Safety of the proposed amendment of the specifications for enzymatically produced steviol glycosides (E 960c): Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract

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Abstract

The EFSA Panel on Food Additives and Flavourings (FAF Panel) provides a scientific opinion on the safety of a proposed amendment of the specifications of enzymatically produced steviol glycosides (E 960c) with respect to the inclusion of rebaudioside D produced via enzyme-catalysed bioconversion of purified stevia leaf extract. Rebaudioside D (95% on dry basis) is produced via enzymatic bioconversion of purified stevia leaf extract using uridine diphosphate (UDP)-glucosyltransferase (UGT) and sucrose synthase enzymes produced by the genetically modified yeast K. phaffii UGT-A, that facilitates the transfer of glucose to purified stevia leaf extract via glycosidic bonds. The same enzymes from K. phaffii UGT-A may be used in the manufacturing process of the food additive, rebaudioside M produced via enzyme modification of steviol glycosides from stevia (E 960c(i)). The Panel considered that separate specifications would be needed for this food additive produced via the manufacturing process described in the current application, aligned with those already established for E 960c(i). The Panel concluded that there is no toxicological concern for Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract using UDP-glucosyltransferase and sucrose synthase produced by a genetically modified strain of the yeast K. phaffii. However, based on the available data, the Panel could not exclude the possibility that some residual amount of DNA coding for the kanamycin resistance gene could remain in the final product. Should this gene propagate in microbiota due to the presence of recombinant DNA in the final product, this would be of concern. Therefore, the Panel concluded that the safety of Rebaudioside D produced via this enzymatic bioconversion was not sufficiently demonstrated with the available data given that the absence of recombinant DNA was not shown.

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Keywords: steviol glycoside preparations, rebaudioside D, enzymatic bioconversion, yeast K. phaffii

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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 Panel) was asked to provide a scientific opinion on the safety of a proposed amendment of the specifications of the food additive enzymatically produced steviol glycosides (E 960c) to include Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract, 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 Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract as provided by the applicant in a newly submitted dossier and additional information submitted by the applicant during the assessment process in response to requests from EFSA.

Rebaudioside D (with a purity assay of not less than 95% on dry basis) is produced via enzymatic bioconversion of purified stevia leaf extract using uridine diphosphate (UDP)-glucosyltransferase (UGT) and sucrose synthase enzymes produced by the genetically modified yeasts K. phaffii UGT-A that facilitates the transfer of glucose to purified stevia leaf extract via glycosidic bonds. The same enzymes from K. phaffii UGT-A may be used in the manufacturing process of the food additive rebaudioside M produced via enzyme modification of steviol glycosides from stevia (E 960c(i)). The food additive manufactured according to the method described in the present application contains no less than 95% of rebaudioside D and minor amount of other steviol glycosides, such as rebaudioside M, A, E and B, may also be present in the final product. The Panel considered that the food additive manufactured according to the method described in the present application and Rebaudioside M produced via enzyme modification of steviol glycosides from stevia (E 960c(i)) are two different steviol glycosides preparations in terms of chemical composition and enzymes involved in the manufacturing. The Panel, therefore, considered that separate specifications would be needed for the food additive produced via the manufacturing process described in the current application, aligned with those already set for rebaudioside M produced via enzyme modification of steviol glycosides from stevia (E 960c(i)) in Regulation (EU) No 231/2012.

The Panel noted that adequate analytical data supporting the compliance with the provision for residual protein specifications were provided by the applicant. However, the Panel noted that the K. phaffii production strain contains a gene conferring resistance to kanamycin due to the genetic modification introduced. This is considered to be a possible safety concern because this gene could be able to spread in the environment due to the presence of viable cells of the production strain or its DNA in the final product. The applicant confirmed the absence of viable cells in the food additive. However, some positive signals were detected when looking for the presence of DNA in the food additive. Although those signals might be due to a laboratory contamination, they were observed in different independent experiments. Therefore, uncertainty remains with respect to the possibility that some residual amounts of DNA coding for the kanamycin resistance gene could remain in the final product, which would give rise to safety concerns.

Regarding toxic elements, the Panel noted that, based on the analytical data provided, the proposed maximum limits for lead, mercury and cadmium are adequate. For arsenic, the Panel noted that a lower maximum limit than the one proposed by the applicant should be set in the specifications for the food additive.

The absence of kaurenoic acid in three batches of rebaudioside D produced by enzymatic bioconversion from purified stevia leaf extract has been demonstrated using a HPLC method with an adequate detection limit (< 0.1 mg/kg).

The in vitro anaerobic metabolism of ‘bioconversion rebaudioside D’ was investigated in pooled human faecal homogenates. The authors concluded that the metabolism of ‘bioconversion rebaudioside D’ in this study indicated rapid deglycosylation to a final steviol metabolite.

In a 28-day dietary rat study retrieved from the literature (Nikiforov et al., 2013), no adverse effects were reported for rebaudioside D up to the highest dose tested, i.e. 2,000 mg/kg body weight (bw) per day, comparable to the outcome of a previous 90-day dietary rat study with rebaudioside A. Considering the similarity of the chemical structures and the toxicokinetics of rebaudioside D and rebaudioside A along with the limited toxicity data from the 28-day study on rebaudioside D, the Panel considered that a read-across from rebaudioside A to D is justified. Therefore, no additional toxicity studies are needed.

The existing ADI of 4 mg/kg bw per day can also be applied to Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract as described in the present opinion.
The Panel concluded that there is no toxicological concern for Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract using UDP-glucosyltransferase and sucrose synthase produced by a genetically modified strain of the yeast *K. phaffii*. However, based on the available data, the Panel could not exclude the possibility that some residual amount of DNA coding for the kanamycin resistance gene could remain in the final product. Should this gene propagate in microbiota due to the presence of recombinant DNA in the final product, this would be of concern. Therefore, the Panel concluded that the safety of Rebaudioside D produced via this enzymatic bioconversion was not sufficiently demonstrated with the available data given that the absence of recombinant DNA was not shown.
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1. Introduction

The present opinion deals with the safety evaluation of a proposed modification of the specifications following a new production process of an already authorised food additive, i.e. enzymatically produced steviol glycosides (E 960c).

1.1. Background and Terms of Reference as provided by the European Commission

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. 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 in food 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.

Steviol glycoside (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 specification 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 recrystallisation of the steviol glycoside.

The European Commission received a request vis-à-vis an amendment of the present specification of Steviol glycoside (E 960) to include a new manufacturing process for rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract (≥ 95% steviol glycosides).

