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Safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive: Rebaudioside M produced via enzyme-catalysed bioconversion of purified stevia leaf extract

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Abstract

The EFSA Panel on Food Additives and Flavourings (FAF) provides a scientific opinion on the safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive, in particular related to rebaudioside M produced via enzyme-catalysed bioconversion of purified stevia leaf extract. Rebaudioside M (95% on dry basis) is produced via enzymatic bioconversion of purified stevia leaf extract using uridine diphosphate (UDP)-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffii* UGT-a and *K. phaffii* UGT-b, that facilitates the transfer of glucose to purified stevia leaf extract via glycosidic bonds. The Panel considered that the parental strain *K. phaffii* ATCC 20864 qualifies for the qualified presumption of safety (QPS) approach for safety assessment and, therefore, is considered to be safe for production purposes. The Panel concluded that there is no safety concern for Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffii* UGT-a and *K. phaffii* UGT-b, to be used as a food additive. However, the Panel recommended that the European Commission considers establishing separate specifications for Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract in Commission Regulation (EU) No 231/2012.

Requestor: European Commission

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Summary

Following a request from the European Commission to the European Food Safety Authority (EFSA), the Panel on Food Additives and Flavourings (FAF) was asked to provide a scientific opinion on the safety of a proposed amendment of the specifications of the food additive steviol glycosides (E 960), in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

The present evaluation is based on the data on steviol glycosides in a newly submitted dossier by the applicant and additional information submitted by the applicant during the assessment process in response to a request by EFSA.

Rebaudioside M (95% on dry basis) is produced via enzymatic bioconversion of purified stevia leaf extract using uridine diphosphate (UDP)-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffii* UGT-a and *K. phaffii* UGT-b, that facilitates the transfer of glucose to purified stevia leaf extract via glycosidic bonds.

The Panel considered that the manufacturing process applied to the production of Rebaudioside M, which is the subject of this application under evaluation, involves a step of enzymatic bioconversion of purified stevia leaf extract which may result in impurities different from those that may be present in steviol glycosides (E 960) obtained from water extraction of the leaves of the Stevia rebaudiana followed by recrystallisation. The Panel, therefore, considered that separate specifications would be needed for the food additive produced via the manufacturing process described in the current application, which should also contain additional parameters related to the specific genetically modified microorganism used for its production.

The specifications for Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract should include parameters relevant for this specific manufacturing process:

- assay referring to the purity of Rebaudioside M (not less than 95% Rebaudioside M),
- proper definition to describe the manufacturing process,
- the absence of recombinant DNA in the final product demonstrated, using an analytical method with a limit of detection not higher than 10 ng DNA/g product,
- the absence of protein to ensure the absence of enzymes in the final product, using an analytical method with a limit of detection not higher than 5 mg/kg.

The Panel considered that the parental strain *K. phaffii* ATCC 20864 qualifies for the qualified presumption of safety (QPS) approach for safety assessment and therefore is considered to be safe for production purposes. The strains used for the production of the fusion enzymes *K. phaffii* UGT-A and *K. phaffii* UGT-B share identity with the parental strain. The introduced genetic modifications include a gene conferring resistance to the antibiotic kanamycin. Since no viable cells nor their DNA remained in the final product, this manufacturing process does not raise a safety concern.

The *in vitro* anaerobic metabolism of 'bioconversion rebaudioside M' was investigated in pooled human faecal homogenates (BRI, 2019, Documentation provided to EFSA n. 2). The authors concluded that the metabolism of 'bioconversion rebaudioside M' in this study indicated rapid deglycosylation of the 'bioconversion rebaudioside M' to a final steviol metabolite.

In the 2010 evaluation on steviol glycosides, the panel on Food additives and Nutrient Sources (ANS) noted that '*in vitro* studies demonstrated that human digestive enzymes are not capable of hydrolysing β-glycosidic bonds of steviol glycosides. However, the intestinal microflora of humans (and rats) is able to convert steviol glycosides to steviol. In addition, in the Caco-2 cell model the apparent permeability value of steviol was found to be 200 to 300-times higher than that of stevioside or rebaudioside A. Other *in vitro* studies assessing the metabolic transformation of steviol showed a similar formation of hydroxy-metabolites of steviol in the presence of rat or human liver microsomes' (EFSA ANS Panel, 2010).

The Panel note that the metabolism of Reb M from leaves of the Stevia was evaluated previously by the ANS Panel (EFSA ANS Panel, 2015a) and concluded that it was extensively cleaved to steviol *in vitro* following incubation with gastrointestinal microbiota for 24 h at concentrations of 0.2 mg/mL, based on the results of the Purkayastha et al. (2014) study.

The results from an *in vitro* anaerobic metabolism study with 'bioconversion rebaudioside M' were in line with those from a previous study on the metabolism pathway of Rebaudioside M from leaves of the Stevia and already evaluated by the EFSA ANS Panel.

Considering that no safety concern was identified from the manufacturing process of Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract and that this is the same

compound as Rebaudioside M extracted from of the leaves of the Stevia rebaudiana Bertoni plant, biological and toxicological data previously assessed by the ANS Panel for steviol glycosides (E960) were considered to support its safety. Therefore, no additional toxicological data were required. The existing acceptable daily intake (ADI) of 4 mg/kg body weight (bw) per day can also be applied to Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract.

The Panel concluded that there is no safety concern for Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffii* UGT-a and *K. phaffii* UGT-b, to be used as a food additive. However, the Panel recommended that the European Commission consider establishing separate specifications for Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract in Commission Regulation (EU) No 231/2012.

