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SCIENTIFIC OPINION

# Safety evaluation of the food additive steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Yarrowia lipolytica* VRM

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## Abstract

The EFSA Panel on Food Additive and Flavourings (FAF Panel) provides a scientific opinion on the safety of a new process to produce steviol glycosides by fermentation of simple sugars using a genetically modified strain of *Yarrowia lipolytica* (named *Y. lipolytica* VRM). The manufacturing process may result in impurities different from those that may be present in the other steviol glycosides E 960a-d, therefore the Panel concluded that separate specifications are required for the food additive produced as described in the current application. Viable cells and DNA from the production strain are not present in the final product. The Panel considered that the demonstration of the absence of kaurenoic acid in the proposed food additive, using a method with a limit of detection (LOD) of 0.3 mg/kg, is adequate to dispel the concerns for potential genotoxicity. Given that all steviol glycosides follow the same metabolic pathways, the Panel considered that the current steviol glycosides would fall within the same group of substances. Therefore, the Panel considered that the already existing data on rebaudioside M and structurally related steviol glycosides are sufficient, and a similar metabolic fate and toxicity is expected for the food additive. The results from the bacterial reverse mutation assay and the *in vitro* micronucleus assay were negative and indicated absence of genotoxicity from the food additive. The existing acceptable daily intake (ADI) of 4 mg/kg body weight (bw) per day, expressed as steviol equivalents, was considered to be applicable to the proposed food additive. The Panel concluded that there is no safety concern for steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. lipolytica* VRM, to be used as a food additive at the proposed uses and use levels.

**KEYWORDS**

enzymatic bioconversion, kaurenoic acid, rebaudioside M, Steviol glycoside preparations, *Yarrowia lipolytica* VRM

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## SUMMARY

The European Commission requested the European Food Safety Authority (EFSA) to provide a scientific opinion as regards 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.[1]

The Panel noted that since the receipt of the present mandate, both Regulation (EC) No 1333/2008 and Regulation (EU) No 231/2012 have been amended with respect to the entry for the food additive 'Steviol glycosides (E 960)', as referred to in the Terms of Reference of the present mandate, currently replaced by the three entries 'Steviol glycosides from Stevia (E 960a), 'Enzymatically produced steviol glycosides (E 960c)' and 'Glucosylated steviol glycosides (E 960d)'.

In the present scientific opinion, the Panel has therefore evaluated the latest proposal submitted by the applicant for the modification of the specification of the already authorised food additives, steviol glycosides (E 960a-960d), to include a new manufacturing process (Documentation provided to EFSA No. 1).

The Panel considered that the manufacturing process applied to the production of steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. lipolytica* VRM, involves bioconversion of sugars by fermentation using a non-toxigenic non-pathogenic strain of the yeast *Y. lipolytica* VRM to obtain a steviol glycoside mixture containing not less than 88% of rebaudioside M, which may result in impurities different from those that may be present in steviol glycosides E 960a-d. The Panel considered that separate specifications are 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 microorganism used for its production.

The production process comprises two main phases: the first involves the fermentation of a simple sugar source by a non-toxigenic non-pathogenic strain of *Y. lipolytica* VRM that has been genetically modified with heterologous genes to overexpress steviol glycosides. After removal of the biomass by solid–liquid separation and heat treatment, the second phase involves the purification and concentration of rebaudioside M with optional decolourisation/crystallisation, resulting in a final product containing not less than 95% rebaudioside M and minor amounts of other steviol glycosides.

Steviol glycosides produced using *Y. lipolytica* is produced by fermentation with the genetically modified production strain named '*Y. lipolytica* VRM'. Both the parental and recipient *Y. lipolytica* strains are considered to be safe and having qualified presumption of safety (QPS) status. As the genetic modification does not give rise to safety concern, the QPS approach can be extended to the production strain *Y. lipolytica* VRM. The Panel noted that the applicant proposed variability in the distribution of the steviol glycoside composition which is the result of the processes at industrial scale. The variability was confirmed by monitoring the batch-to-batch variability in five production batches: the final product was consistently characterised by a steviol glycoside content above 95% (comprising rebaudiosides M, D, A and B).

The Panel noted that the specifications proposed by the applicant contain parameters related to the specific genetically modified *Y. lipolytica* VRM used to produce the food additive (i.e. absence of both viable cells and DNA from the production strain; no more than 20 mg/kg of residual protein), are aligned with the EU specifications for E 960b, as laid down in Monograph 26 (JEFCA, 2021). Neither viable cells nor their DNA were present in the final product.

Based on the data on particle size distribution submitted by the applicant and the criteria set in the EFSA Guidance-TR (EFSA Scientific Committee, 2021), the Panel concluded that the presence of small particles in the pristine food additive, including nanoparticles, cannot be excluded. The Panel noted that the maximum permitted use levels for Steviol E 960a-960d for most food categories do not exceed 350 mg/L (expressed as steviol equivalents). For food categories FC 5.2, 5.3, 17.1 and 17.2 the food additive is allowed at maximum use levels that are in the range of 670–3300 mg/L and for table-top sweeteners the additive is allowed *quantum satis*. The Rebaudioside M and Rebaudioside D preparations have a steviol equivalency factor of 0.25 and 0.29 respectively, therefore MPL values expressed as steviol equivalents correspond to approximately four-fold higher concentration of the preparation. Taking into account the MPLs and the reported solubility – ranging from 1.61 to 1.89 g/L – the Panel considered that full dissolution of the proposed food additive is to be expected in foods and/or in the GI tract and that ingested particles (if any) would not persist. Therefore, the Panel concluded there is no concern with regard to the potential presence of small particles, including nanoparticles, in the proposed food additive and considered that the risk assessment can be performed following the EFSA Guidance for submission for food additive evaluations (EFSA ANS Panel, 2012).

Regarding the toxic elements lead, mercury, cadmium and arsenic, the Panel noted that, based on the analytical data provided, the proposed maximum limits for lead, mercury and cadmium are adequate and that the presence of the toxic elements in the food additive would not give rise to concern except for arsenic, whose margin of exposure (MOE) values were insufficient i.e. below the target value of 1000.

The absence of kaurenoic acid was shown in five batches of the proposed food additive, in which kaurenoic acid measured through liquid chromatography–mass spectrometry (LC–MS) was not detected in the tested samples (LOD of 0.3 mg/kg). Based on the available data, a genotoxic potential from kaurenoic acid in the proposed food addtive could not be ruled out and therefore the Panel considered appropriate to apply the TTC approach for this contaminant. Therefore, the threshold value of 0.0025 µg/kg bw, considered appropriate for potential DNA-reactive mutagens and/or carcinogens, was used in this assessment. The Panel noted that the exposure calculations at the 95th percentile showed a small exceedance

[1]Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008.

of this threshold of toxicological concern (TTC) value. However, taking into account that the conservative exposure assessment likely resulted in an overestimation, the Panel considered that the demonstration of the absence of kaurenoic acid in the proposed food additive, using a method with an LOD of 0.3 mg/kg, is adequate to dispel the concerns for potential genotoxicity in this case. The Panel recommends introducing a specific entry for kaurenoic acid in the final product specifications.

The Panel considered that the metabolic fate of steviol glycosides, including steviol glycosides obtained via fermentation, leads to the aglycone which is absorbed. Given that all steviol glycosides follow the same metabolic pathways, the Panel considered that the current steviol glycosides would fall within the same group of substances (EFSA ANS Panel, 2010; EFSA FAF Panel, 2020, 2022), and the group approach would be applicable. Therefore, the Panel considered that the already existing data on rebaudioside M and structural-related steviol glycosides (EFSA ANS Panel, 2010; EFSA FAF Panel, 2019, 2020, 2021, 2022), along with new supportive toxicity data on rebaudioside A produced fermentatively by *Y. lipolytica* (same manufacturing process of the proposed food additive), are sufficient. Therefore, no additional toxicity studies are required.

