result from an *in vitro* test in mammalian cells may be considered of limited or even no relevance if the effect was observed only at highly cytotoxic concentrations (EFSA Scientific Committee, 2011).

The Applicant concluded that 'Based on the results of the in vitro bacterial and mammalian genotoxicity assays performed according to standardised testing guidelines and GLP, and considering the corroborative evidence from the literature demonstrating negative results at non-cytotoxic test concentrations, it is concluded that kaurenoic acid is not genotoxic.'

The AEJEG requested further information on the toxicity profile of kaurenoic acid. They requested that further scientific sources that had not been identified in the initial literature review by the Applicant be reviewed.

The Applicant in response presented their literature search methodology for their previously conducted search.

The Applicant stated they had performed two additional literature reviews in an effort to identify any new studies.

The Applicant stated they had identified one additional source which investigated potential reproductive toxicity of kaurenoic acid conducted by Cunha *et al*. (2011).

The study consisted of dosing pregnant Swiss albino mice (12-17 in number) with kaurenoic acid isolated from *Copifera langsdorffii,* at doses of either 25-50 mg/kg bw by oral gavage between days 1-7 or 8-15 of gestation. The authors utilised drinking water as a negative control and drinking water with 5% DMSO as a vehicle control. The Applicant summarised and discussed the study as follows: ‘Weights were recorded on Days 1, 10, and 20 of gestation. Necroscopy was conducted in dams on Days 10 or 20 of gestation and assessed for uterine implantation sites, resorptions, and the number of corpora lutea in ovaries. Dams undergoing necroscopy at Day 20 of gestation were assessed for the number of live and dead foetuses, and the foetuses were weighed and examined for visceral alterations. In mice treated during Days 1 to 7 of gestation, the number of mated females that did not conceive were significantly higher in those provided with 50 mg/kg body weight/day kaurenoic acid relative to negative or vehicle controls and the low-dose kaurenoic acid treatment animals. High-dose mice also had lower number of implantation sites and corresponding number of corpora lutea. Reduced weight gain was reported in both groups given kaurenoic acid with a greater effect noted in the high-dose group.

In mice treated during Days 8 to 15 of gestation, the number of mated females that did not conceive was significantly higher than those given control (water or vehicle) or low dose kaurenoic acid. Reduced weight gain was reported in mice of the high-dose group relative to both controls and low-dose mice. Increased level of foetal loss due to resorption in high-dose mice relative to both control and low-dose group was reported. While mice of the low-dose group had higher fetal loss rates relative to both control groups, these effects did not reach statistical significance. No treatment-related effects were reported in pup weight, placental weight, and male to female ratio of the pups. Mice given kaurenoic acid during Day 8 to 15 of gestation showed retarded ossification of sternebra and metacarpals, and skeletal variations in cervical ribs, thoracic vertabra, and limb defects at 5% and 32% in mice of the low-dose and high-dose group, respectively. No skeletal malformations were reported in mice of either control groups. Overall, the authors noted that high doses of kaurenoic acid at 50 mg/kg body weight/day resulted in impaired fertility'.

The Applicant stated that EFSA had established an ADI of 4 mg/kg bw for steviol equivalents applicable to all 60 steviol glycosides. The Applicant further stated 'Using this ADI and the proposed kaurenoic acid specification of 300 mg/kg, a level of intake of 0.0012 mg kaurenoic acid/kg body weight/day should be considered. Moreover, based on the average kaurenoic acid content of 5 representative lots of steviol glycosides from fermentation (Reb M) (….)150.2 ppm (....) it could be reasonably expected that consumers would be exposed to kaurenoic acid at 0.0006 mg kaurenoic acid/kg body weight/day within the EFSA ADI for steviol glycosides; a value that is over 41,000-fold less than the low-dose level evaluated in this study and was not observed to have toxic effects.'

The AEJEG considered that the manifestations present in the study identified by the Applicant were severe, however it considered that at the low levels of exposure to this contaminant from the specification proposed in the intended product, they would not be of concern.