The requested amendments are identical to those described in EFSA scientific opinion on rebaudioside M produced via enzyme-catalysed bioconversion of purified stevia leaf extract (EFSA FAF Panel, 2019), however, with the addition of rebaudioside D as an end product of the enzymatic bioconversion manufacturing process.

The enzymes (uridine 5’-diphospho(UDP)-glucosyltransferase and sucrose synthase) are derived from two strains of Komagataella 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 D. The resulting rebaudioside D undergoes a series of purification and isolation steps to produce the final rebaudioside D (≥ 95%) determined to be 200 times sweeter than sucrose.

Rebaudioside D is a minor glycoside that is present at very low levels in the stevia leaf but has more favourable sensory characteristics when compared to the major glycosides (i.e. Stevioside, rebaudioside A) and a taste profile that is more reflective of sucrose.

1.1.2. Terms of Reference

The European Commission request the European Food Safety Authority (EFSA) to perform a risk assessment to provide a scientific opinion on the safety of the proposed amendment of the specifications of the food additive Steviol glycoside (E 960), in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

1.2. Interpretation of the Terms of Reference

In the present scientific opinion, the Panel has evaluated the latest proposal submitted by the applicant to add rebaudioside D to the entry currently present in the EU specifications for \textit{rebaudioside D produced via enzyme-catalysed bioconversion of purified stevia leaf extract}. The Panel has replaced reference to E 960 with E 960a or E 960c, whenever appropriate.
M produced via enzyme modification of steviol glycosides from stevia (E 960c(i))’ (Documentation provided to EFSA n. 4).

1.3. Information on existing evaluations and authorisations

Steviol glycosides from Stevia (E 960a) are an authorised food additive in the EU according to Regulation (EC) No 1333/2008 on food additives. The food additive is obtained by water extraction of the leaves of the Stevia rebaudiana Bertoni plant. According to the specifications defined in Commission Regulation (EU) No 231/2012, it is described as: ‘containing not less than 95% of the identified 11 related steviol glycosides steviolbioside, rubusoside, dulcoside A, stevioside, rebaudiosides A, B, C, D, E, F and M on the dried basis, in any combination and ratio’.

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, was established, 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 (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 latest exposure assessment to steviol glycosides (E 960) was carried out by the EFSA ANS Panel in 2015 (EFSA ANS Panel, 2015b).

Rebaudioside A is authorised also as an EU flavouring substance ([FL-no: 16.113]) according to Regulation (EC) No 1334/2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods5 and it was assessed within Flavouring Group Evaluation 310 (FGE.310), considering the toxicological data set available for E 960 (EFSA CEF Panel, 2011).

In 2020, the Panel evaluated an application to amend the existing EU specifications for steviol glycosides to allow for the inclusion of 60 steviol glycosides identified in S. rebaudiana Bertoni leaves, including both ‘major’ and ‘minor’ steviol glycosides, that may comprise the assay value of not less than 95% total steviol glycosides. The Panel concluded that the overall metabolic fate of these steviol glycosides is the same, and therefore, it would be acceptable to use a read-across approach for the safety assessment of the 60 steviol glycosides and the ADI of 4 mg/kg bw per day would apply to all those steviol glycosides. However, the Panel noted at that time that the proposed change from 11 to 60 specified steviol glycosides, while maintaining an assay value of not less than 95% as proposed by the applicant, would allow less pure preparations of the food additive onto the market. According to the proposed change in specifications, there would remain a small but not insignificant fraction of the additive that was undefined and therefore could not be evaluated by the Panel. Therefore, while inclusion of the 60 steviol glycosides in the specifications for steviol glycoside (E 960) would not be of safety concern, the FAF Panel could not conclude on the safety of the proposed amendment to the specifications for steviol glycosides (E 960) as food additive if the purity assay value of not less than 95% for the total content of steviol glycosides was maintained (EFSA FAF Panel, 2020).

A new entry for 'enzymatically produced steviol glycosides (E 960c)' was added to Annex II to Regulation (EC) No 1333/2008 as amended by Commission Regulation (EU) 2021/1156 of 13 July 2021.6 This amendment to the Regulation is based on the conclusions from EFSA on the safety of a proposed amendment of the specifications of the food additive steviol glycosides (E 960) concerning rebaudioside M produced by enzyme modification of steviol glycosides, using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts K. phaffii UGT-A and K. phaffii UGT-B (EFSA FAF Panel, 2019). Regulation (EU) No 231/2012 was also amended accordingly, with the inclusion of a new entry for 'E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from Stevia'.

Assessment of a new application requesting an amendment of the specifications of steviol glycosides in order to include the enzymatic conversion of highly purified rebaudioside A and/or stevioside leaf extracts from the stevia plant to minor glycosides that are present in the leaf, including rebaudioside AM, was also completed by the Panel (EFSA FAF Panel, 2021) since receipt of the present mandate.

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5 Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354 31.12.2008, p. 34.
6 Commission Regulation (EU) 2021/1156 of 13 July 2021 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council and the Annex to Commission Regulation (EU) No 231/2012 as regards steviol glycosides (E 960) and rebaudioside M produced via enzyme modification of steviol glycosides from Stevia. OJ L 249, 14.7.2021, p. 87--98.
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, 2008, 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 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).

In 2017, JECFA 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). These specifications have been superseded in 2019 by new tentative JECFA specifications adopted jointly with a framework approach based on the different methods of production applied to the manufacturing of steviol glycosides, i.e. water extraction, fermentation, bioconversion and glucosylation (FAO and WHO, 2020).

The framework adopted in 2019 has been subsequently amended by JECFA at its 91st meeting in February 2021. Specifications for steviol glycosides manufacturing using four different methods have been established, including specifications for ‘Enzyme modified Steviol Glycosides’ (FAO and WHO, 2021).

2. Data and methodologies

2.1. Data

The present evaluation is based on the data submitted in the application dossier ('Documentation provided to EFSA' No 1) and additional information submitted by the applicant following requests by EFSA (Documentation provided to EFSA No 2-4).

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 Guidance documents 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 the 'Statement on the characterisation of microorganisms used for the production of food enzymes’ (EFSA CEP Panel, 2019) have been followed by the FAF 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 proposed food additive

The present evaluation deals with a new manufacturing process proposed by the applicant, which foresees the enzymatic bioconversion of purified *Stevia rebaudiana* Bertoni leaf extract (≥ 95% steviol glycosides), to obtain a high purity rebaudioside D preparation (containing not less than 95% of rebaudioside D). The enzymes involved are UDP-glucosyltransferase (UGT) and sucrose synthase which are produced by a genetically modified strain of the yeast *Komagatella phaffii* (*K. phaffii* formerly known as *Pichia pastoris*) that facilitates the transfer of glucose to purified stevia leaf extract via glycosidic bonds (Documentation provided to EFSA n. 1).