Newly generated studies on genotoxicity of steviol glycosides produced by fermentation using *Y. lipolytica* VRM were submitted by the applicant. The results from the bacterial reverse mutation assay and the *in vitro* micronucleus assay were negative and indicated absence of genotoxicity of the food additive.

For the other toxicity endpoints, no new studies were performed with the food additive, and no new studies relevant for the risk assessment were submitted by the applicant. Nonetheless, the Panel is of the opinion that a read-across with regard to toxicity is applicable, considering the availability of toxicity studies on other previously evaluated steviol glycosides (EFSA ANS Panel, 2010; EFSA FAF Panel, 2020, 2022). Therefore, no additional toxicity studies are required.

The existing ADI of 4 mg/kg bw per day (expressed as steviol equivalents) can also be applied to steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM as described in the present opinion.

The Panel concluded that there is no safety concern for steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM to be used as a food additive at the proposed uses and use levels, taking into account the existing ADI of 4 mg/kg bw per day (expressed as steviol equivalents). Separate specifications for steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM should be considered in Commission Regulation (EU) No 231/2012, since the manufacturing process may lead to impurities different from those that may be present in the other, already authorised, steviol glycosides (E 960a-960d).

# 1 | INTRODUCTION

The present opinion deals with the safety evaluation of the food additive steviol glycosides composed predominantly of Rebaudioside M, manufactured by a new process by fermentation of simple sugars using a genetically modified strain of *Yarrowia lipolytica* (named *Y. lipolytica* VRM).

## 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.[2] 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.[3]

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 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 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 production process that covers a purified steviol glycoside mixture primarily comprised of rebaudiose M produced by fermentation of simple sugars using a *Yarrowia lipolytica* production strain. The *Y. lipolytica* organism has been genetically modified to express the steviol glycoside synthesis pathway of the plant *Stevia rebaudiana*.

### 1.1.2 | Terms of Reference

The European Commission requests 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.[4]

### 1.1.3 | Interpretation of the Terms of Reference

The Panel noted that since the receipt of the present mandate, both Regulation (EC) No 1333/2008 and Regulation (EU) No 231/2012 have been amended with respect to the entry for the food additive 'Steviol glycosides (E 960)', as referred to in the Terms of Reference of the present mandate, currently replaced by the three entries 'Steviol glycosides from Stevia (E 960a), 'Enzymatically produced steviol glycosides (E 960c)' and 'Glucosylated steviol glycosides (E 960d)'. In the present scientific opinion, the Panel has therefore evaluated the latest proposal submitted by the applicant for the modification of the specification of the already authorised food additives, steviol glycosides (E 960a-960c), to include a new manufacturing process (Documentation provided to EFSA No. 1).

The Panel considered that the manufacturing process applied to the production of Rebaudioside M produced by *Y. lipolytica* VRM, which is the subject of this application under evaluation, involves enzymatic bioconversion of simple sugars by fermentation using a strain of the non-toxigenic non-pathogenic yeast *Y. lipolytica* to obtain a steviol glycoside mixture containing not less than 88% of rebaudioside M. This production method may result in impurities different from those that may be present in the other steviol glycosides E 960a-d. The Panel, therefore, considered that this issue needs to be addressed in the evaluation.

## 1.2 | Information on existing evaluations and authorisations

Steviol glycosides from Stevia (E 960a) is 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: 'not less than 95%

[2]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.

[3]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.

[4]Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008.

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).

In 2020, the FAF 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 into 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 be not 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 of steviol glycosides (E 960) as a 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).

In July 2021, a new entry for 'enzymatically produced steviol glycosides (E 960c)' was added to Annex II to Regulation (EC) No 1333/2008.[5] 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 uridine diphosphate (UDP)-glucosyl transferase and sucrose synthase enzymes produced by the genetically modified yeasts *Komagaetella 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'.

In October 2022, Regulation (EU) No 231/2012 was further amended, with the inclusion of the following new entries: 'E 960c(ii) Rebaudioside M produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extracts', 'E 960c(iii) Rebaudioside D produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extracts' and 'E960c(iv) rebaudioside AM produced via enzymatic conversion of highly purified stevioside stevia leaf extracts' This amendment to the Regulation was based on evaluations by the FAF Panel (EFSA FAF Panel, 2020, 2021).

In 2022, a new opinion on the safety of an additional proposed amendment to the specifications of the food additive steviol glycosides (E 960) was published and regarded rebaudioside D produced by enzymatic bioconversion of purified *S. rebaudiana* Bertoni leaf extract, using UDP glucosyltransferase (UGT) and sucrose synthase produced by a genetically modified strain of the yeast *K. phaffii* (EFSA FAF Panel, 2022).

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[6] expressed in *Y. 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 *Y. Lipolytica* for the yeast-derived product, recognising that it was manufactured by a distinctly different, biosynthetic process compared with stevia leaf-derived products (JECFA, 2016a).

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 at its 87th meeting 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, enzymatic modification and glucosylation (JECFA, 2019). The framework adopted in 2019 has been subsequently revised by JECFA at its 91st meeting in February 2021 and the tentative specifications prepared at its 87th meeting were replaced. Specifications for steviol glycosides manufactured using four different methods have been established, including specifications for 'Steviol Glycosides from Fermentation', covering also the case of steviol glycosides from *Y. Lipolytica* (JECFA, 2021).

In the United States (U.S.), steviol glycosides from fermentation (Reb M) produced by *Y. Lipolytica* has Generally Recognised as Safe (GRAS) status for food and beverage uses with no objection from the U.S. FDA (FDA, 2019).

[5]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.

[6]According to JECFA specifications: 'Rebaudioside A is obtained from the fermentation of a non-toxigenic non-pathogenic strain of *Yarrowia lipolytica* that is genetically modified with heterologous genes from multiple donor organisms to [over]express steviol glycosides'.

The U.S. FDA has provided no objections to the GRAS status of several steviol glycoside preparations for use as general purpose sweeteners in foods and beverages, including steviol glycosides (≥ 95% purity) extracted from the plant *S. rebaudiana*, enzyme modified steviol glycosides, steviol glycosides produced via microbial fermentation or steviol glycosides produced via enzymatic bioconversion (U.S. FDA, 2021).

In Australia and New Zealand, FSANZ has included steviol glycosides in the Australia New Zealand Food Standards Code as an intense sweetener under the food additive code number 960, adopting an ADI of 4 mg/kg bw for all steviol glycosides (FSANZ, 2017). Specifications for steviol glycoside preparations currently include 'ebaudioside M', 'steviol glycoside mixtures containing rebaudioside M', 'steviol glycosides from *S. rebaudiana* Bertoni' and 'steviol glycosides from fermentation', expressing biosynthesis pathway genes and must all contain no less than 95% steviol glycosides on a dry weight basis (FSANZ, 2020).

In Canada, permitted sources of steviol glycosides to be used as sweeteners are limited to: *S. rebaudiana* Bertoni; *Saccharomyces cerevisiae* CD15380; *S. cerevisiae* CD15407; *S. cerevisiae* Y63348 (Health Canada, 2020a). Steviol glycosides from these sources are permitted for use in food at the same maximum use levels: up to a maximum level of 0.35% in finished products as per the List of Permitted Sweeteners (Health Canada, 2020b).