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1. Introduction

The present scientific opinion deals with the safety evaluation for a modification of the specifications following a new production process of an already authorised food additive, steviol glycosides (E 960).

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

The use of food additives is regulated under the European Parliament and Council Regulation (EC) No 1333/2008 on food additives.[1] Only food additives that are included in the Union list, in particular in Annex II to that regulation, may be placed on the market and used as in foods under the conditions of use specified therein. Moreover, food additives shall comply with the specifications as referred to in Article 14 of that Regulation and laid down in Commission Regulation (EU) No 231/2012[2].

Steviol glycosides (E 960) is an authorised food additive in the European Union for use in several food categories and specifications have been adopted for it. Presently, those specifications stipulate that 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, and the second involving recrystallinisation of the steviol glycosides.

The European Commission received a request vis-à-vis an amendment of the present specifications of Steviol glycosides (E 960) to include a new production process for rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract (≥ 95% steviol glycosides). The enzymes (Uridine 5′-diphospho(UDP)-glucosyltransferase and sucrose synthase) are derived from two strains of *K. phaffii* that have been genetically modified, and undergo fermentation. Following the fermentation step, the enzymes are isolated from the production microorganisms and are mixed with purified stevia leaf extract (≥ 95% steviol glycosides) to generate rebaudioside M. The resulting rebaudioside M undergoes a series of purification and isolation steps to produce the final rebaudioside M (≥ 95%) determined to be 200 times sweeter than sucrose.

Although rebaudioside M is a minor glycoside present at very low levels (< 1%) in the stevia leaf, it has more favourable sensory characteristics when compared to the major glycosides (i.e., stevioside and rebaudioside) and a taste profile that is more reflective of sucrose.

1.1.2. Terms of reference

In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002 the European Commission requests to the European Food Safety Authority to perform a risk assessment and to provide a scientific opinion on the safety of a proposed amendments of the specifications of the food additive Steviol Glycosides (E 960) in accordance with Regulation (EC) 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.[3]

1.1.3. Interpretation of the Terms of reference

The Panel considered that the manufacturing process applied to the production of rebaudioside M, which is the subject of this application under evaluation, involves a step of enzymatic bioconversion of purified stevia leaf extract which may result in impurities different from those that may be present in steviol glycosides (E 960) obtained from water extraction of the leaves of the Stevia rebaudiana followed by recrystallisation. The Panel, therefore, considered that separate specifications could be needed for the food additive produced via the manufacturing process described in the current application, which could also contain additional parameters related to the specific genetically modified microorganism used for its production.

1.2. Information on existing evaluations and authorisations

Steviol glycosides (E 960) from water extraction of the leaves of the Stevia rebaudiana Bertoni plant and described as 'not less than 95% steviolbioside, rubusoside, dulcoside A, stevioside,

[1] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008.

[2] Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) no 1333/2008 of the European Parliament and of the Council. OJ L 83, 22.3.2012.

[3] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December establishing a common authorisation procedure for food additives, food eznymes and food flavourings. OJ L 354, 31.12.2008.

rebaudiosides A, B, C, D, E, F and M on the dried basis, in any combination and ratio' is an authorised food additive in the European Union (EU) according to Regulation (EC) No 1333/2008 on food additives and specifications have been defined in the Commission Regulation (EU) No 231/2012.

The safety of steviol glycosides as a food additive was evaluated by EFSA in 2010 and an acceptable daily intake (ADI) of 4 mg/kg body weight (bw) per day, expressed as steviol equivalents, based on application of a 100-fold uncertainty factor to the no observed adverse effect level (NOAEL) from a 2-year carcinogenicity study in the rat was established (EFSA ANS Panel, 2010). Following the EFSA assessment in 2015 (EFSA ANS Panel, 2015a), rebaudioside D and M were included in the specifications for steviol glycosides (E 960).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established an ADI for steviol glycosides of 0–4 mg/kg bw per day, expressed as steviol (JECFA, 2009).

In 2016, JECFA confirmed that rebaudioside A from multiple gene donors expressed in Yarrowia lipolytica is included in the ADI of 0–4 mg/kg bw, expressed as steviol (JECFA, 2016). JECFA has prepared new specifications for Rebaudioside A from Multiple Gene Donors Expressed in Yarrowia lipolytica) for the yeast-derived product, recognising that it was manufactured by a distinctly different, biosynthetic process compared with stevia leaf-derived products (JECFA, 2016).

JECFA recently issued new specifications for 'Steviol Glycosides from *Stevia rebaudiana* Bertoni' that consist of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose and deoxyglucose) in any of the orientations occurring in the leaves of *S. rebaudiana* Bertoni, provided that the total percentage of steviol glycosides is not less than 95% (JECFA, 2017).

At the JECFA meeting in 2019, a framework was adopted for developing specifications for steviol glycosides by four different methods of production among them 'enzyme modified steviol glycosides (new specifications)' that include a process in which steviol glycosides that have been extracted from the leaves of Stevia rebaudiana Bertoni undergo enzyme conversion of major steviol glycoside to minor ones (JECFA, 2019).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of its application for the request to amend the specifications for the use of steviol glycosides (E 960) with respect to a new manufacturing process (Documentation provided to EFSA No. 1). Following a request from EFSA, additional data were generated by the applicant (Documentation provided to EFSA No. 3, 4, 5 and 6).