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7 According to JECFA specifications: ‘Rebaudioside A is obtained from the fermentation of a non-toxicogenic non-pathogenic strain of *Yarrowia lipolytica* that is genetically modified with heterologous genes from multiple donor organisms to [over]express steviol glycosides’.
Rebaudioside D is a minor naturally occurring steviol glycoside present in the leaves of *Stevia rebaudiana* Bertoni. It is an ent-kaurane diterpene glycoside with a steviol backbone conjugated to five glucose units, an ether at position C-13 and an ester at position C-19 (see Figure 1).

![Chemical structure of Rebaudioside D](image)

**Figure 1:** Chemical structure of Rebaudioside D

The following information on the chemical identity of rebaudioside D was provided by the applicant (Documentation provided to EFSA n. 1):

- **Chemical name**: 13-[(O-β-d-Glucopyranosyl-3-O-β-d-glucosylpyranosyl-β-d-glucosylpyranosyl)oxy]-kaur-16-en-18-oic acid, 2-O-β-d-glucosylpyranosyl-β-d-glucosylpyranosyl ester.
- **Common name**: Steviol glycosides.
- **Synonyms**: Rebaudioside D, Reb D, Stevia Reb D.
- **Chemical formula**: C\textsubscript{50}H\textsubscript{80}O\textsubscript{28}.
- **Molecular weight**: 1,129.15 Daltons.
- **CAS number**: 63279-13-0.

According to the applicant, the proposed food additive is a white to off-white powder, 202 times sweeter than sucrose and it meets the existing solubility requirement for steviol glycosides of ‘freely soluble to slightly soluble in water’ as per Commission Regulation (EU) No 231/2012 (Documentation provided to EFSA n. 1).

The applicant provided data on the steviol glycosides composition in five non-consecutive batches of the proposed food additive, analysed in triplicate, in accordance with a modified JECFA high performance liquid chromatography (HPLC) method (JECFA, 2010). According to these data, the content of rebaudioside D was over 98% and small amounts of other minor steviol glycosides were also detected in some samples e.g. rebaudioside A (< 2%) and rebaudioside B (< 0.5%) (Documentation provided to EFSA n. 1). The applicant also provided the analysis of five further batches using the most recent JECFA method (JECFA, 2017) which foresees the determination of major steviol glycosides by HPLC using commercially available standards (method A) and determination of minor steviol glycosides by HPLC/MS (method B) (Documentation provided to EFSA n. 3). In the five further batches, the content of rebaudioside D was > 98%, rebaudioside M was approximately 1.5% and the rebaudioside E peak areas were not quantifiable. The applicant indicated that the presence and identity of other minor steviol glycosides may stem from small variations in the manufacturing and purification steps and from variations in the composition of the starting stevia leaf extract material (≥ 95% steviol glycosides). In any case, the final product will always contain not less than 95% of rebaudioside D and minor amount of other steviol glycosides, such as rebaudioside M, A, E and B (Documentation provided to EFSA n. 1 and 3).
The Panel noted that the method of analysis used for the characterisation of the second set of five samples was not fully in accord with the latest JECFA HPLC method (JECFA, 2017) since it was lacking quantification of the minor peaks using the appropriate standards but, instead, quantification was based on the area percentage of the peaks in the HPLC chromatograms. Despite this and other minor shortcomings, the Panel considered overall that the data are sufficiently robust to demonstrate that the rebaudioside D purity in the final product is consistently higher than 95%.

The Panel noted that the yeast strain A (K. phaffii UGT-A) and the UGT-A fusion enzyme used in the present manufacturing process are identical to those previously assessed by the Panel for the safety evaluation of rebaudioside M produced by enzymatic bioconversion from purified stevia leaf extract (EFSA FAF Panel, 2019) and that also the same fermentation conditions are applied (i.e. [insert specific conditions]). Therefore, further information was sought by the applicant to understand how the bioconversion is selectively driven to produce predominantly rebaudioside D (and no other steviol glycosides).

According to the applicant, the raw starting material used as substrate for the enzymatic bioconversion is a stevia leaf extract containing ≥ 95% total steviol glycosides (primarily stevioside and/or rebaudioside A). The applicant stated that the UGT-A enzyme specifically converts the rebaudioside A (with four glucose molecules bound to the steviol backbone), present in the starting stevia leaf extract, to rebaudioside D (with five glucose molecules) in a single step by addition of a single glucose unit to rebaudioside A. Therefore, according to the applicant, if the starting stevia leaf extract contains a higher content of rebaudioside A, the bioconversion to rebaudioside D will be more efficient. The applicant also indicated that the reaction mixture resulting from the enzymatic bioconversion of the stevia extract consists mainly of rebaudioside D and may also contain trace amounts of other steviol glycosides such as rebaudioside A, E, B and M. By exploiting the difference in solubility of the different rebaudiosides in the solvent used in the crystallisation step, the predominant rebaudioside D compound can be crystallised while removing the other minor steviol glycosides (rebaudioside A, E, B and M) (Documentation provided to EFSA n. 2).

### 3.1.2. Proposed amendment to the specifications

The Panel noted that specifications have been set in Regulation (EU) No 231/2012 for ‘E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from stevia’ (see Table 1).

The applicant indicated that, since the same enzyme produced from the same production strain (i.e. UGT-A fusion enzyme from K. phaffii UGT-A) is used to manufacture both rebaudioside M and rebaudioside D, it is proposed that rebaudioside M and rebaudioside D would be captured under the same specifications, ‘E 960c(i) Rebaudioside M and D produced via enzyme modification of steviol glycosides from stevia’ (Documentation provided to EFSA n. 4).

| Table 1: Specifications for ‘E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from stevia’ as set in Commission Regulation (EU) No 231/2012 and proposed amendment to the specifications (Documentation provided to EFSA n. 4) |
|-------------------------------------------------|-------------------------------------------------|
| **Existing specifications for E 960c(i) as set in Commission Regulation (EU) No 231/2012** | **Proposed amendment to the specifications** |
| **E 960c(i)** | Rebaudioside M produced via enzyme modification of steviol glycosides from Stevia | Rebaudioside M and D produced via enzyme modification of steviol glycosides from Stevia |
| **Definition** | Rebaudioside M is a steviol glycoside composed predominantly of rebaudioside M with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside B, rebaudioside D, rebaudioside I and stevioside. | Rebaudioside M is a steviol glycoside composed predominantly of rebaudioside M with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside B, rebaudioside D, rebaudioside I and stevioside. Rebaudioside D is a steviol glycoside composed predominantly of rebaudioside D with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside B and rebaudioside M. |
Existing specifications for E 960c(i) as set in Commission Regulation (EU) No 231/2012

Rebaudioside M is obtained via enzymatic bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides) of the Stevia rebaudiana Bertoni plant using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts K. phaffi (formerly known as Pichia pastoris) UGT-A and K. phaffi UGT-B that facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds.