# 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 on additional information, following requests by EFSA, submitted by the applicant in May 2022 (Documentation provided to EFSA No. 2), in September 2022 (Documentation provided to EFSA No. 3) and in June 2023 (Documentation provided to EFSA No. 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 'Scientific Guidance for the submission of dossiers on Food Enzymes' (EFSA CEP Panel, 2021) have been followed by the FAF Panel for evaluating the proposed change in manufacturing process and changes in the specifications. In addition, the EFSA Scientific Committee 'Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles' (EFSA Scientific Committee, 2021) has been followed by the FAF Panel.

# 3 | ASSESSMENT

## 3.1 | Technical data

### 3.1.1 | Identity of the proposed food additive

The present opinion deals with the safety evaluation of the food additive Steviol glycosides predominantly Rebaudioside M, produced via a new process by fermentation of simple sugars using a genetically modified strain of *Y. Lipolytica* VRM (Documentation provided to EFSA No. 1–4).

Other than rebaudioside M, the resulting mixture contains about 10% of the minor rebaudiosides D, A and B (Table 1) (Documentation provided to EFSA No. 3). Steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. Lipolytica* VRM meets the ≥ 95% purity definition for Steviol Glycosides from Fermentation established by JECFA (JECFA, 2021). Information on the composition of the proposed preparations was provided by the applicant following an additional data request from EFSA (Documentation provided to EFSA No. 3). The steviol glycoside mixture was characterised using an high performance liquid chromatography – ultraviolet (HPLC-UV) method (at 210 nm) developed by the applicant and adapted from the JECFA method for measuring steviol glycosides (JECFA, 2021). The HPLC analysis, performed to determine the concentrations of the individual steviol glycosides, was carried out by an external laboratory; the certificates of analysis were submitted (Documentation provided to EFSA No. 3). The applicant informed that the quantification of the individual rebaudiosides was performed through individual calibration curves (from commercial reference standards) prepared for rebaudiosides M, D, A and B. In detail, the composition of five non-consecutive batches of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM, produced over a 2-months period, was analysed using the method of assay described by JECFA (2021). Reb M was the dominant form, in the range of 87.7%–89.7%. Reb D was the second most

dominant, at 8.5%–9.5%. Reb A was in the range 0.17%–0.27%, and Reb B in the range of < 0.1%–0.22%. The sum of these four steviol glycosides was greater than 95% (dry weight basis) for all the five batches: the range being 96.4%–98.9% (dry weight basis). Corresponding chromatograms for each batch were provided along with the Certificates of analysis confirming that the five batches matched the proposed specifications (Section 3.1.2).

The CAS numbers, molecular formulae, molecular weights, and R1 and R2 groups for the individual steviol glycosides that are present in the mixture are summarised in Table 1. According to the applicant, all constituents of the steviol glycosides share the same backbone structure (Figure 1).

**TABLE 1** Molecular weight and formula, and R-groups in backbone structure of the Steviol glycosides.

| Steviol glycoside | CAS number | Molecular weight | Molecular formula | R-groups in backbone structure | |
|---|---|---|---|---|---|
| | | | | $R_1$ | $R_2$ |
| Rebaudioside A (Reb A) | 58543-16-1 | 967.01 | $C_{44}H_{70}O_{23}$ | β-Glc | Glcβ(1–2)[Glcβ(1–3)]Glcβ1- |
| Rebaudioside B (Reb B) | 58543-17-2 | 804.88 | $C_{38}H_{60}O_{18}$ | H | Glcβ(1–2)[Glcβ(1–3)]Glcβ1- |
| Rebaudioside D (Reb D) | 63279-13-0 | 1129.15 | $C_{50}H_{80}O_{28}$ | B-Glc-β-Glc(2–1) | Rhaα(1–2)[Glcβ(1–3)]Glcβ1- |
| Rebaudioside M (Reb M) | 1220616-44-3 | 1291.3 | $C_{56}H_{90}O_{33}$ | Glcβ(1–2)[Glcβ (1–3)]Glcβ1- | Glcβ(1–2)[Glcβ(1–3)]Glcβ1 |

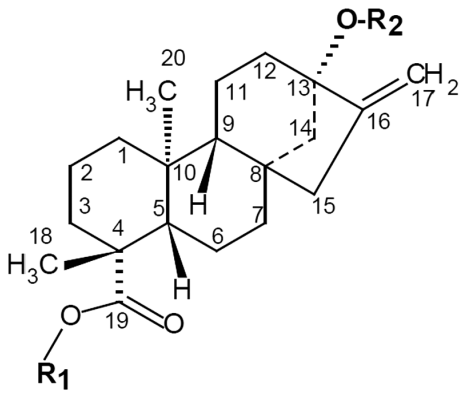


**FIGURE 1** Backbone structure of Steviol glycosides.

According to the applicant, the preparation Steviol glycosides produced by fermentation using *Y. lipolytica* VRM is a white to off-white powder with a characteristic sweet taste and mild odour (Documentation provided to EFSA No. 1).

### 3.1.2 | Proposed specifications

The applicant provided the product specification data and reported that the food additive is manufactured within its proposed specifications (Documentation provided to EFSA No. 1–4). Information on the parameters considered to be relevant for the specifications has been compiled by the Panel in Table 2.

The Panel noted that the JECFA Specifications for 'E 960b Steviol Glycosides from Fermentation' with a genetically modified strain of *Y. lipolytica* are available, and a comparison between the proposal from the applicant and the existing JECFA specifications is presented in Table 2.

**TABLE 2** Specifications as proposed by the applicant for 'Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Yarrowia lipolytica* VRM', and for '*Steviol Glycosides from Fermentation*' as set in the JECFA Monographs 26 (Documentation provided to EFSA No. 1–4).

| | **Proposed specification by the applicant for Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Yarrowia lipolytica* VRM** | | | **JECFA specifications for Steviol glycosides from fermentation (JECFA, 2021)** | | |
|---|---|---|---|---|---|---|
| **SYNONYMS** | n.a. | | | INS No. 960b | | |
| **DEFINITION** | Steviol glycosides from *Yarrowia lipolytica* consist of a mixture predominantly composed of rebaudioside **M**, with some rebaudioside **D**, and smaller amounts of rebaudioside **A** and rebaudioside **B**. The manufacturing process comprises two main phases. The first phase involves fermentation of a non-toxigenic non-pathogenic strain of *Y. ipolytica* (VRM) that has been genetically modified with heterologous genes to overexpress steviol glycosides. Removal of biomass by solid–liquid separation and heat treatment is followed by concentration of the steviol glycosides. The second phase involves purification by employing ion exchange chromatography, followed by recrystallisation of the steviol glycosides resulting in a final product containing not less than 95% of rebaudiosides **M**, **D**, **A** and **B**. Viable cells of *Y. lipolytica* or DNA from the production organism shall not be detected in the food additive | | | Steviol glycosides from fermentation consist of a mixture of compounds containing a steviol backbone conjugated to various sugar moieties (e.g. glucose or sucrose) depending on the specific production organism and fermentation conditions used<br>Steviol glycosides from fermentation are obtained from the fermentation of non-toxigenic non-pathogenic strains of *Yarrowia lipolytica* and *Saccharomyces cerevisiae* that have been genetically modified with heterologous genes from multiple donor organisms to overexpress steviol glycosides. After removal of the biomass by solid–liquid separation and heat treatment, the process involves concentration of the steviol glycosides (e.g. by resin adsorption), followed by purification of the desired steviol glycosides by crystallisation and drying. Ion exchange resins may be used in the purification process. The final product may be spray dried. Commercial products are primarily composed of either rebaudioside **A**, rebaudioside **M** or a combination of rebaudioside **M** and rebaudioside **D**; additional minor steviol glycosides may be present | | |
| Chemical names | 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<br>Rebaudioside **B**: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid<br>Rebaudioside **D**: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester<br>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 **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<br>Rebaudioside **D**: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester<br>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 and conversion factor | **Trivial name** | **Formula** | **Conversion factor** | **Trivial name** | **Formula** | **Conversion factor** |
| | Rebaudioside A | $C_{44}H_{70}O_{23}$ | **0.33** | Rebaudioside A | $C_{44}H_{70}O_{23}$ | 0.33 |
| | Rebaudioside B | $C_{38}H_{60}O_{18}$ | **0.44** | | | |
| | Rebaudioside D | $C_{50}H_{80}O_{28}$ | **0.29** | Rebaudioside D | $C_{50}H_{80}O_{28}$ | 0.28 |
| | Rebaudioside M | $C_{56}H_{90}O_{33}$ | **0.25** | Rebaudioside M | $C_{56}H_{90}O_{33}$ | 0.25 |
| CAS No. and Molecular weight (g/mol) | **Trivial name** | **CAS No.** | **Molecular weight** | **Trivial name** | **CAS No.** | **Molecular weight** |
| | Rebaudioside A | 58543-16-1 | 967.0 | Rebaudioside A | 58543-16-1 | 967 |
| | Rebaudioside B | 58543-17-2 | 804.9 | | | |
| | Rebaudioside D | 63279-13-0 | 1129.2 | Rebaudioside D | 63279-13-0 | 1129 |
| | Rebaudioside M | 1220616-44-3 | 1291.3 | Rebaudioside M | 1220616-44-3 | 1291 |
| Assay | Not less than 95% of rebaudioside M, rebaudioside D, rebaudioside A and rebaudioside B, on a dried basis | | | Not less than 95% of total of steviol glycosides, on the dried basis | | |
| **DESCRIPTION** | White to light yellow powder, approximately between 200 and 350 times sweeter than sucrose (at 5% sucrose equivalency) | | | White to light yellow powder, odourless or having a slight characteristic odour. About 200–300 times sweeter than sucrose | | |
| **FUNCTIONAL USES** | n.a. | | | Sweetener | | |
| **CHARACTERISTICS** | | | | | | |
| Solubility | Freely soluble to slightly soluble in water | | | Very slightly soluble to freely soluble in water; slightly soluble to freely soluble in a mixture of ethanol and water (50:50 v/v) | | |