The AEJEG requested clarification on the use of the ADI of steviol glycosides and the proposed limit for kaurenoic acid to derive an exposure to kaurenoic acid as presented above. The Applicant responded with a detailed description of how they had utilised the limit for kaurenoic acid to derive an exposure assessment for kaurenoic acid from steviols. The Applicant reiterated their exposure assessment for steviol glycosides which used a read-across approach to estimate worst case scenario exposure assuming that kaurenoic acid was present at the proposed regulatory limit of 300 mg/kg (0.03%). This is discussed further within the exposure assessment section of this safety advice document. The Applicant did not clarify further the reason for discussing the ADI of steviol equivalents in relation to the proposed limit for kaurenoic acid.

2.8.8.3 Establishing a safe human exposure level

The Applicant discussed how they utilised a threshold of toxicological concern (TTC) to identify a safe human exposure level for kaurenoic acid based on its structure classification. This is due to a lack of scientific data on the compound. Originally, based on structural analysis utilising the OECD QSAR toolbox (see below), kaurenoic acid was classified as a class I Cramer structure, the least toxic grouping of chemicals within the Cramer grouping system.

The COT noted that some, albeit limited, scientific data are available for kaurenoic acid, and that any substance specific-ADI would be higher that the respective TTC value. The AEJEG noted the Applicant had initially not provided SAR reports for the Cramer classification of kaurenoic acid, these were then provided.

The Applicant stated ‘A safe exposure of 1800 µg/person/day or 30 µg/kg body weight/day may be assumed for compounds which are Cramer Class I but not genotoxic, assuming an average body weight of 60 kg/person (Kroes *et al*., 2004; EFSA Scientific Committee, 2019).’

The Applicant concluded that ‘Based on the above analysis, using data from a robust and reliable human pharmacokinetic study (Choi *et al*., 2018), systemic exposure to kaurenoic acid from consumption of Amyris’ steviol glycosides from fermentation (Reb M) is expected to be extremely low; therefore, there is low potential for systemic toxicity hazard or reproductive / developmental toxicity hazard associated with kaurenoic acid as a non-genotoxic impurity of steviol glycosides from fermentation (Reb M).’

From the above information and that within the dossier and annexes the Applicant concludes the following regarding kaurenoic acid in their products: ‘Using the TTC approach and given the lack of genotoxic potential for kaurenoic acid which may be present at a maximum concentration of 300 ppm [mg/kg] in Amyris’ steviol glycosides from fermentation (Reb M), it can be concluded that there is an adequate margin of safety for the exposure to this potential impurity from the daily intake of Amyris’ steviol glycosides from fermentation (Reb M) and that the consumption of this potential impurity does not present a human health hazard.’

Following investigation, The AEJEG were not satisfied with the Cramer classification provided by the Applicant and requested that the Applicant reinvestigate the Cramer classification.

The Applicant responded detailing their approach to utilising the QSAR toolbox. They discussed the potential inconsistencies between the OECD Toolbox v4.5 and Toxtree v3.1.0 when evaluating diterpenes such as kaurenoic acid. The Applicant then assured that the highest mean and 95th percentile relative exposure of kaurenoic acid at 0.72 and 1.29 µg/kg bw/day respectively (for toddlers aged 12-35 months, 97.5th percentile of exposure; Table 10) would be below the threshold of toxicological concern for a Cramer Class 3 compound (1.5 µg/kg bw/day). The Applicant stated that these estimates were calculated from the upper limit of steviol glycoside consumption across European populations and were considered sufficiently conservative for the risk assessment.

The AEJEG noted that the Applicant had only considered exposure to kaurenoic acid from consumption of steviol glycosides within their exposure assessment.

The Applicant added that ‘systemic exposure to kaurenoic acid following oral consumption is expected to be far less than the worst-case exposure estimates summarised above (Choi *et al*., 2018). To more accurately estimate systemic exposure to kaurenoic acid following oral consumption, absorption factors ranging from 4.7% to 12.5% could be applied to the exposure estimates; however, these factors were not applied to the data herein for simplicity (Choi *et al*., 2018).

Based on the data presented above, maximum exposure estimates for kaurenoic acid as an impurity in Amyris’ steviol glycosides from fermentation (Reb M) do not exceed the TTC for both Cramer Class I and III compounds.’

The AEJEG and COT did not agree with the Applicant’s use of absorption factors to refine their exposure assessment as discussed above and within proposed uses and exposure assessment.