After removal of the enzymes by solid–liquid separation and heat treatment, the purification involves concentration of the rebaudioside M resulting in a final product containing not less than 95% of rebaudioside M. Viable cells of the yeasts K. phaffi UGT-A and K. phaffi UGT-B or their DNA shall not be detected in the food additive.

Proposed amendment to the specifications

Rebaudioside M or D is obtained via enzymatic bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides) of the Stevia rebaudiana Bertoni plant using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts K. phaffi (formerly known as Pichia pastoris) UGT-A and/or K. phaffi UGT-B that facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds.

Rebaudioside M is produced using enzymes from both yeasts, whereas rebaudioside D is produced using enzymes only from K. phaffi UGT-A.

After removal of the enzymes by solid–liquid separation and heat treatment, the purification involves concentration of the rebaudioside M or D by resin adsorption, followed by recrystallisation of rebaudioside M resulting in a final product containing not less than 95% of rebaudioside M or not less than 95% rebaudioside D. Viable cells of the yeasts K. phaffi UGT-A and/or K. phaffi UGT-B or their DNA shall not be detected in the food additive.

### Chemical name

| Chemical name | 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 | 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-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester |
|----------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|

### Molecular formula

| Molecular formula | Trivial name | Formula | Conversion factor | Trivial name | Formula | Conversion factor |
|------------------|--------------|---------|------------------|--------------|---------|------------------|
| Rebaudioside M   | C_{56} H_{90} O_{33} | 0.25 | Rebaudioside D   | C_{50} H_{80} O_{28} | 0.29 |
| Rebaudioside M   | C_{56} H_{90} O_{33} | 0.25 |

### Molecular weight and CAS No

| Molecular weight and CAS No | Trivial name | CAS number | Molecular weight (g/mol) | Trivial name | CAS number | Molecular weight (g/mol) |
|-----------------------------|--------------|------------|--------------------------|--------------|------------|--------------------------|
| Rebaudioside M              | C_{56} H_{90} O_{33} | 1,291.29 | Rebaudioside D           | 63279-13-0   | 1,129.15   |
| Rebaudioside M              | C_{56} H_{90} O_{33} | 1,291.29 |

### Assay

| Assay | Not less than 95% rebaudioside M on the dried basis. | Not less than 95% rebaudioside M on the dried basis or not less than rebaudioside D, on the dried basis. |
|-------|------------------------------------------------------|---------------------------------------------------------------------------------------------------|
The applicant submitted analytical data from the analyses of five non-consecutive batches of the final rebaudioside D product (Documentation provided to EFSA n. 1). Based on the data submitted, the Panel considered that rebaudioside D produced by via enzymatic bioconversion of purified stevia leaf extracts is consistently produced and compliant with the proposed specifications, as outlined in Table 1.

The Panel noted the proposal from the applicant for expanding existing EU specifications for ‘E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from stevia’ to add rebaudioside D produced by via enzymatic bioconversion of purified stevia leaf extracts (see Table 1). In this respect, the Panel considered that a separate entry for rebaudioside D produced via enzymatic bioconversion of steviol glycosides from stevia should be established in the applicable legislation. The food additive (i.e. rebaudioside D) manufactured according to the proposed production method and rebaudioside M produced via enzyme modification of steviol glycosides from stevia are two different steviol glycoside preparations in terms of chemical composition and enzymes involved in the manufacturing (the proposed food additive is produced using enzymes only from K. phaffii UGT-A, whereas rebaudioside M production process makes use of enzymes from K. phaffii UGT-A and/or K. phaffii UGT-B).

According to the applicant, the food additive manufactured according to the method described in the present application contains no less than 95% of rebaudioside D. Minor amounts of other steviol glycosides, such as rebaudioside M, A, E and B, may also be present in the final product. The Panel noted that the data submitted from the analysis of steviol glycosides composition in 10 batches of the proposed food additive (see Section 3.1.1) fulfill such declared purity (Documentation provided to EFSA n. 1 and 3). Therefore, the Panel considered the proposed purity assay of ‘not less than 95% of rebaudioside D’ adequate.

Regarding toxic elements, the Panel noted that analytical data on the content of arsenic, lead, cadmium and mercury were provided for five batches of the proposed food additive (Documentation provided to EFSA n. 1). Based on these data, the Panel considered the proposed maximum limits for lead, mercury and cadmium to be adequate. For arsenic, the Panel noted that a lower maximum limit than the one proposed by the applicant should be set in the EU specifications for the food additive. The potential exposure to these impurities were compared against the available RPs and HBGVs (see Section 3.3.1) (Table 3).

Food-grade ethanol is used as a desorption and crystallisation solvent during the manufacturing process for rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract. The Panel noted that, in five non-consecutive batches of the proposed food additive, the residual levels of ethanol were consistently less than 20 mg/kg, which is well below the proposed maximum limit of 5,000 mg/kg.

Five non-consecutive batches of rebaudioside D obtained by enzymatic bioconversion were analysed for the presence of possible microbiological contaminants. Total plate count, yeast and mould, total coliforms and individual types of microorganisms, including E. coli and Salmonella, were consistently not detectable in any tested sample (Documentation provided to EFSA n. 1).
The proposed food additive has been tested for residual proteins using the BCA assay with a limit of detection (LOD) of 5 mg/kg to confirm that the processing enzymes (UDP-glucosyltransferase and sucrose synthase), or associated protein impurities, have been effectively removed from the finished product (Documentation provided to EFSA n. 1). No protein residues were detected above the LOD in five non-consecutive batches of the proposed food additive. The Panel noted that a provision for residual proteins is already included in the existing specifications for E 960c(i), i.e. not more than 5 mg/kg.

Data on the absence of viable cells and recombinant DNA of the enzyme production yeast strain in the end product were provided by the applicant (Documentation provided to EFSA n. 1-4) and summarised below in Section 3.1.3.3.