(Continues)

**TABLE 2** (Continued)

| | Proposed specification by the applicant for Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Yarrowia lipolytica* VRM | JECFA specifications for Steviol glycosides from fermentation (JECFA, 2021) |
|---|---|---|
| HPLC chromatographic profile | n.a. | The main peaks in a chromatogram obtained by analysing a sample following the procedure in Method of Assay correspond to steviol glycosides |
| 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°, 2 h) | Not more than 6% (105°, 2 h) |
| Residual solvents | Not more than 5000 mg/kg ethanol | Not more than 200 mg/kg methanol<br>Not more than 5000 mg/kg ethanol |
| Arsenic | Not more than 0.1 mg/kg | Not more than 1 mg/kg |
| Lead | Not more than 0.1 mg/kg | Not more than 1 mg/kg |
| Cadmium | Not more than 0.01 mg/kg | |
| Mercury | Not more than 0.05 mg/kg | |
| Microbiological criteria | Total (aerobic) plate count: Not more than 1000 CFU/g | Total (aerobic) plate count: Not more than 1000 CFU/g |
| | Yeasts and moulds: Not more than 100 CFU/g<br>*E. coli*: Negative in 1 g<br>Salmonella: Negative in 25 g | Yeasts and moulds: Not more than 200 CFU/g<br>*E. coli*: Negative in 1 g<br>Salmonella: Negative in 25 g |
| Residual protein | Not more than 20 mg/kg | |

Abbreviations: CFU, colony forming unit; n.a., not available.

The applicant submitted analytical data from the analyses of five non-consecutive batches of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM (Documentation provided to EFSA No. 1–3). Based on the data submitted, the Panel considered that the proposed food additive is consistently produced and compliant with the proposed specifications, as outlined in Table 2.

The proposed food additive contains not less than 95% of Rebaudiosides M, D, A and B, with Reb M being the most abundant compound. The Panel noted that the data submitted from the analysis of steviol glycosides in five batches of the proposed food additive (see Section 3.1.1) fulfil such declared purity (Documentation provided to EFSA No. 1–3). The Panel considered adequate the proposed purity assay of '*Rebaudiosides M, D, A and B to account for not less than 95%*' of the final product *Y. lipolytica* VRM (dry basis). The assay value of not less than 95% for total steviol glycosides should be limited to the four named glycosides that are included in the specification proposed by the applicant.

The Panel noted that the conversion factor for Reb D provided by the applicant (i.e. 0.29) differs slightly from the conversion factor found in JECFA Monographs 26 (i.e. 0.28). However, this difference was not considered to impact on the safety assessment.

The Panel noted that the phrase 'overexpress steviol glycosides' proposed by the applicant for inclusion in the specifications is incorrect and should read 'overexpress genes which are involved in the synthesis of steviol glycosides.'

The Panel recommended to specify in the definition the production strain, *Y. lipolytica* VRM, (DS 82603), which was deposited in the Culture Collection of the Westerdijk Fungal Biodiversity Institute (CBS, The Netherlands) with deposition number CBS 147477.

The applicant has not specified the functional uses of the proposed food additive, although these are indirectly given in the entry description, where the product is described as up to 350 times sweeter than sucrose. The Panel recommended including the functional uses in the final proposed specifications. In addition, the Panel noted that in the JECFA's specification, E 960b is described as up to 300 times sweeter than sucrose, whereas the applicant considers the proposed food additive as up to 350 times sweeter than sucrose.

The proposed food additive is described by the applicant as *'freely soluble to slightly soluble in water'*; however, this description is not supported by the experimental data submitted to EFSA (see section on Solubility and particle size), according to which Steviol glycosides produced by fermentation using *Y. lipolytica* VRM had a solubility ranging from 1.61 to 1.89 g/L. Therefore, the Panel recommends restricting the description of water solubility as 'slightly soluble'.

The Panel noted that the applicant did not make any proposal for the specifications' entry 'HPLC chromatographic profile'. Nevertheless, this is not considered of relevance for the safety assessment.

In the initial specifications proposed by the applicant, the solvents used for producing the proposed food additive were not included. Following an additional data request from EFSA, the applicant included the maximum levels for residual solvents (Documentation provided to EFSA No. 4): not more than 5000 mg/kg of ethanol. The applicant did not propose a limit for the solvent methanol since it is not used in the production of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM.

Regarding the toxic elements, following an additional data request from EFSA, the applicant provided analytical data on the content of arsenic, lead, cadmium and mercury in five batches of the proposed food additive (Documentation provided to EFSA No. 2). The analyses were performed by an external laboratory using inductively coupled plasma–mass spectrometry (ICP–MS) through method: AOAC 2015.01 [2232] and the certificates of analyses were provided. All five batches had levels of toxic elements below the limits of quantification (LOQ) which were 0.01, 0.01, 0.001 and 0.005 mg/kg for As, Pb, Cd and Hg, respectively. Based on the obtained data, the applicant proposed as limits in the product's specifications the values of 0.1 mg/kg (for As and Pb), 0.01 mg/kg (for Cd) and 0.05 mg/kg for Hg. Although the JECFA's specifications do not have limits for Cd and Hg, the Panel recommended the inclusion of also these two toxic elements in the product specifications, and considered the proposal made by the applicant in line with the data obtained.