The AEJEG noted that the Applicant had performed initial profiling of kaurenoic acid however queried why the Applicant had not performed (Q)SAR analysis on the genotoxic potential of kaurenoic acid and requested it be performed.

The Applicant stated they had intended to only utilise the QSAR toolbox to classify kaurenoic acid under the Cramer system and justified their previous threshold of toxicological concern approach quoting evaluations by EFSA and the WHO (2016) and the EFSA Scientific Committee (2019).

The Applicant provided the additional (Q)SAR analyses requested utilising ToxTree (v3.1.0) software, supplying the full outputs. A range of models were applied to kaurenoic acid including ‘structure alerts for the in vivo micronucleus assay in rodents, in vitro mutagenicity [Ames test] alerts by ISS [Istituto Superiore di Sanità],

carcinogenicity, DNA binding, etc' in an effort to explore the genotoxic potential of the compound. The Applicant reported no alerts for either the *in vivo* micronucleus or the *in vitro* mutagenicity (Ames) modules. In addition, the Applicant reported no alerts for carcinogenicity or mutagenicity across other modules. The Applicant stated that through utilising the Kroes TTC decision tree and the maximum estimated kaurenoic acid exposure from proposed uses of steviol glycosides from fermentation, kaurenoic acid is not expected to present a safety concern. The Applicant added there had been no DNA binding alerts for kaurenoic acid.

The Applicant continued by stating that *in silico* methods such as those above are typically only used when there is a lack of available scientific data available. The Applicant stated they had performed several pivotal studies to evaluate the genotoxic potential of kaurenoic acid *in vitro*. The Applicant proceeded to describe the results of the *in vitro* genotoxicity studies they had performed, described earlier in this opinion.

The Applicant concluded that they had previously not considered the (Q)SAR analysis necessary due to the presence of *in vitro* data, referencing the Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment (EFSA, 2019) however they considered that the *in silico* data provided may be used as corroborative evidence supporting kaurenoic acid not exhibiting genotoxic or mutagenic activity.

The AEJEG considered the Applicant's derivation of a safe human exposure level consisting of a TTC for kaurenoic acid as a Cramer class 3 compound following negative genotoxicity testing. The AEJEG noted that the Applicant had presented the highest 95th percentile exposure to kaurenoic acid to be a maximum of 1.29 µg/kg bw/day (Table 10) which was lower than the TTC value of 1.5 µg/kg bw/day for a Cramer class 3 compound. The AEJEG accepted the claim that the Applicant's exposure assessment was conservative. Overall, The AEJEG were satisfied by the information received from the Applicant regarding the derivation of a safe human exposure level for kaurenoic acid.

2.9 Overall conclusions on the safety of rebaudioside M produced by fermentation with *S. cerevisiae* for use in foods

The Applicant reiterated that rebaudioside M produced by fermentation is chemically identical to the rebaudioside M extracted from the leaves of *S. rebaudiana* Bertoni. The Applicant listed the safety opinions presented by differing regulatory and advisory agencies and discussed the similarities in metabolism to currently regulated steviol glycosides. The Applicant described again their read across approach to assessing the safety of their product.

The Applicant stated that their Reb M product will not contain any remnants of the genetically modified production strain or its DNA. The Applicant discussed the genotoxicity testing performed on their product. The Applicant also stated that the kaurenoic acid present in the product does not present a safety concern based on the low (<0.16 µg/kg body weight/day) estimated exposure to the contaminant.

The AEJEG reviewed the safety of the proposed modification to the specifications of steviol glycosides.

The AEJEG considered that Reb M produced through the modification of the manufacturing process for steviol glycosides to include manufacture via fermentation with a genetically modified production strain of *S. cerevisiae* as described within this Application would not present a chemical risk to health from the proposed uses.

3. Conclusions

To support the FSA and FSS in evaluating the dossier, the Additives and Enzymes Joint Expert Group (AEJEG) were asked to review the dossier submitted by the Applicant and the subsequent additional information requested and advise the FSA and FSS.

The Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) also reviewed the AEJEG advice and agreed with the conclusions of the AEJEG.

The FSA and FSS agreed on the conclusions of the AEJEG in that the proposed change in the steviol glycoside specification to include a production method using *Saccharomyces cerevisiae* to convert sugar into Reb M via fermentation is safe under the proposed conditions of use and at the anticipated levels of intake.