2.2. Methodologies

This opinion was formulated following the principles described in the EFSA Guidance of the Scientific Committee on transparency with regard to scientific aspects of risk assessment (EFSA Scientific Committee, 2009) and following the relevant existing Guidances from the EFSA Scientific Committee.

The current 'Guidance for submission for food additive evaluation' (EFSA ANS Panel, 2012), 'Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use' (EFSA GMO Panel, 2011) and 'Guidance on the characterisation of microorganisms used as feed additives or as production organisms' (EFSA FEEDAP Panel, 2018) have been followed by the ANS Panel for evaluating the proposed change in manufacturing process and changes in the specifications.

3. Assessment

3.1. Technical data

3.1.1. Identity of the substance

According to the applicant, rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract using UDP-glucosyltransferase (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13) enzymes produced by strains of the yeast *Komagaetella phaffii* (*K. phaffii* formerly known as *Pichia pastoris*) that facilitates the transfer of glucose to purified stevia leaf extract via glycosidic bonds.

The Panel noted that a more precise name for the description of this manufacturing process would be rebaudioside M produced via enzyme-catalysed bioconversion of purified stevia leaf extract.

Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract contains at least 95% rebaudioside M on dry basis. The Panel noted that the applicant submitted results of the analyses of five non-consecutive batches of the final rebaudioside M product in support of its purity.

The following information was provided by the applicant:

Chemical name	13-[(O-β-D-Glucopyranosyl-(1-2)-O-[β-D-glucosylpyranosyl-(1-3)]-β-Dglucosylpyranosyl)oxy]-kaur-16-en-18-oic acid (4-)-O-β-D-glucosylpyranosyl-(1-2)-O-[β-Dglucosylpyranosyl-(1-3)]-β-D-glycosylpyranosyl ester
Synonyms	Rebaudioside M, Reb M, Stevia Reb M
CAS No.	1220616-44-3
Chemical formula	$C_{56}H_{90}O_{33}$
Molecular weight:	1,291.29 Da

The structural formula of rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract as provided by the applicant is shown in Figure 1.

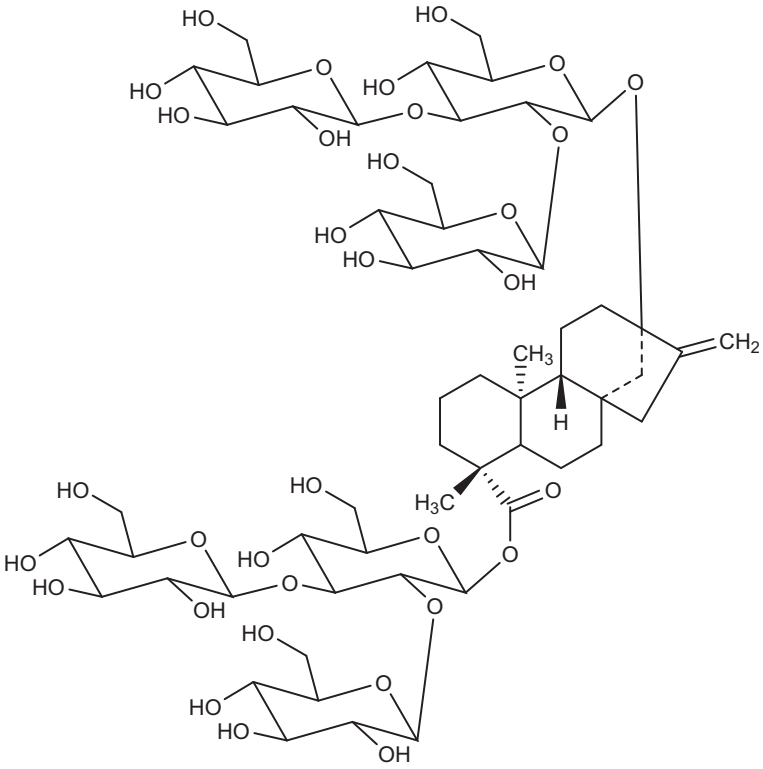

Figure 1: Structural formula of rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract, as provided by the applicant

In the original dossier, three minor peaks corresponding to Iso-rebaudioside M, rebaudioside I and an unidentified minor steviol glycoside were observed by high-performance liquid chromatography (HPLC) in five samples. Additional information submitted (Documentation provided to EFSA n. 1) in five new samples analysed using the same HPLC method (modified JECFA 2010 method) identified as minor peaks: rebaudioside D (0.4–1.1%) in all samples, rebaudioside B (0.4–1.2%) in all samples, rebaudioside I (0.2–1.1%) in two samples and rebaudioside A (0.01%) in one sample (Documentation provided to EFSA n. 3).

3.1.2. Manufacturing process

According to the applicant Rebaudioside M (≥ 95%) is manufactured through a multistep enzymatic process using UDP-glucosyltransferase and sucrose synthase enzymes produced by genetically

modified strains of the yeast *K. phaffii*. These enzymes facilitate the transfer of glucose to steviol glycosides present in purified stevia leaf extract via glycosidic bonds.

Stage 1 – Enzyme Production

The first stage of the manufacturing process involves preparation of the enzymes that are utilised as processing aids in stage 2. The enzymes are generated by strains of *K. phaffii* that express UDP glucosyltransferase and sucrose synthase as a fusion enzyme necessary to convert steviol glycosides present in purified stevia leaf extract to rebaudioside M. The 2 strains are *K. phaffii* UGT-a and *K. phaffii* UGT-b carrying the UGT-A and UGT-B fusion enzymes (*i.e.* glucosyltransferase fused with sucrose synthase), respectively.