The Panel noted that kaurenoic acid is formed along the biosynthetic pathway of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM, as outlined in the technical dossier (Documentation provided to EFSA No. 1). Following an additional data request from EFSA, the applicant provided analytical data on five batches of the proposed food additive showing that kaurenoic acid was not detected in the tested samples (LOD 0.3 mg/kg). The analytical report was submitted to EFSA (Documentation provided to EFSA No. 2). An LC–MS system was employed to analyse Steviol glycosides produced by fermentation using *Y. lipolytica* VRM for the presence of kaurenoic acid. The MS was operated in negative electrospray ionisation ($ESI^-$) mode with selective ion recording (SIR) of the deprotonated molecule $[M\text{-}H]^-$ for kaurenoic acid at 301.4 *m/z*. The quantification was performed using a kaurenoic acid reference standard obtained commercially. The analytical recovery was 82%–110% for samples spiked with kaurenoic acid at 2.5 mg/kg. The method had an LOD of approximately 0.3 mg/kg and an LOQ of 1 mg/kg.

Results of the batch analyses showed that the final product meets the updated Assay requirements and the microbiological limits suggested in the proposed specifications (Documentation provided to EFSA No. 1–4). Testing was performed on five non-consecutive batches of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM. The analysis of toxic elements was performed with the same five batches as those used for batch analysis in the original technical dossier; residual protein analysis was also based on the five batches used for batch analysis presented in the original technical dossier (Documentation provided to EFSA No. 2). However, in order to assess the solubility of the final preparation of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM, a more recent set of five batches was used, with the results of the testing being submitted to EFSA (Documentation provided to EFSA No. 2).

Five non-consecutive batches of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM were analysed for the presence of microbiological contaminants. Total plate count, yeasts and moulds, total coliforms and individual types of microorganisms, including *E. coli* and *Salmonella*, were not detected in the tested samples (Documentation provided to EFSA No. 2–3). In detail, the total (aerobic) plate count was < 10 colony forming unit (CFU)/g, yeasts and moulds < 10 CFU/g, *E. coli* absent in 1 g and *Salmonella* absent in 25 g.

Data on the absence of viable cells and recombinant DNA from the production strain in the final product were provided by the applicant (Documentation provided to EFSA No. 3) and summarised in Section 3.1.3.4.

The Panel noted that the absence of viable cells/residual DNA from the production strain is included in the proposed definition, where it is stated '*Viable cells of Y. lipolytica or DNA from the production organism shall not be detected in the food additive*'. All the microbiological analyses were supported by the relevant certificate of analyses. In general, the microbiological specification parameters and limits proposed by the applicant were consistent with those published by JECFA for 'Steviol Glycosides from fermentation' (JECFA, 2021), and were considered by the Panel in line with the data provided.

In the proposed specifications the applicant reported that 'not more than 20 mg/kg' of residual protein are expected to be found in the proposed food additive. The Panel noted that the analytical data provided by the applicant comply with the proposed specification limit for residual protein (i.e. < 20 mg/kg).

The applicant was requested to demonstrate the absence of DNA from the production microorganism, in compliance with requirements of Section 1.3.4.2 of EFSA's Scientific Guidance 2021 (EFSA CEF Panel, 2021). The applicant replied submitting a study performed on three non-consecutive batches of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM. No amplification signal indicative of contamination with genomic DNA was reported in any of the test samples, thus confirming the absence of DNA from the production organism in the final product. The test was performed by an external laboratory, and the study report was submitted to EFSA (Documentation provided to EFSA No. 4).

*Solubility*

The applicant provided information on the water solubility for five batches of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM, determined by applying the OECD TG 105 (shake flask method) (Documentation provided to EFSA No. 2). The solubility of the tested Steviol glycosides produced by fermentation using *Y. lipolytica* VRM at 22 ± 0.5°C, at pH 6.5 was ranging from 1.61 to 1.89 g/L across five samples.

The Panel considered that the water solubility tests reported in the information submitted by the applicant have not been performed in full accordance with the requirements of the EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021). The Panel recommended describing the water solubility in the proposed specifications as 'slightly soluble'.

*Particle size distribution*

The applicant provided information on particle size distribution (PSD) of five batches of steviol glycosides from *Y. lipolytica* VRM determined by scanning electron microscopy (SEM) analysis (Documentation provided to EFSA No. 2). The dry samples of steviol glycosides from *Y. lipolytica* VRM were dispersed in cold (4°C) isopropanol as the substance is poorly soluble in this alcohol. Once dried on a silicon wafer, the samples were readily imageable by SEM. The detected constituent particles were of polygon shape with no obvious aggregation. The particle size was determined by measuring minimal Feret diameter ($Feret_{min}$) of the particles (by using an image analysis software) as requested in the EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021). For each batch of steviol glycosides produced by fermentation using *Y. lipolytica* VRM, 15 image areas were randomly selected and a total of circa 1000 representative particles were analysed. The number-based size histograms and some descriptive statistics (mean and minimum $Feret_{min}$ diameter) were calculated for each batch. The mean diameter of the particles analysed in the batches were in the range of 2033–3249 nm and the minimum diameter was in the range of 270–405 nm. The applicant stated that the SEM results revealed that all analysed samples contained particles below 500 nm but that in none of the five batches particles smaller than 250 nm were detected.

The Panel noted that the magnifications used for SEM analysis, i.e. x2000, and the resolution of the images estimate by the applicant to be of order 100 nm does not allow to fully characterise the fraction of small particles or nanoparticles. Therefore, based on the data provided, the presence of a fraction of small particles including nanoparticles cannot be excluded.

## 3.1.3 | Manufacturing process

### 3.1.3.1 | *Identity of raw materials and processing aids*

Information regarding the raw materials, processing aids and equipment used to manufacture Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. lipolytica* VRM were provided by the applicant (Documentation provided to EFSA No. 1–4). All raw materials, processing aids and equipment are food-grade and comply with the relevant *Food Chemicals Codex* (FCC) standards.

The Panel noted that in the production of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM is used a boiler chemical, namely Nalco® 359, which comprises diethylethanolamine (CAS no.: 100-37-8); thus, the applicant was requested to demonstrate the absence of diethylethanolamine in the final product. It was reported that the boiler chemical is used as an antiscalant agent to protect the steam piping system employed in the manufacturing process (Documentation provided to EFSA No. 3). In addition, the applicant developed a method to test the proposed food additive for diethylethanolamine. The analysis was performed by an external accredited laboratory on three production batches; the study report and the certificate of analysis were submitted to EFSA. Results of the analysis showed the absence of diethylethanolamine in the tested samples of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM (i.e. below LOD of 2.2 ppb in solution, corresponding to a diethylethanolamine level of < 1.6 ppm in the steviol glycoside powdered samples).

### 3.1.3.2 | *Description of manufacturing process*

Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. lipolytica* VRM is a purified steviol glycoside mixture that is produced via fermentation of simple sugars using a *Y. lipolytica* strain that has been engineered to produce steviol glycosides. Steviol glycosides produced by fermentation using *Y. lipolytica* VRM are manufactured in accordance with cGMP. The *Y. lipolytica* VRM production strain is added to the fermentation medium and is allowed to produce steviol glycosides under aerobic conditions. The fermentation is stopped via heat treatment to inactivate yeast cells, then, the biomass is separated from the steviol glycosides by microfiltration. The steviol glycosides are purified in accordance with the methodologies outlined in the CTA published by JECFA for steviol glycosides (JECFA, 2016b), which includes the use of filtration aids, purification resins and crystallisation. The final product is comprised mostly of rebaudioside M and contains a mixture of the following glycosides at various concentrations: rebaudiosides A, B and D, such that the total steviol glycosides' content is not less than 95%.