The Panel noted that the absence of viable cells/residual DNA of the enzymes production microorganism in the final product is captured in the proposed definition, where it is stated 'Viable cells of the yeasts K. phaffii UGT-A (…) or DNA shall not be detected in the food additive'. The absence of viable cells was adequately demonstrated by the applicant. Experiments to confirm the absence of recombinant DNA were inconclusive, with K. phaffii UGT-A DNA encoding the kanamycin resistance gene still detected in some of the samples tested (see Section 3.1.3.2). Since uncertainty remains on the possible presence of DNA in the final rebaudioside D preparation, the Panel could not exclude the possibility that some residual amount of DNA coding for the kanamycin resistance gene could remain in the final product. Should this gene be propagated in microbiota due to its presence in the final product, this would be of concern.

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 E 960 took place in 2018. No kaurenoic acid was detected in three samples of rebaudioside D produced by enzymatic conversion from purified stevia leaf extracts analysed by HPLC- Ultraviolet (UV) with a limit of detection (LOD) of 0.1 mg/kg. (Documentation provided to EFSA n. 1).

In view of the botanical origin of the stevia extract used as starting material, limits for the presence of pesticides should be considered. Five non-consecutive batches of rebaudioside D produced by enzymatic bioconversion from purified stevia leaf extracts were analysed for the presence of residues of commonly used pesticides; no pesticide residues were detected (Documentation provided to EFSA n. 1). The Panel noted that maximum residue levels (MRLs) for pesticides set under Regulation (EC) 396/2005 apply to stevia (Stevia rebaudiana), as 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.

### 3.1.3. Manufacturing process

Rebaudioside D, subject of the present assessment, is manufactured by enzymatic bioconversion of purified stevia leaf extracts (≥ 95% steviol glycosides). The enzymes involved, UDP-glucosyltransferases (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13), are derived from a genetically modified strain of Komagaetella phaffii; its characterisation data are reported in Section 3.1.3.3.

UDP-glucosyltransferase facilitates the transfer of glucose from an activated donor molecule (e.g. UDP-glucose) to the acceptor molecule steviol transfer glucose (Richman et al., 2005). Sucrose synthase ensures the availability of UDP-glucose by catalysing the conversion of UDP and sucrose to fructose and UDP-glucose (Wang et al., 2016).

According to the applicant, all raw materials, processing aids and purification equipment used in the manufacturing process are compliant with internationally recognised specifications standards (e.g. JECFA, CODEX, EU and US Pharmacopeia) (Documentation provided to EFSA n. 1).

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8 [https://www.efsa.europa.eu/en/events/event/180611-0](https://www.efsa.europa.eu/en/events/event/180611-0)
9 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, p. 1-16.
3.1.3.1. Description of the manufacturing process

Stage 1 – Enzymes 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 the steviol glycosides present in purified stevia leaf extract to rebaudioside D.

The strain and the UGT-A fusion enzyme are identical to those previously assessed by the Panel for the safety evaluation of rebaudioside M (EFSA FAF Panel, 2019).

Stage 2 – Rebaudioside D production

A) Bioconversion of Purified Stevia Leaf Extract to Rebaudioside D

For the catalytic reaction needed to convert purified stevia leaf extract to rebaudioside D, UGT-A fusion enzyme is mixed with the reaction buffer under 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 D is collected in a storage tank and is heated ( ) to denature the enzymes. The mixture is filtered to remove the denatured enzymes.

B) Rebaudioside D Purification

The filtrate is loaded onto columns containing a macroporous resin. The column is rinsed with a series of buffer solutions and rebaudioside D 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 D to crystallise and precipitate from the solution. The wet crystals are collected, washed and dissolved in ethanol. The redissolved rebaudioside D is treated with activated charcoal to remove remaining impurities, recrystallised, dried and processed to the final high-purity rebaudioside D product (≥ 95%).

3.1.3.2. Raw materials and processing aids

Carbohydrate Source

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

Resins

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

3.1.3.3. Characterisation of the enzymes production organism

The enzymes UDP glucosyltransferase (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13) (EC 2.4.1.13) are produced by a genetically modified Komagaetella phaffii strain. The enzymes are used to convert purified stevia leaf extract to rebaudioside D.

The UGT-A fusion enzyme is obtained from the genetically modified K. phaffii strain A. The strain is deposited in the China Center for Type Culture Collection as K. pastoris UGT-A, with deposition number CCTCC M2017681. The strain was identified by sequence analysis of the 26S rRNA gene, the mitochondrial small subunit rRNA gene, partial sequence of the sequence with accession EF547706.1, partial sequence of the translation elongation factor Tf 1α- gene, the RNA polymerase I gene and partial sequence of the sequence with GQ327957.1 (Documentation provided to EFSA n. 1-2).

Characteristics of the recipient strain

The parental microorganism is the yeast K. phaffii ATCC20864. The recipient strain was obtained by selection for a spontaneous mutant with resistance to 10% steviol. 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, 2022).

Characteristics of the donor sequences

The UGT-A fusion enzyme consists of a sequence encoding UDP glucosyltransferase from barley (Hordeum vulgare) (catalyses the conversion of stevioside to rebaudioside E) in frame with the SUS gene from bean (Vigna radiata) catalysing the glycosylation of UDP. The expression cassette UGT-A contains the AOX1 (alcohol oxidase) promoter, the α-factor signal for protein secretion from
**Sachharomyces cerevisiae**, in frame with the GCW61 gene encoding a cell wall protein from *K. phaffii* and the AOX1 terminator from *K. phaffii*.

The plasmid vector pHKA-UGTA derives from the expression vector pPIC9A and contains five copies of the expression cassette of UGT-A, 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 in *E. coli*.

**Description of the genetic modification process**

The plasmid pHKA-UGTA was linearised, transformed into the recipient strain and integrated into the HIS4 locus of the recipient strain. After screening for positive transformants, the production strain *K. phaffii* strain A was obtained.

**Safety aspects of the production strains**

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

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

**Absence of viable cells of the production strains in the end product**

The absence of the production microorganisms in the final rebaudioside D 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 (Documentation provided to EFSA n. 1-2).

**Absence of DNA of the production strain in the end product**

The absence of recombinant DNA in the rebaudioside D product was analysed by polymerase chain reaction (PCR) analysis of five batches, tested in triplicate, with primers that would amplify a 750 bp fragment specific for the kanamycin resistance gene (Documentation provided to EFSA n. 2). The tests were designed to have a limit of detection in the range of 10 ng spiked DNA of the production strain/g of product. Recombinant DNA was detected, including in the blank samples with water, and therefore, the tests were considered invalid. The applicant was requested to provide a second set of experiments; however, the same problem persisted (Documentation provided to EFSA n. 3). A third set of experiments also reported similar inconclusive results, with recombinant DNA still detected in some of the samples tested (Documentation provided to EFSA n. 4).