The AEJEG advised the FSA and FSS that sufficient information had been provided to allow for an evaluation of the proposal for modification of the manufacturing specifications of steviol glycosides from a genetically modified production strain of *Saccharomyces cerevisiae*, there were no concerns over safety of the proposed process.

Regarding the impurity kaurenoic acid which may be present at a maximum concentration of 300 mg/kg in steviol glycosides from fermentation. The FSA and FSS, on advice from the AEJEG, were satisfied by the information received from the Applicant regarding the derivation of a safe human exposure level for kaurenoic acid.

The FSA and FSS therefore conclude in this assessment that the modification of the manufacturing specifications of steviol glycosides from a genetically modified production strain of *Saccharomyces cerevisiae* as described within this application would not pose a risk to health. Therefore, there were no concerns over safety of the proposed process.

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5. Abbreviations

µg	Microgram
ADI	Acceptable daily intake
AEJEG	Additives, Enzymes and other Regulated Products Joint Expert Group
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANS Panel	Scientific Panel on Food Additives and Nutrient Sources added to Food
AOAC	Association of Analytical Communities
AUC	Area under the curve
BMI	Body Mass Index
bw	Bodyweight

CAS	Chemical Abstracts Service
CFU	Colony forming units
C_{max}	Maximum serum concentration
cGMP	Current Good Manufacturing Practice
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
E 960	Steviol Glycosides
EFSA	European Food Safety Authority
EFSA SC	EFSA Scientific Committee
EPC	Endothelial progenitor cells
EU	European Union
FAF Panel	The Panel on Food Additives and Flavourings
FAO	Food and Agriculture Organisation of the United Nations
FDA	United States Food and Drug Administration
FEEDAP Panel	The Panel on Additives and Products or Substances used in Animal Feed
FSA	Food Standards Agency
FSANZ	Food Standards Australia New Zealand
FSSAI	Food Standards and Safety Authority of India
g	Gram
GALT	Gut associated lymphoid tissue
GIP	Glucose dependent insulinotropic polypeptide
GLP	Good laboratory practice
GMO	Genetically Modified Organism
GRAS	Generally recognised as safe
h	Hour
HACCP	Hazard analysis critical control point
HbA1c	Glycated haemoglobin
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HDPE	High density polyethylene

HOMA	homeostatic model assessment
HPLC	High performance liquid chromatography
HPLC-ESI-MS/MS	High-performance liquid chromatography/electrospray ionization tandem mass spectrometry high-performance liquid chromatography with electrospray ionization tandem mass spectrometry
HPLC-UV	High performance liquid chromatography with Ultraviolet detection
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INS	International numbering system
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KA	Kaurenoic Acid
kg	Kilogram
LC_{50}	Lethal concentration (50%)
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low density lipoprotein
LLOQ	Lower limit of Quantification
m/z	Mass-to-charge-ratio
Mg	Milligram
MOHFW	Ministry of Health and Family Welfare
Mol	Mole
MPL	Maximum Permitted Level
MS	Mass Spectrometry
n	number
nm	Nanometre
NMT	No more than
NNS	Non-nutritive sweeteners
^oC	Celsius
OECD	Organisation for Economic Co-operation and Development

OECD TG	Organisation for Economic Co-operation and Development test guideline
OGTT	Oral glucose tolerance test
QDa	Quadrupole dalton
QSAR	Quantitative structure analysis
Reb M	Rebaudioside M
RP-HPLC-DAD	Reverse-phase high-performance liquid chromatography coupled to a diode array detector
RP-HPLC-UV	Reversed phase high performance liquid chromatography with ultraviolet detection
S. cerevisiae	Saccharomyces cerevisiae
S. rebaudiana	Stevia rebaudiana
T1R2	Taste receptor type 1 subunit 2
T1R3	Taste receptor type 1 subunit 3
Tmax	Time to peak drug concentration
TNF-α	Tumour necrosis factor
TTC	Threshold of toxicological concern
UK	United Kingdom
UPLC	Ultra performance liquid chromatography
UPLC MS/MS	Ultra performance liquid chromatography -tandem mass spectrometry
USP	United States Pharmacopeia
Vz/f	Volume of distribution
w/v	Weight per volume
w/w	Weight per weight
WHO	World Health Organisation

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