Following an additional data request from EFSA (Documentation provided to EFSA No. 2), the applicant reported that the variability in the distribution of the steviol glycoside composition, in the proposed food additive, is the result of the processes being performed at industrial scale. The applicant reported that manufacturing steps such as crystallisation (used for concentration and purification purposes) might cause variability in the distribution of the steviol glycosides. This was confirmed by monitoring the batch-to-batch variability in five production batches: the final product was consistently characterised by a steviol glycosides content above 95% (comprising rebaudiosides M, D, A and B) (Documentation provided to EFSA No. 1–4).

### 3.1.3.3 | *Characterisation of the production organism*

*Characteristic of the GMM production strain*

The production strain of the steviol glycosides is the genetically modified yeast *Y. lipolytica* VRM, (DS 82603), which was deposited in the Culture Collection of the Westerdijk Fungal Biodiversity Institute (CBS, The Netherlands), with deposition number CBS 147477. The production strain was taxonomically identified as *Y. lipolytica* by 18S rRNA gene sequence analysis.

*Characteristics of the recipient strains*

According to the applicant, the three parental strains (ATCC 76861, ATCC76982 and ATCC 201249) were obtained directly from the American Type Culture Collection (ATCC, USA) and given the internal names ML305, ML311 and ML326, respectively. These three parental strains were used to generate strain ML350 through two rounds of mating and sporulation. Strains ML326 and ML350 were used as starting strains in the construction of the production strain because they had opposite mating types (so allowing subsequent mating) and natural polymorphic variation.

*Characteristics of the inserted sequences*

A total of 20 different coding sequences were inserted. All of them, with the exception of [redacted], are involved in an artificially designed metabolic pathway to produce steviol glycosides, including genes for increasing the flux of carbon through the mevalonate pathway and for the transport of glycosides from the cell. The inserted sequences are derived from the yeasts [redacted], the filamentous fungi [redacted], the bacteria [redacted]. and the plants [redacted]. The plant and fungal coding sequences were flanked by *Y. lipolytica* promoter and terminator sequences and optimised for expression in *Y. lipolytica*. Genes conferring resistance to kanamycin/G450, hygromycin or nourseothricin were included in expression cassettes and used as selectable markers.

*Description of the genetic modification*

The genetic modifications were described in the application. The expression cassettes were introduced in the recipient strains using different transformation systems and randomly or targeted integrated into the genome. When the expression cassette included a marker gene, this was later removed.

Using the methods above, the recipient strains ML326 and ML350 were subjected to several consecutive transformations, and the resulting strains were mated and sporulated. Haploid spores were selected and this process was repeated, in combination with a conventional mutagenesis step. The final strain resulting from this process was selected as the production strain *Y. lipolytica* VRM.

At different stages of the process, plasmids were used that carried genes conferring resistance to different antibiotics including ampicillin.

As a consequence of the genetic modifications, the production strain has an enhanced mevalonate pathway leading to overproduction of geranyl pyrophosphate, which is then converted into steviol glycosides.

*Safety of the genetic modification*

The parental strains and recipient strains qualify for the QPS approach for safety assessment, and therefore are considered as safe (EFSA BIOHAZ Panel, 2020).

The production strain differs from the recipient strains in its enhanced mevalonate pathway and its ability to synthesise steviol glycosides. The absence of antimicrobial resistance genes kanamycin, hygromycin, nourseothricin and ampicillin, used during the genetic modification, was confirmed by WGS investigation. The genetic modification does not give rise to safety concern. Therefore, the QPS approach can be extended to the genetically modified strain *Y. lipolytica* VRM (EFSA BIOHAZ Panel, 2020).

On the basis of the data provided by the applicant, the Panel concluded that the genetic modification of the production strain does not give rise to safety concerns.

#### 3.1.3.4 | *Absence of viable cells of the production strain in the final product*

The absence of viable cells of the production strain was shown in three batches of the proposed food additive, each tested in triplicate. One gram of sample was plated on selective medium and incubated at 30°C for 4 days. No colonies were produced. A positive control was included (Documentation provided to EFSA No 2).

#### 3.1.3.5 | *Absence of DNA in the final product*

The absence of DNA of the production strain from the steviol glycosides was shown in three batches, each tested in triplicate. No amplification was observed using primers that would amplify a 679-bp fragment of the [redacted], with a limit of detection of 10 ng control DNA/g of product as demonstrated using spiked controls (Documentation provided to EFSA No. 2–4).

### 3.1.4 | Method(s) of analysis in food

No information on a method of analysis for this proposed additive in food was provided by the applicant. However, the Panel assumes that the methods of analysis available for the other steviol glycosides preparations will be applicable.

### 3.1.5 | Stability, reaction and fate in food of the proposed food additive

Two studies with Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Yarrowia lipolytica* VRM were performed by the applicant, in order to evaluate the stability of the proposed food additive under conventional and accelerated storage conditions, for up to 9 months. The studies were performed with three non-consecutive batches of Steviol glycosides produced by fermentation using *Y. lipolytica* VRM. The samples were analysed in duplicate to monitor the content of the different steviol glycosides (Reb M, Reb A, Reb B and Reb D), and the loss on drying at 0, 3, 6 and 9 months.

The first study consists of a conventional storage stability study carried out at 25°C and at 60% of relative humidity. The second study consists of an accelerated storage stability study that was performed at 40°C and at 75% of relative humidity. Based on the data obtained, the applicant concluded that Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. lipolytica* VRM remained stable, under the tested conventional and accelerated conditions, for up to 9 months.

## 3.2 | Proposed uses and use levels

Maximum levels of steviol glycosides (E 960a-d) expressed as steviol equivalents are defined in Annex II to Regulation (EC) No 1333/2008.[7]

Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. lipolytica* VRM is proposed for use in food and beverages under the same conditions as those already approved for steviol glycosides (E 960a-d) in the EU (Documentation provided to EFSA No. 1).

## 3.3 | Exposure data

Because the proposed uses and use levels of Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. lipolytica* VRM are the same as the already authorised food additive steviol glycosides (E 960a-d), the applicant did not provide an exposure estimate but referred to the latest estimated exposure to E 960 (EFSA ANS Panel, 2015b).

The Panel considers that if steviol glycosides would be replaced by Steviol glycosides produced by fermentation using *Y. lipolytica* VRM, the exposure to rebaudiosides M and D (expressed as steviol equivalents) 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 impurities

The potential exposure to impurities from the use of Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. lipolytica* VRM as a food additive can be calculated by assuming that the impurity is present in the food additive up to a limit value and then by calculation pro-rata to the estimates of exposure to the food additive itself.

For the current assessment, previous exposure estimates performed by the ANS Panel (EFSA ANS Panel, 2015b) were considered. The highest exposure levels to steviol glycosides for the mean and 95th percentile among the different population groups were considered, i.e. 2.4 and 4.3 mg/kg bw per day respectively, for toddlers.

The current application concerns Steviol glycosides produced by fermentation using *Yarrowia lipolytica* VRM that contains Rebaudiosides M and D (up to approximately 99% on a dry basis). Since steviol itself has a molecular weight of 318.5 g/mol, whereas Rebaudiosides M and D have molecular weights of 1291.3 and 1129.15 g/mol, respectively, the steviol equivalencies of Rebaudiosides M and D are 0.25 and 0.29, respectively (conversion factor, see Table 2). Therefore, considering their concentration in the proposed food additive, an exposure of 2.4 and 4.3 mg/kg bw per day expressed as steviol equivalents equates to 9.3 and 16.6 mg/kg bw per day (Table 4) for the highest mean and highest 95th percentile, respectively, of steviol glycosides produced using the proposed production process.

The level of the impurities in the food additive combined with the estimated intakes of the food additive result in an exposure which can be compared with the following reference points (RP), or health-based guidance values (HBGV) for the undesirable impurities potentially present in the food additive.

[7]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.