Both strains are harvested separately by centrifugation, resuspended in a reaction buffer and are passed through a homogeniser operated at minimum pressure to release the enzymes present on the cell surface without lysing the cells. The enzymes are separated from the yeast cells via centrifugation, and the supernatants containing the UGT-A and UGT-B fusion enzymes are collected and used in the bioconversion.

Stage 2 – Rebaudioside M Production

A) Bioconversion of purified stevia leaf extract to rebaudioside M

For the catalytic reaction needed to convert purified stevia leaf extract to rebaudioside M, UGT-A and UGT-B fusion enzymes are mixed together with slow agitation. Purified stevia leaf extract (95% steviol glycosides) is fed into the tank to allow the reaction to proceed.

The reaction mixture containing mainly rebaudioside M is collected in a storage tank and is heated to denature the enzymes. The mixture is filtered to remove the denatured enzymes.

B) Rebaudioside M Purification

The supernatant is loaded onto large columns containing a macroporous resin. The supernatant flows through the column by gravity and is bound to the resin. The column is rinsed with a series of buffer solutions and rebaudioside M is eluted with food-grade ethanol numerous times. The eluent is collected and condensed in a wipe-film evaporator. The condensate is chilled to allow rebaudioside M to crystallise and precipitate from the solution. The wet crystals are collected, washed and dissolved in ethanol. The redissolved rebaudioside M is treated with activated charcoal to remove remaining impurities, recrystallised, dried and processed to the final high-purity rebaudioside M product ($\geq$ 95%).

Raw materials and processing aids are discussed briefly below.

Carbohydrate Source

Sucrose and UDP-glucose are added to the reaction mixture as sources of glucose for the bioconversion of stevia extract to rebaudioside M.

Food Enzymes

The UGT-A and UGT-B fusion enzymes used in stage 2 of the production process are comprised of the food enzymes UDP-glucosyltransferase and sucrose synthase. The enzymes are used to facilitate the transfer of glucose to purified stevia leaf extract via glycosidic bounds. The enzymes are obtained from two genetically modified strains of the yeast *K. phaffii*, which is characterised in Section 3.1.3.

Resins

According to the application dossier, macroporous resin column is used for the purification of the rebaudioside M.

3.1.3. Characterisation of the production organism

The UGT-A and UGT-B fusion enzymes are obtained from the genetically modified strains *K. phaffii* UGT-a and *K. phaffii* UGT-b, respectively. The strains are deposited in the China Center for Type Culture Collection as *K. pastoris* UGT-a and *K. pastoris* UGT-b with the deposition numbers CCTCC M2017681 and CCTCC M2017682, respectively. The strains were identified as *K. phaffii* by sequence analysis of the 26S rRNA gene, partial mitochondrial small subunit rRNA gene, partial Tf 1-α gene and partial RNA polymerase I gene.

Characteristics of the parental and recipient microorganisms

The parental microorganism is the yeast *K. phaffii* ATCC20864. *K. phaffii* is recommended for the Qualified Presumption of Safety (QPS) status, with the qualification that the species is used for production purposes, as is the case here (EFSA BIOHAZ Panel, 2019). *K. phaffii* ATCC20864 was selected based on tolerance towards 10% steviol glycosides in liquid media.

Characteristics of the inserted sequences

The UGT-A and UGT-B fusion proteins are encoded by three different chimeric genetic sequences (one for UGT-A and two for UGT-B), all with the same structure. They consist of a sequence encoding UDP glucosyltransferase fused in frame with the *SUS* gene (encoding sucrose synthase) from mung bean (*Vigna radiata*). For UGT-A, the UDP glucosyltransferase coding sequence ([redacted]) derives from barley (*Hordeum vulgare*), whereas for UGT-B, UDP glucosyltransferase is encoded by two different variants ([redacted] and [redacted]) of the gene from *Stevia rebaudiana*. The chimeric genes were synthetic and codon optimised for expression in *K. phaffii*.

From each chimeric gene, an expression cassette was constructed consisting of the AOX1 (alcohol oxidase) promoter, the α-factor signal for protein secretion from *Saccharomyces cerevisiae*, the corresponding chimeric gene, the GCW61 gene (encoding a cell wall protein) from *K. phaffii*, and the AOX1 terminator from *K. phaffii*.

The plasmid vectors pHKA-UGT-A and pHKA-UGT-B derive from the expression vector pPICZαA, and contain the pUC origin of replication, the HIS4 gene (involved in histidine biosynthesis) from the expression vector pPIC9K, and a kanamycin resistance gene used as selectable marker for transformation. Plasmid pHKA-UGT-A carries five copies of the expression cassette of UGT-A, whereas pHKA-UGT-B carries two copies of each of the two expression cassettes of UGT-B.

Description of the genetic modification process

The plasmids pHKA-UGT-A and pHKA-UGT-B were linearised and transformed separately into the recipient strain. Both plasmids were integrated into the HIS4 locus of the recipient strain. After screening for positive transformants, the production strains *K. phaffii* UGT-a and *K. phaffii* UGT-b were obtained.

Safety aspects of the production strain

The parental strain *K. phaffii* ATCC 20864 qualifies for the QPS approach for safety assessment and therefore is considered to be safe. The production strains *K. phaffii* UGT-a and *K. phaffii* UGT-b contain the full vector sequences inserted into their genome, including a gene conferring resistance to kanamycin. The insertion of the plasmids was confirmed by whole-genome sequence analysis.