On the basis of the data provided, the Panel was unable to assess whether recombinant DNA of the production strain remains in the food additive. The Panel noted that the production strain carries an antimicrobial resistance (AMR) gene.

**3.1.4. Method(s) of analysis in food**

While no information on a method of analysis for rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract in food was provided by the applicant, the Panel assumed that methods of analysis available for other steviol glycosides preparations would also be applicable.

**3.1.5. Stability, reaction and fate in food of the proposed food additive**

A 6-month accelerated stability study was conducted on five batches of rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract (testing conditions: samples stored at 40 ± 2°C at a relative humidity of 75 ± 5%). The proposed food additive was reported to be stable in terms of appearance, moisture content and percentage of rebaudioside D, measured by HPLC, under the tested conditions and time span (Documentation provided to EFSA n. 1).
3.2. Proposed uses and use levels

Maximum levels of steviol glycosides (E 960) expressed as steviol equivalents are defined in Annex II to Regulation (EC) No 1333/2008.10 Rebaudioside D 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 (Documentation provided to EFSA n. 1).

3.3. Exposure data

Because the proposed uses and use levels for rebaudioside D 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 (EFSA ANS Panel, 2015b).

The Panel considers that if steviol glycosides would be replaced by rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract, exposure to rebaudioside D (expressed as steviol equivalent) will not be higher than the last EFSA estimate of exposure to steviol glycosides (E 960) (EFSA ANS Panel, 2015b). At that time, based on the maximum permitted levels (MPLs), the ANS Panel concluded that the conservative estimates of the exposure (mean, 95th percentile) to steviol glycosides (E 960) were below the ADI of 4 mg/kg bw per day in all population groups, except for toddlers at the upper range of the exposure estimates in one country (4.3 mg/kg bw per day).

3.3.1. Anticipated exposure to toxic elements from proposed specifications

The applicant provided analytical data on the content of arsenic (As, < 0.02–0.09 mg/kg), lead (Pb, 0.10–0.16 mg/kg), cadmium (Cd, < 0.01 mg/kg) and mercury (Hg, < 0.01 mg/kg) and proposed maximum limits for these elements to be included in the proposed amendment of the specifications for the food additive (see Table 1). The potential exposure to the toxic elements from the use of the proposed food additive can be calculated by assuming contamination of the additive may be up to (i) the highest reported analytical data or (ii) the specification limit values proposed, and then by calculation pro-rata to the estimate of exposure to the proposed food additive itself (EFSA ANS Panel, 2015b).

As noted above, if steviol glycosides would be replaced by the rebaudioside D preparations as described in the present application, exposure (expressed as steviol equivalent) will not be higher than the last EFSA estimate of exposure to steviol glycosides (E 960a). The scenario calculated by the ANS Panel using MPLs and the proposed extension of use (toddlers, 95th percentile) resulted in a highest estimated exposure of 4.3 mg/kg bw per day expressed as steviol equivalents (EFSA ANS Panel, 2015a,b).

The current application concerns high purity Rebaudioside D preparations containing not less than 95% of Rebaudioside D. Since steviol itself has a molecular weight of 318.45 Da and Rebaudioside D has a MW of 1,129 Da, the steviol equivalency of Rebaudioside D is 0.29 (conversion factor, see Table 1). Therefore, an exposure of 4.3 mg/kg bw per day expressed as steviol equivalents equates to approximately 15 mg/kg bw per day expressed as Rebaudioside D.

The level of the toxic element in the food additive combined with the estimated intakes of steviol glycosides, presented in Table 3, could result in an exposure which can be compared with the following reference points (RPs) or health-based guidance values (HBGVs) (Table 2).

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10 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.
The risk assessment of the undesirable impurities helps inform whether there could be a possible health concern if these impurities and constituents would be present at the limit values in the food additive. The assessment is performed by calculating the MOE by dividing the RP (e.g. BMDL, see Table 2) by the exposure estimate (see Table 3), or by estimating the contribution of the use of E 960 to the HBGV (expressed as percentage of the HBGV).

The Panel emphasised that the choice of maximum limits for toxic elements in the specifications is in the remit of risk management. The numbers used here were merely taken to support the risk assessment of these toxic elements as presented below.

Table 2: Reference points/health-based guidance value for toxic elements present in (E 960c)

| Impurity/constituent/ HBGV/RP (µg/kg bw) | Basis/Reference                                                                 |
|-----------------------------------------|---------------------------------------------------------------------------------|
| Lead (Pb)/ 0.5 (BMDL01)                | The reference point is based on a study demonstrating perturbation of intellectual development in children with the critical response size of 1 point reduction in IQ. The EFSA CONTAM Panel mentioned that a 1 point reduction in IQ is related to a 4.5% increase in the risk of failure to graduate from high school and that a 1 point reduction in IQ in children can be associated with a decrease of later productivity of about 2%. A risk cannot be excluded if the exposure exceeds the BMDL01 (MOE lower than 1). EFSA CONTAM Panel (2010) |
| Mercury (Hg)/ 4 (TWI)                  | The HBGV was set using kidney weight changes in male rats as the pivotal effect. Based on the BMDL10 of 0.06 mg/kg bw per day, expressed as mercury, and an uncertainty factor of 100 to account for inter- and intra-species differences, with conversion to a weekly basis and rounding to one significant figure, a TWI for inorganic mercury of 4 µg/kg bw per week, expressed as mercury was established. EFSA CONTAM Panel (2012) |
| Cadmium (Cd)/ 2.5 (TWI)               | The derivation of the reference point is based on a meta-analysis to evaluate the dose–response relationship between selected urinary cadmium and urinary beta-2-microglobulin as the biomarker of tubular damage recognised as the most useful biomarker in relation to tubular effects. A group-based BMDL3 of 4 µg Cd/g creatinine for humans was derived. A chemical-specific adjustment factor of 3.9 was applied to account for human variability in urinary cadmium within each dose-subgroup in the analysis resulting in a reference point of 1.0 µg Cd/g creatinine. In order to remain below 1 µg Cd/g creatinine in urine in 95% of the population by age 50, the average daily dietary cadmium intake should not exceed 0.36 µg Cd/kg bw, corresponding to a weekly dietary intake of 2.5 µg Cd/kg bw. EFSA CONTAM Panel (2009a) |
| Arsenic (As)/ 0.3-8 (BMDL01)           | The reference point is based on a range of benchmark dose lower confidence limit (BMDL01) values between 0.3 and 8 µg/kg bw per day identified for cancers of the lung, skin and bladder, as well as skin lesions. In general, the MOE should be at least 10,000 if the reference point is based on carcinogenicity in animal studies. However, as the BMDL for As is derived from human studies, an interspecies extrapolation factor (i.e. 10) is not needed. EFSA CONTAM Panel (2009b), EFSA Scientific Committee (2012) |

HBGV: health-based guidance value; RP: reference point; BMDL01: benchmark dose (lower confidence limit); bw: body weight; TWI: Tolerable Weekly Intake; TDI: Tolerable Daily Intake; MOE: margin of exposure.
For arsenic, the calculated MOE values can fall below the target of 1,000 (lower bounds in Table 3); therefore, the Panel considered that a lowering of the proposed maximum limit for arsenic would be appropriate and, based on the analytical data provided, this seems also technologically achievable.