**TABLE 3** Reference points/health-based guidance values for impurities present in Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Yarrowia lipolytica* VRM.

| Impurity/constituent/HBGV/RP (μg/kg bw) | Basis/reference |
|---|---|
| Lead (Pb)/0.5 ($BMDL_{01}$) | 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 $BMDL_{01}$ (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 $BMDL_{10}$ 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 $BMDL_5$ 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 per 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 ($BMDL_{01}$) | The reference point is based on a range of benchmark dose lower confidence limit ($BMDL_{01}$) 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, i.e. a MOE of 1000 would be sufficient. EFSA CONTAM Panel (2009b), EFSA Scientific Committee (2012) |

Abbreviations: bw, body weight; $BMDL_{01}$, benchmark dose (lower confidence limit); HBGV, health-based guidance value; MOE, margin of exposure; RP, reference point; TWI, Tolerable Weekly Intake.

The risk assessment of the undesirable impurities helps to determine whether there could be a possible health concern if these impurities would be present at the limit values in the food additive. The assessment is performed by calculating the MOE (margin of exposure) by dividing the reference point (i.e. BMDL, Table 3) by the exposure estimate (see Section 3.3), or by estimating the contribution of the use of the food additive to the HBGV (expressed as percentage of the HBGV).

#### 3.3.1.1 | *Toxic elements*

The Panel noted that the occurrence data on toxic elements submitted by the applicant are lower than the limits in the proposed specifications (Table 2; Documentation provided to EFSA No. 2).

The results of the analyses for lead, mercury, cadmium and arsenic in five samples of the proposed food additive were reported (Section 3.1.2). Information on the LOQs and the analytical method used to quantify the toxic elements was provided.

The Panel assessed the risk that would result if these toxic elements were present in the food additive at the maximum limit as proposed in the specifications by the applicant.

The outcome of the risk assessment is illustrated in Table 4.

**TABLE 4** Risk assessment for toxic elements.

| Exposure to Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. Lipolytica* VRM (mg/kg bw per day) | Considering the presence of toxic elements at the proposed specification limits in Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. Lipolytica* VRM | | | |
|---|---|---|---|---|
| | MOE for Pb at 0.1 mg/kg | % of the TWI for Hg at 0.05 mg/kg | % of the TWI for Cd at 0.01 mg/kg | MOE for As at 0.1 mg/kg |
| Mean: 9.3[a] | 538 | 0.08 | 0.03 | 323–8602 |
| 95th percentile: 16.6[a] | 301 | 0.15 | 0.05 | 181–4819 |

Abbreviations: bw, body weight; MOE, margin of exposure.

[a]Estimated exposure converted from steviol equivalents (EFSA ANS Panel, 2015b) taking into account Reb M and Reb D at concentrations of ~89% and 9%, respectively, and with the conversion factors of 0.25 and 0.29, respectively.

When considering the limits proposed for the specifications (Table 2), the Panel concluded that for arsenic the lower end of the ranges of the calculated MOE values were insufficient, i.e. below the target value of 1000. For the other three toxic elements (cadmium, lead and mercury), the proposed specification values do not give rise to safety concerns.

The Panel considered that the choice of maximum limits for toxic elements in the specifications is in the remit of risk manager(s). The numbers used here were merely taken to support the risk assessment of these toxic elements as presented above.

### Kaurenoic acid

Several publications assessing the genotoxicity of kaurenoic acid *in vitro* and *in vivo* were retrieved from the literature. In the bacterial reverse mutation assay, kaurenoic acid showed negative results (Damasceno et al., 2019; Pezzuto et al., 1985, 1986). In other *in vitro* studies (micronucleus and comet assay), positive results were reported at high concentrations; however, the level of cytotoxicity was not appropriately estimated (Cavalcanti et al., 2006; Cavalcanti et al., 2010; Cardoso et al., 2017; Pasqualli et al., 2019). When non-cytotoxic concentrations of kaurenoic acid were tested, negative results were observed (Cano et al., 2017; Damasceno et al., 2019; Dalenogare et al., 2019; Pezzuto et al., 1985, 1986). Only the study by Cano et al., 2017 was performed according to a modified OECD test guideline 487 (2014), while the other studies were not performed according to OECD test guidelines.

In the *in vivo* study of Dalenogare et al., 2019, kaurenoic acid was administered at a dose of 1 mg/kg bw by gavage for 7 days in male and female Swiss mice. The study assessed comet assay parameters in liver and blood and the presence of micronuclei in bone marrow. No evidence of bone marrow exposure was provided. No genotoxicity was observed at the dose used in the study.

Cavalcanti et al. (2010) reported positive results *in vivo* in a micronucleus test and in a comet assay in Swiss male mice. The Panel noted some shortcomings of the study, which included that kaurenoic acid was administered by intraperitoneal injection, a route not recommended by OECD TG; that the high doses tested in the micronucleus assay (25, 50 and 100 mg/kg bw) caused high bone marrow toxicity; and that in the comet assay no information on local toxicity (histopathological analysis) was reported. Notwithstanding these shortcomings, the Panel could not dismiss the positive findings of this *in vivo* micronucleus assay.

In light of the uncertainties in the genotoxicity data, the Panel considered the TTC approach to conduct a risk assessment for kaurenoic acid as an impurity. Given the indications of a possible genotoxic potential reported in the Cavalcanti et al., 2010 publication, the Panel considered kaurenoic acid as a potential DNA-reactive mutagen and/or carcinogen, for which a TTC of 0.15 μg/person per day or 0.0025 μg/kg bw per day is applicable (EFSA Scientific Committee, 2019).

Kaurenoic acid was not detected in the proposed food additive and the Panel performed the calculation of potential exposure to kaurenoic acid as if it were present in the food additive at the LOD (0.3 mg/kg) of the analytical methods used by the applicant. The outcome of this calculation is given in Table 5.

**TABLE 5** Potential exposure to kaurenoic acid from Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Yarrovia lipolytica*.

| Exposure to Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. Lipolytica* VRM (mg/kg bw per day) | Exposure to kaurenoic acid if at 0.3 mg/kg in the proposed food additive |
|---|---|
| Mean: 9.3[a] | 0.00279 μg/kg bw per day |
| 95th percentile: 16.6[a] | 0.00498 μg/kg bw per day |

Abbreviation: bw, body weight.

[a]Estimated exposure converted from steviol equivalents (EFSA ANS Panel, 2015b) taking into account Reb M and Reb D at concentrations of approx. 89% and 9%, respectively, and with the conversion factors of 0.25 and 0.29, respectively.

The calculated mean potential exposure to kaurenoic acid is 0.00279 μg/kg bw per day and at the 95th percentile is 0.00498 μg/kg bw per day. The Panel noted that these calculations indicate a potential for exposure at up to two times the TTC value of 0.0025 μg/kg bw per day. However, the Panel considered that this concern is mitigated by (i) a likely overestimation of kaurenoic acid exposure due to the use of a conservative estimate for exposure to the proposed food additive itself and the conservative assumption that the concentration of KA is at the LOD and (ii) the uncertainties in the genotoxicity data.

Taking these aspects into account, the Panel considered that the demonstration of the absence of kaurenoic acid in the proposed food additive, using a method with an LOD of 0.3 mg/kg, is adequate to dispel the concerns for potential genotoxicity in this case.

The Panel recommends introducing a specific entry for kaurenoic acid in the final product specifications.

## 3.4 | Biological and toxicological data

Within the application dossier, scientific publications and original study reports considered by the applicant relevant to the safety of steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM were submitted (Documentation provided to EFSA No. 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, 2022). According to these

opinions, steviosides and steviol glycosides are not hydrolysed by digestive enzymes of the upper gastrointestinal tract due to the presence of ß-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 & 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 M and 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, 2022). In all these studies, the deglycosylation of the steviol glycosides to the final steviol metabolite was shown to occur within the first 12h of metabolic incubation.