The presence of the antimicrobial resistance gene in the production strains of the fusion enzymes is a possible safety concern related to the genetic modification which is further discussed in this opinion.

Absence of viable cells and recombinant DNA

The absence of the production microorganisms in the final rebaudioside M product was demonstrated in three independent batches analysed in triplicate. One gram of product was plated on selective medium plates and incubated at 30°C for 3 days. No colonies were produced.

The absence of recombinant DNA in the rebaudioside M product was demonstrated by polymerase chain reaction (PCR) analysis of three batches. No DNA was detected with primers that would amplify a 0.75 Kb fragment specific for the kanamycin resistance gene, with a limit of detection of 10 ng spiked DNA/g product (Documentation provided to EFSA n. 3).

Since the production strain and its DNA were not detected in the final rebaudioside M product, the final product does not raise environmental safety concern on these aspects.

Additional information on the potential safety concerns from the use of enzymes

Several steps are described in the manufacturing process to inactivate and remove the enzyme system, including heating, treatment with activated carbon, resin purification, crystallisation and filtration. The final rebaudioside M product was tested for residual protein using the bicinchoninic acid (BCA) assay to ensure that the processing enzymes were effectively removed, and no residual protein was detected (limit of detection = 5 ppm).

To confirm that the UGT-A and UGT-B fusion enzymes do not harbour any toxic potential, the Basic Local Alignment Search Tool (BLAST) program was used to conduct a sequence alignment query of the UGT-A and UGT-B fusion enzyme FASTA protein sequences against downloaded protein sequences

obtained from a curated database of venom proteins and toxins maintained by UniProt (UniProtKB/Swiss-Prot Tox-Prot12). Neither UGT-A nor UGT-B fusion enzyme sequences aligned significantly with the queried toxins and virulence factors.

A sequence homology search conducted according to the approach outlined by the FAO/WHO (FAO/WHO, 2001) and the Codex Alimentarius (2009) using the AllergenOnline Database version 17[4] was submitted in the application dossier.

In addition, and in accordance with the FAO/WHO guideline, the database was searched using a sliding window of 80 amino acid sequences derived from the full-length amino acid sequences. Using this search strategy, no identity matches of greater than 35% were identified for either of the enzyme sequences.

Both search strategies indicated an unlikely potential for cross-reactivity to the allergens held in the databases.

3.1.4. Specifications

According to the applicant (Documentation provided to EFSA n. 5), to encompass all steviol glycosides derived from *Stevia rebaudiana* Bertoni under the same specification (i.e. steviol glycosides manufactured by extraction from the leaf or enzymatic bioconversion of purified stevia leaf extract), the following two changes to the specifications for E 960 steviol glycosides are proposed (see Appendix A):

1) An expansion of the definition to include enzymatic bioconversion of purified stevia leaf extract as a permitted manufacturing method for rebaudioside M. The following text is proposed to be added to the current definition for E960: 'In order to produce a higher yield of rebaudioside M, purified steviol glycoside leaf extracts may be subject to enzymatic bioconversion, utilising UDP-glucosyltransferase and sucrose synthase enzymes derived from strains of *K. phaffii* that facilitate the transfer of glucose to steviol glycosides *via* glycosidic bonds'.
2) For rebaudioside M produced by enzymatic bioconversion of purified stevia leaf extract, an additional purity parameter is proposed to be added to ensure that no residual DNA arising from the use of enzymatic processing aids derived from *K. phaffii* (i.e. UDP-glucosyltransferase and sucrose synthase enzymes derived from strains of *K. phaffii* is present in the final product. The following text is proposed to be added to the end of the current list of purity parameters: 'Residual DNA, Not more than 10 ng DNA/g product [*only applicable to rebaudioside M produced by enzymatic bioconversion of purified stevia leaf extract*]'.

'The Panel noted that the following definition is proposed 'purified steviol glycoside leaf extracts may be subject to enzymatic bioconversion, utilising UDP-glucosyltransferase and sucrose synthase enzymes derived from strains of K. *phaffii* that facilitate the transfer of glucose to steviol glycosides via glycosidic bonds'. Information on all the sources (e.g. transfer of glucose from sucrose and UDP-glucose) for this manufacturing process as well as the specific strains of K. *phaffii* (*K. phaffii* UGT-a and *K. phaffii* UGT-b) should be mentioned in the definition.

The Panel noted that the limit for residual DNA would be valid only for rebaudioside M produced by enzymatic bioconversion of purified stevia leaf extract and it is not applicable for steviol glycosides extracted from the leaves of Stevia rebaudiana. This observation reconfirms the need for separate specifications. According to the applicant, residual DNA from the microorganisms used in the manufacturing process may be analysed using PCR according to EFSA's '*Guidance on the characterisation of microorganisms used as feed additives or as production organisms*' (EFSA FEEDAP Panel, 2018).

The Panel noted that the applicant submitted results on possible microbiological contaminants in rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract. Total plate count, total coliforms and yeast and mould in all batches were consistently below the defined specifications of $< 1{,}000$, < 3 and < 50 colony forming units (CFU)/g, respectively. Individual species of microorganisms, including *Escherichia coli* and *Salmonella*, were absent in all test samples (i.e. not detected).