For lead, the calculated MOE is well above 1 in both scenarios, i.e. considering the analytical data and the proposed specification limits (see Table 3).

For Hg and Cd, the resulting estimates of exposure are only a marginal fraction of their TWIs (see Table 3).

### 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 (Documentation provided to EFSA n. 1).

#### 3.4.1. Absorption, distribution, metabolism and excretion (ADME)

Data on ADME of some of the steviol glycosides currently listed in the EU specifications have been considered and summarised in previous EFSA opinions (EFSA ANS Panel 2010, 2015a; EFSA FAF Panel, 2019, 2020, 2021). According to these opinions, steviosides and steviol glycosides are not hydrolysed by digestive enzymes of the upper gastrointestinal tract due to the presence of β-glycosidic bonds. After entering the colon intact, steviol glycosides are subject to microbial degradation by the gut microbiome, resulting in the release of the aglycone steviol which is then absorbed. In rats and humans, absorbed steviol is glucuronidated; steviol glucuronide is then excreted in the urine and partly via bile into the faeces.

The microbial hydrolysis of different steviol glycosides, in particular rebaudiosides A, B, C, D, E, F, M, steviolbioside and stevioside (with different purity levels or purity not specified) has been investigated in vitro with human faecal incubations (Purkayastha et al., 2014, 2016; Purkayastha and Kwok, 2020). The results demonstrate efficient deglycosylation/hydrolysis of these steviol glycosides in the presence of colonic microbiota collected from adults or children to the final stable metabolite steviol.

In vitro metabolic studies in human faecal homogenate samples incubated with different steviol glycosides preparations, including rebaudioside D, have been previously assessed by the Panel for the evaluation of other proposed amendments to the specifications of the food additive steviol glycosides (E 960) (EFSA FAF Panel, 2019, 2020, 2021). In all these studies, the deglycosylation of the steviol glycosides to the final steviol metabolite was shown to happen within the first 12 h of metabolic incubation.

**In vitro study submitted by the applicant**

In support of the current application, data from an in vitro metabolic study in human faecal homogenate samples performed with bioconversion rebaudioside D and bioconversion rebaudioside M have been submitted (Documentation provided to EFSA n. 1). In the study, bioconversion rebaudioside D (purity 98.2%) and rebaudioside M (purity 97.8%) were incubated with adult male and...
adult female pooled faecal homogenate samples (six male and six female donors) at concentrations of 0.2 mg/mL under anaerobic conditions at 37°C for 4–72 h. Rebaudioside A was used as a metabolic activity positive control in parallel to ensure that the experimental incubation conditions were satisfactory. Liquid chromatography-mass spectrometry (LC/MS) analysis was used to provide metabolic mass balance on the molar equivalent formation of the steviol metabolite over the time course. According to the study authors, no apparent difference in the extent of deglycosylation was noted in the pooled faecal homogenates between adult male and adult female donors.

The authors concluded that the metabolism of 'bioconversion rebaudioside D' in pooled male and female human faecal homogenates indicated rapid deglycosylation of the steviol glycosides to the final metabolite steviol over the first 8 h of metabolic incubation.

The Panel noted these results are consistent with those previously considered in other scientific opinions (EFSA FAF Panel, 2019, 2020, 2021) and obtained under the same experimental conditions with rebaudioside D and related steviol glycosides from stevia leaf extract.

Additional information from literature

In addition to the data provided by the applicant, the Panel considered the results from a published study comparing the metabolism and toxicological profile of rebaudioside D and rebaudioside A in several in vitro models and in a 28-day toxicity study (Nikiforov et al., 2013). The study by Nikiforov et al. (2013) supports the information as presented in the section above.

3.4.2. Toxicological data

No toxicity studies on Rebaudioside D from stevia extract or produced via the enzymatic bioconversion of purified leaf extract described in the present application were submitted. The metabolic fate of steviol glycosides, including rebaudioside D, leads to the aglycone which is absorbed. Given the similarities in metabolic fate of steviol glycosides, a read-across with regard to toxicity was considered applicable considering the availability of toxicity studies on other previously evaluated steviol glycosides (EFSA ANS Panel, 2010).

To further support the read-across of data from rebaudioside A to rebaudioside D, the Panel considered data from a published 28-day dietary toxicity study in rats performed in accordance to U.S Food and Drugs Administration Redbook 2000 guidelines and the Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (Nikiforov et al., 2013). The absence of adverse effects of rebaudioside D in this 28-day study (using doses up to 2,000 mg/kg bw per day) along with the similarity of the toxicokinetic data of rebaudioside D and rebaudioside A would support the read-across.

3.4.3. Other studies

The applicant has provided a selection of publications investigating the protective effects of steviol glycosides in different experimental studies (Philippaert et al. (2017); El-Mesallamy et al., 2018; Zhao et al., 2018), as well as effects on food intake in goats (Han et al., 2019). The Panel noted that none of the endpoints reported in these publications provided information that was considered relevant for the current safety assessment.

3.5. Discussion

The subject of the present application is a proposal for amending the existing EU specifications of the food additive E 960c(i) to include a new manufacturing process, which foresees the enzymatic bioconversion of purified Stevia rebaudiana Bertoni leaf extract (≥ 95% steviol glycosides), to obtain a high purity rebaudioside D preparation (containing not less than 95% of rebaudioside D). Rebaudioside D is a minor steviol glycoside present in the leaves of the Stevia plant. The enzymes involved are uridine diphosphate (UDP)-glucosyltransferase and sucrose synthase which are produced by a genetically modified strain 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.