Regarding toxic elements, in addition to the maximum limits for lead and arsenic already included in the current EU specifications for E 960, results for the analysis of cadmium and mercury were also reported (0.015 mg/kg for cadmium and 0.07 mg/kg for mercury). Based on the analytical data, the Panel noted that maximum limits for cadmium and mercury should be added to the proposed specifications. Additionally, lower levels of the maximum limits for lead and arsenic could be considered

[4] Available at http://www.allergenonline.org; updated January 18, 2017.

in the proposed specifications since the results obtained in the five batches analysed were 0.015 mg/kg for arsenic and 0.2 mg/kg for lead.

In view of the botanical origin of the stevia, limits for the presence of pesticides should be considered. The same samples were analysed for pesticide residues that cover a range of commonly used pesticides. No pesticide residues were detected in any of the finished products. The Panel noted that maximum residue levels (MRLs) for pesticides set under Regulation (EC) 396/2005[5] apply to stevia (Stevia rebaudiana) is listed in Part B of Annex I. Thus, MRLs established for this commodity code equally apply to Stevia rebaudiana. For processed products derived from stevia, the provisions of Article 20 are applicable, meaning that the changes in the levels of pesticide residues caused by processing need to be taken into account.

Despite particle size not being part of the current specifications for steviol glycosides (E 960), the applicant stated that 100% of the particles in the final material are larger than 74 μm using a mesh #200 sieve with a particle size limit of 74 μm (Documentation provided to EFSA n. 5).

Ethanol is a potential impurity in the final product as ethanol is used in the manufacturing process as a desorption solvent and a crystallisation solvent to purify rebaudioside M. The results of the analysis showed that the residual levels of ethanol are consistently less than 200 mg/kg, which is below the specified limit of 5,000 mg/kg.

The final rebaudioside M product has been tested for residual protein using the BCA assay with a limit of detection of 5 ppm to confirm the absence of protein residues in the final product. The Panel considers that to ensure the absence of enzymes or associated impurities in the final product, a limit indicating the absence of protein (e.g. below limit of detection) should be included in the proposed specifications.

An ad hoc meeting between EFSA and industry on the food additive steviol glycosides (E 960) to talk about the possible presence of kaurenoic acid as an impurity in the food additive E960 took place in 2018.[6] No kaurenoic acid was detected in the two analysed samples of Rebaudioside M produced by enzymatic bioconversion from purified stevia leaf extract analysed by HPLC (limit of detection of 0.5 mg/kg) (Documentation provided to EFSA n. 5).

3.1.5. Methods of analysis in food

No information on a method of analysis of rebaudioside M in food was provided.

3.1.6. Stability of the substance, and reaction and fate in food

A 6-month accelerated stability study was conducted on three lots of rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract. The samples were stored at 40 ± 2°C at a relative humidity of 75 ± 5%. Rebaudioside M was reported to be stable over the course of the accelerated stability study, based on appearance, moisture content and percent rebaudioside M content measured by HPLC compared to baseline (Documentation provided to EFSA n. 1).

3.2. Proposed uses and use levels

Maximum level of steviol glycosides (E 960) expressed as steviol equivalents are defined in Annex II to Regulation (EC) No 1333/2008.[1]

Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract is proposed for use as high-intensity sweetener in food and beverages under the same conditions as those already approved for steviol glycosides (E 960) in the EU (Regulation (EC) No 1333/2008) (Documentation provided to EFSA n. 1).

3.3. Exposure data

Because the proposed uses and use levels for rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract are the same as the already authorised food additive steviol glycosides (E 960), the applicant did not provide an exposure estimate but made reference to the latest estimated exposure to E 960 (e.g. EFSA ANS Panel, 2015b).

[5] Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. OJ L 70, 16.3.2005.

[6] https://www.efsa.europa.eu/en/events/event/180611-0

The Panel considers that if steviol glycosides would be replaced by rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract, exposure to rebaudioside M (expressed as steviol equivalent) will not be higher than the last EFSA estimate of exposure to steviol glycosides (E 960) (EFSA ANS Panel, 2015b).

3.4. Biological and toxicological data

Within the application dossier, scientific publications considered by the applicant relevant to the safety of steviol glycosides were submitted.

Biological and toxicological studies not performed with rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract or rebaudioside M from stevia extract were not considered in the current assessment by the Panel since they are not related to the substance under evaluation.

3.4.1. Absorption, distribution, metabolism and excretion

The *in vitro* anaerobic metabolism of 'bioconversion rebaudioside M', 'bioconversion rebaudioside D' and rebaudioside A (as control) was investigated in pooled human faecal homogenates from samples from 12 healthy subjects (6/sex) (BRI, 2019, Documentation provided to EFSA n. 2). The test materials were incubated at a concentration of 0.2 mg/mL with the incubation time points of 0, 4, 8, 16, 24 and 72 h. The rate and level of disappearance of the rebaudiosides and formation of steviol were analysed using liquid chromatography–mass spectrometry (LC/MS). The authors concluded that the metabolism of 'bioconversion rebaudioside M' in pooled male and female human faecal homogenate indicated rapid deglycosylation of the steviol glycosides to a final steviol metabolite over the first 8 h of metabolic incubation.

3.4.2. Toxicity data

No toxicity studies on Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract or Rebaudioside M from stevia extract were submitted.

3.5. Discussion

Rebaudioside M (95% on dry basis) is produced via enzymatic bioconversion of purified stevia leaf extract using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffii* UGT-a and *K. phaffii* UGT-b, that facilitates the transfer of glucose to purified stevia leaf extract via glycosidic bonds.

The Panel considered that the manufacturing process applied to the production of Rebaudioside M, which is the subject of this application under evaluation, involves a step of enzymatic bioconversion of purified stevia leaf extract which may result in impurities different from those that may be present in steviol glycosides (E 960) obtained from water extraction of the leaves of the Stevia rebaudiana followed by recrystallisation. The Panel, therefore, considered that separate specifications would be needed for the food additive produced via the manufacturing process described in the current application, which should also contain additional parameters related to the specific genetically modified microorganism used for its production.

The Panel noted that a more precise name for the description of this manufacturing process would be rebaudioside M produced via enzyme-catalysed bioconversion of purified stevia leaf extract.

The specifications for Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract should include parameters relevant for this specific manufacturing process:

- assay referring to the purity of Rebaudioside M (not less than 95% Rebaudioside M),
- proper definition to describe the manufacturing process,
- the absence of recombinant DNA in the final product demonstrated, using an analytical method with a limit of detection not higher than 10 ng DNA/g product (EFSA FEEDAP Panel, 2018),
- the absence of protein to ensure the absence of enzymes in the final product using an analytical method with a limit of detection not higher than 5 mg/kg.

The Panel considered that the parental strain *K. phaffii* ATCC 20864 qualifies for the QPS approach for safety assessment and therefore is considered to be safe for production purposes. The strains used for the production of the fusion enzymes *K. phaffii* UGT-A and *K. phaffii* UGT-B share identity with the parental strain. The introduced genetic modifications include a gene conferring resistance to the

antibiotic kanamycin. Since no viable cells nor their DNA remained in the final product, this manufacturing process does not raise a safety concern.

The *in vitro* anaerobic metabolism of 'bioconversion rebaudioside M' was investigated in pooled human faecal homogenates (BRI, 2019, Documentation provided to EFSA n. 2). The authors concluded that the metabolism of 'bioconversion rebaudioside M' in this study indicated rapid deglycosylation of the 'bioconversion rebaudioside M' to a final steviol metabolite.

In the 2010 evaluation on steviol glycosides, the ANS Panel noted that '*in vitro* studies demonstrated that human digestive enzymes are not capable of hydrolysing β-glycosidic bonds of steviol glycosides. However, the intestinal microflora of humans (and rats) is able to convert steviol glycosides to steviol. In addition, in the Caco-2 cell model the apparent permeability value of steviol was found to be 200 to 300-times higher than that of stevioside or rebaudioside A. Other *in vitro* studies assessing the metabolic transformation of steviol showed a similar formation of hydroxy-metabolites of steviol in the presence of rat or human liver microsomes' (EFSA ANS Panel, 2010).

The Panel noted that the metabolism of Reb M from leaves of the Stevia was evaluated previously by the ANS Panel (EFSA ANS Panel, 2015a) and concluded that it was extensively cleaved to steviol *in vitro* following incubation with gastrointestinal microbiota for 24 h at concentrations of 0.2 mg/mL, based on the results of the Purkayastha et al. (2014) study.

The Panel noted that the results from the BRI (2019, Documentation provided to EFSA n. 2) study are in line with those from Purkayastha et al. (2014) investigating the metabolism pathway of Rebaudioside M from leaves of the Stevia (EFSA ANS Panel, 2015a).

Considering that no safety concern was identified from the manufacturing process of Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract and that this is the same compound as Rebaudioside M extracted from of the leaves of the Stevia rebaudiana Bertoni plant, biological and toxicological data previously assessed by the ANS Panel for steviol glycosides (E960) were considered to support its safety. Therefore, no additional toxicological data were required. The existing ADI of 4 mg/kg bw per day can also be applied to Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract.

4. Conclusions

The Panel concluded that there is no safety concern for Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffii* UGT-a and *K. phaffii* UGT-b, to be used as a food additive. However, the Panel recommended that the European Commission consider establishing separate specifications for Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract in Commission Regulation (EU) No 231/2012.

Documentation provided to EFSA

1) Dossier 'Application for a change in the steviol Glycoside specification in the specification in the European Union to include a new manufacturing method for steviol glycosides including rebaudioside M'. January 2018. Submitted by SweeGen, Inc.
2) BRI Report no. SWE-2018-001, 2019. *In Vitro* Anaerobic Metabolism of Bioconversion Rebaudioside D and Rebaudioside M in Pooled Human Fecal Homogenates from Healthy Male and Female Adult Subjects. Unpublished report. Submitted within the application dossier.
3) Additional information on 14th January 2019. Submitted by SweeGen, Inc in response to a request from EFSA.
4) Additional information on 25th January 2019. Submitted by SweeGen, Inc in response to a request from EFSA.
5) Additional information on 9th April 2019. Submitted by SweeGen, Inc in response to a request from EFSA.
6) Additional information on 11th June 2019. Submitted by SweeGen, Inc in response to a request from EFSA.

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Abbreviations

ADI	acceptable daily intake
ANS	EFSA Panel on Food Additives and Nutrient Sources added to Food
BCA	bicinchoninic acid
BIOHAZ	EFSA Panel on Biological Hazards
BLAST	Basic Local Alignment Search Tool
bw	body weight
CAS	Chemical Abstracts Service
CFU	colony forming units
FAF	EFSA Panel on Food Additives and Flavourings
FAO	Food and Agriculture Organisation
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed

GMO	EFSA Panel on Genetically Modified Organism
HPLC	high-performance liquid chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC/MS	liquid chromatography–mass spectrometry
NOAEL	no observed adverse effect level
PCR	polymerase chain reaction
QPS	qualified presumption of safety
UDP	uridine diphosphate
UGT	UDP-glucuronosyltransferase
WHO	World Health Organization