Such rebaudioside D preparation is primarily comprised by rebaudioside D (≥ 95%) and may also contain trace amounts of other minor steviol glycosides, such as rebaudioside A, E, B and M, which occur in the starting stevia leaf extract material. The presence and identity of these other minor steviol glycosides is depending upon small variations in the manufacturing and purification steps and from variations in the composition of the starting stevia leaf extract material.
The Panel considered that the proposed manufacturing process applied to the production of rebaudioside D subject of the present evaluation involves enzymatic bioconversion steps of purified stevia leaf extract. This process may result in impurities different from those that may be present in steviol glycosides obtained by water extraction of the leaves of the Stevia plant (E 960a). In this respect, the Panel noted the proposal by the applicant to amend the entry in the existing applicable legislation for ‘E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from stevia’. The Panel would rather recommend the European Commission to consider whether a separate entry for Rebaudioside D produced via enzymatic bioconversion of steviol glycosides from stevia should be established in the applicable legislation. The food additive manufactured according to the method described in the present application and Rebaudioside M produced via enzyme modification of steviol glycosides from stevia (E 960c(i)) are two different steviol glycoside preparations in terms of chemical composition and enzymes involved in the manufacturing (the proposed food additive is produced using enzymes only from K. phaffii UGT-A, whereas Rebaudioside M using enzymes from K. phaffii UGT-A and/or K. phaffii UGT-B).

The Panel noted that the amendment to the specifications proposed by the applicant also contains parameters related to the specific genetically modified microorganism used to produce the enzyme involved in the bioconversion steps (i.e. absence of viable cells of the yeast K. phaffii UGT-A and/or K. phaffii UGT-B or their DNA; not more than 5 mg/kg of residual protein), which are aligned with the EU specifications for ‘E 960c(i) rebaudioside M produced via enzyme modification of steviol glycosides from stevia’, as laid down in Regulation (EU) No 231/2012.

The Panel noted that adequate analytical data supporting the compliance with the provision for residual protein specifications were provided by the applicant. However, the Panel noted that the K. phaffii production strain contains a gene conferring resistance to kanamycin due to the genetic modification introduced. This is considered to be a possible safety concern because this gene could be able to spread in the environment due to the presence of viable cells of the production strain or its DNA in the final product. The applicant confirmed the absence of viable cells in the food additive. However, some positive signals were detected when looking for the presence of DNA in the food additive. Although those signals might be due to a laboratory contamination, they were observed in different independent experiments. Therefore, uncertainty remains with respect to the possibility that some residual amounts of DNA coding for the kanamycin resistance gene could remain in the final product, which would give rise to safety concerns.

The Panel noted that the data submitted from the analysis of steviol glycosides composition in 10 batches of the proposed food additive fulfilled the declared purity of ‘not less than 95% of rebaudioside D’. Therefore, the Panel considered the proposed purity assay to be adequate.

Regarding toxic elements, the Panel noted that based on the analytical data provided, the proposed maximum limits for lead, mercury and cadmium are adequate. For arsenic, the Panel noted that a lower maximum limit than the one proposed by the applicant should be set in the EU specifications for the food additive. The potential exposure to these impurities were compared against the available RPs and HBGVs (Tables 2 and 3).

The absence of kaurenoic acid in three batches of rebaudioside D produced by enzymatic bioconversion from purified stevia leaf extract has been demonstrated using a HPLC method with an adequate detection limit (< 0.1 mg/kg).

The in vitro metabolic study of ‘bioconversion rebaudioside D’ was investigated in pooled human faecal homogenates. The authors concluded that the metabolism of ‘bioconversion rebaudioside D’ in this study indicated rapid deglycosylation to a final steviol metabolite. These results are consistent with those previously considered in other scientific opinions of the Panel (EFSA FAF Panel, 2019, 2020, 2021) and obtained under the same experimental conditions – with rebaudioside D isolated from stevia leaf extract (Purkayasta et al. 2014).

In a 28-day dietary rat study (Nikiforov et al., 2013), no adverse effects were reported for rebaudioside D up to the highest dose tested, i.e. 2,000 mg/kg bw per day, comparable to the outcome of a previous 90-day dietary rat study with rebaudioside A (Nikiforov and Eapen, 2008).

Considering the similarity of the chemical structures and the toxicokinetics of rebaudioside D and rebaudioside A along with the limited toxicity data from the 28-day study on rebaudioside D, the Panel considered that a read-across from rebaudioside A to D is justified. Therefore, no additional toxicity studies are needed.

The existing ADI of 4 mg/kg bw per day can also be applied to Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract as described in the present opinion.
4. Conclusions

The Panel concluded that there is no toxicological concern for Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract using UDP-glucosyltransferase and sucrose synthase produced by a genetically modified strain of the yeast *K. phaffii*. However, based on the available data, the Panel could not exclude the possibility that some residual amount of DNA coding for the kanamycin resistance gene could remain in the final product. Should this gene propagate in microbiota due to the presence of recombinant DNA in the final product, this would be of concern. Therefore, the Panel concluded that the safety of Rebaudioside D produced via this enzymatic bioconversion was not sufficiently demonstrated with the available data given that the absence of recombinant DNA was not shown.

Documentation as provided to EFSA

1) Application for a change in the steviol glycoside specification in the European Union to include a new manufacturing method including Rebaudiose D. Technical Dossier. Sweegen, Inc. October 2019. Including:
2) BRI (Biopharmaceutical Research Inc.), 2019. BRI Report no. RPT-SWE-2018-001 (v1.0), January 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.
3) Additional information submitted by the applicant Sweegen, Inc. following a request from EFSA. January 2021.
4) Additional information submitted by the applicant Sweegen, Inc. following a request from EFSA. June 2021.
5) Additional information submitted by the applicant Sweegen, Inc. following a request from EFSA. January 2022.

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Abbreviations

ADI acceptable daily intake
ADME Absorption, distribution, metabolism and excretion
AMR antimicrobial resistance
ANS Panel Panel on Food Additives and Nutrient Sources added to Food
BMDL benchmark dose (lower confidence limit)
Bw body weight
CAS Chemical Abstract Service
CONTAM Panel Panel on Contaminants in the Food Chain
FAP Panel Panel on Food Additives and Flavourings
FAO/WHO Food and Agriculture Organisation/World Health Organisation
HBGV health-based guidance value
HPLC High Performance Liquid Chromatography
HPLC/MS High Performance Liquid Chromatography/mass spectrometry
HPLC-UV High Performance Liquid Chromatography - Ultraviolet
JECFA Joint FAO/WHO Expert Committee on Food Additives
LC/MS Liquid chromatography-mass spectrometry
LOD limit of detection
MOE margin of exposure
MPLs maximum permitted levels
MRLs maximum residue levels
MS mass spectrometry
NOAEL no observed adverse effect level
QPS Qualified Presumption of Safety
RP reference point
TWI Tolerable Weekly Intake
UDP Uridine diphosphate
UGT UDP glucosyltransferase