Appendix A – Specifications proposed by the applicant

	Steviol glycosides (E 960) (Commission Regulation 231/2012)	**Specifications for E 960 Steviol Glycosides including expansion of the definition to include enzymatic bioconversion of purified stevia leaf extract as another manufacturing method as proposed by the applicant (Documentation provided to EFSA n. 5)**
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)	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. **In order to produce a higher yield of rebaudioside M, purified steviol glycoside leaf extracts may be subject to enzymatic bioconversion, utilising UDP-glucosyltransferase and sucrose synthase enzymes derived from strains of *Pichia pastoris* (QPS) that facilitate the transfer of glucose to steviol glycosides *via* glycosidic bonds** 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-	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-

	Steviol glycosides (E 960) (Commission Regulation 231/2012)			**Specifications for E 960 Steviol Glycosides including expansion of the definition to include enzymatic bioconversion of purified stevia leaf extract as another manufacturing method as proposed by the applicant (Documentation provided to EFSA n. 5)**		
	D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester 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			glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester 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		
Molecular formula	**Trivial name**	**Formula**	**Conversion factor**	**Trivial name**	**Formula**	**Conversion factor**
	Steviol	$C_{20}H_{30}O_3$	1.00	Steviol	$C_{20}H_{30}O_3$	1.00
	Steviolbioside	$C_{32}H_{50}O_{13}$	0.50	Steviolbioside	$C_{32}H_{50}O_{13}$	0.50
	Rubusoside	$C_{32}H_{50}O_{13}$	0.50	Rubusoside	$C_{32}H_{50}O_{13}$	0.50
	Dulcoside A	$C_{38}H_{60}O_{17}$	0.40	Dulcoside A	$C_{38}H_{60}O_{17}$	0.40
	Stevioside	$C_{38}H_{60}O_{18}$	0.40	Stevioside	$C_{38}H_{60}O_{18}$	0.40
	Rebaudioside A	$C_{44}H_{70}O_{23}$	0.33	Rebaudioside A	$C_{44}H_{70}O_{23}$	0.33
	Rebaudioside B	$C_{38}H_{60}O_{18}$	0.40	Rebaudioside B	$C_{38}H_{60}O_{18}$	0.40
	Rebaudioside C	$C_{44}H_{70}O_{20}$	0.34	Rebaudioside C	$C_{44}H_{70}O_{20}$	0.34
	Rebaudioside D	$C_{50}H_{80}O_{28}$	0.29	Rebaudioside D	$C_{50}H_{80}O_{28}$	0.29
	Rebaudioside E	$C_{44}H_{70}O_{23}$	0.33	Rebaudioside E	$C_{44}H_{70}O_{23}$	0.33
	Rebaudioside F	$C_{43}H_{68}O_{22}$	0.34	Rebaudioside F	$C_{43}H_{68}O_{22}$	0.34
	Rebaudioside M	$C_{56}H_{90}O_{33}$	0.25	Rebaudioside M	$C_{56}H_{90}O_{33}$	0.25
Molecular weight and CAS No	**Trivial name**	**CAS Number**	**Molecular weight**	**Trivial name**	**CAS Number**	**Molecular weight**
	Steviol		318.46	Steviol		318.46
	Steviolbioside	41093-60-1	642.73	Steviolbioside	41093-60-1	642.73
	Rubusoside	64849-39-4	642.73	Rubusoside	64849-39-4	642.73
	Dulcoside A	64432-06-0	788.87	Dulcoside A	64432-06-0	788.87
	Stevioside	57817-89-7	804.88	Stevioside	57817-89-7	804.88
	Rebaudioside A	58543-16-1	967.01	Rebaudioside A	58543-16-1	967.01
	Rebaudioside B	58543-17-2	804.88	Rebaudioside B	58543-17-2	804.88
	Rebaudioside C	63550-99-2	951.02	Rebaudioside C	63550-99-2	951.02

Molecular formula	**Trivial name**	**Formula**	**Conversion factor**	**Trivial name**	**Formula**	**Conversion factor**
	Rebaudioside D Rebaudioside E Rebaudioside F Rebaudioside M	63279-13-0 63279-14-1 438045-89-7 1220616-44-3	1,129.15 967.01 936.99 1,291.30	Rebaudioside D Rebaudioside E Rebaudioside F Rebaudioside M	63279-13-0 63279-14-1 438045-89-7 1220616-44-3	1,129.15 967.01 936.99 1,291.30
Assay	Not less than 95% steviolbioside, rubusoside, dulcoside A, stevioside, rebaudiosides A, B, C, D, E, F, and M, on the dried basis, in any combination and ratio			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)			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			Freely soluble to slightly soluble in water		
pH	Between 4.5 and 7.0 (1 in 100 solution)			Between 4.5 and 7.0 (1 in 100 solution)		
Purity						
Total ash	Not more than 1%			Not more than 1%		
Loss on drying	Not more than 6% (105°C, 2 h)			Not more than 6% (105°C, 2 h)		
Residual solvent	Not more than 200 mg/kg methanol			Not more than 200 mg/kg methanol		
	Not more than 5,000 mg/kg ethanol			Not more than 5,000 mg/kg ethanol		
Arsenic	Not more than 1 mg/kg			Not more than 1 mg/kg		
Lead	Not more than 1 mg/Kg			Not more than 1 mg/Kg		
Residual DNA				**Not more than 10 ng DNA/g product [*only applicable to rebaudioside M produced by enzymatic bioconversion of purified stevia leaf extract*]**		