#### ***In vitro* study submitted by the applicant**

In support of the current application, an *in vitro* metabolic study was performed with human faecal homogenate samples and a test item representative of the proposed food additive incubated up to 48h under anaerobic conditions to assess the conversion of steviol glycosides to the final metabolite steviol (Documentation provided to EFSA No. 1).

Pooled faecal homogenates prepared from stools of male and female adults were incubated with steviol glycoside material (namely High Purity Fermentation Derived Reb M and Reb D, consisting of 90.7% Reb M, 8.2% Reb D, 0.9% Reb A and 0.3% Reb B) at 0.2 mg/mL under anaerobic conditions. Parallel incubations with Reb A reference compound or steviol served as positive control for conversion or stability of the final metabolite, respectively. Samples of triplicate incubations per time point (0, 4, 8, 12, 16, 24 and 48 h) with two pooled male (M1, M2) and two pooled female (F1, F2) faecal homogenates were analysed by an LC/MS method to determine the remaining steviol glycoside and the steviol metabolite concentrations (expressed as % molar equivalents).

Based on the data observed for deglycosylation of Reb A (as positive control), which was to a large extent metabolised within 12–16 h in the pooled male and female faecal homogenates, the study authors considered the experimental conditions as appropriate. Data with the fermentation derived steviol glycoside material indicated a similar (slower) time course, yet with a relatively faster deglycosylation in pooled male over female homogenates.

The observed rates of conversion, which involve stepwise hydrolysis of multiple glucose units from Reb M and Reb D (and Reb A) to the final metabolite stevio,l are largely in line with previous studies on these compounds.

### 3.4.2 | Toxicological data

No toxicity studies on the proposed food additive were submitted by the applicant.

However, the Panel considered that the metabolic fate of steviol glycosides, including steviol glycosides obtained via fermentation, leads to the aglycone which is absorbed. Given that all steviol glycosides follow the same metabolic pathways, the Panel considered that the current steviol glycosides would fall within the same group of substances (EFSA ANS Panel, 2010; EFSA FAF Panel, 2020, 2022), and the group approach would be applicable.

### 3.4.3 | Additional information from the literature submitted by the applicant

In line with the group approach, data from previously evaluated steviol glycosides a sub-chronic toxicity study and two genotoxicity studies performed with rebaudioside A produced fermentatively by *Y. lipolytica* (Rumelhard et al., 2016) were considered relevant to be included in this opinion and are summarised below.

#### 3.4.3.1 | *Sub-chronic toxicity*

The 90-day toxicity study was performed following the principles of the test guideline (TG) OECD 408 (OECD, 1998) and in accordance with Good Laboratory Practice (GLP) (Rumelhard et al., 2016).

Sprague–Dawley rats (20/sex per group) were orally (diet) administered with 0, 500, 1000 and 2000 mg/kg bw per day of rebaudioside A produced fermentatively by *Y. lipolytica* (purity > 95%) (concentration in diet not reported). Average compound consumption was 516, 1026 and 2057 mg/kg bw per day in males and 509, 1016 and 2021 mg/kg bw per day in females of the low, mid and high dose group, respectively. The study did not result in any toxicologically relevant changes in clinical observations, clinical chemistry, haematology, ophthalmology, coagulation, urinalysis parameters, organ weights, nor macroscopic or microscopic pathology. Functional observational battery (FOB) and motor activity (MA) were also recorded during week 12 and no adverse effects were observed. Body weight, body weight gain and cumulative body weight gain were, generally, statistically significantly lower in males at the high dose. At the end of the study, the mean body weight of males of the high dose group was 5.9% lower than the controls. Some statistically significant decreases in

body weight were also observed in females in the highest dose group, throughout the course of the study, however, no significant difference was observed in final body weight. The Panel noted that the effects sizes of the decreases in body weights were only small, and therefore not considered adverse. Regarding food consumption, statistically significant increases were observed in females of low and high dose groups from study week 4–5. The effects were not considered treatment related. The study authors concluded that the NOAEL of this study can be set at the highest dose of 2000 mg/kg bw per day. This NOAEL corresponds to approximately 700 mg steviol equivalents/kg bw per day.

The Panel agreed with this conclusion.

The Panel noted that the test item of this study is not fully representative of the proposed food additive, whose main component is rebaudioside M, however the manufacturing process (fermentation by *Y. lipolytica*) is the same, and therefore considered relevant for this safety assessment.

Other sub-chronic toxicity studies were submitted by the applicant (Nettleton et al., 2019; Sanchez-Tapìa et al., 2019; Schiano et al., 2019). However, the Panel considered these studies not relevant for the present assessment because the test material was not representative of the proposed food additive and/or some shortcomings were identified in those studies.

#### 3.4.3.2 | *Genotoxicity*

The mutagenic activity of rebaudioside A (purity > 95%), produced by fermentation by a genetically modified strain of *Y. lipolytica*, was evaluated in a bacterial reverse mutation assay in Salmonella Typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and in *E. coli* WP2 uvrA (Rumelhard et al., 2016). Two separate experiments were conducted using the plate incorporation method, with and without metabolic activation (S9 mix from liver of Sprague–Dawley rat dosed with phenobarbital/β-naphthoflavone). The procedure was mainly in compliance with the OECD TG 471 (1997). The test substance was dissolved in DMSO, and the maximum concentration applied was 5000 µg/plate. No biologically relevant increase in the number of revertant colonies was observed in treated plates when compared with negative controls, while the positive control substances induced the expected effect, demonstrating the functionality of the experimental system.

The same test item was also tested in cultured peripheral human lymphocytes for the induction of micronuclei, in line with the OECD TG 487 (2014) (Rumelhard et al., 2016). The test was conducted with and without metabolic activation (S9 mix from liver of Sprague–Dawley rat dosed with phenobarbital/β-naphthoflavone) up to a maximum concentration of 5000 µg/mL. The cultures were treated for 3 h followed by 24 h of recovery with metabolic activation and for 24 h without metabolic activation. Cytochalasin B was used to allow the induction and the analysis of binucleated cells. Duplicate cultures were used for each concentration level and at least 1000 binucleated cells were analysed per culture. No precipitation and no cytotoxic effect were observed at any concentration tested in the 3-h treatment, while a slight decrease in relative cell growth (17%) was observed after the 24-h treatment. No biologically relevant increase in the number of micronucleated cells was observed at any tested concentration, while the positive controls induced significant effects.

The studies were considered relevant and reliable by the Panel. The results of the two assays do not raise a concern for genotoxicity of steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM.

Two publications reporting on genotoxicity endpoints were submitted in the dossier (Pasqualli et al., 2020; Yilmaz et al., 2020), however they were considered as not reliable and of low relevance due to inconsistent reporting and methodological shortcomings. Therefore, they were not further considered in the genotoxicity assessment of the food additive.

#### 3.4.3.3 | *Reproductive and developmental toxicity*

The applicant provided two publications. Gholizadeh et al. (2019) described the protective effect of Stevia extract (400 mg/kg bw per day, duration of administration not described) on the impaired male fertility in diabetic rats. Li et al. (2020) studied the influence of rebaudioside A for 28 days on the expression of sweet taste receptors (T1R2, T1R3) in the ovary and uterus of peripubertal female guinea pigs. The Panel noted that the endpoints reported in these publications provided information that was considered not relevant for the current assessment.

#### 3.4.3.4 | *Human studies*

Human studies performed with rebaudioside A or commercially available products containing stevia extract (Ajami et al., 2020; Cocco et al., 2019; Higgins and Mattes, 2019; Farhat et al., 2019; Stamataki et al., 2020; Sanchez-Delgado et al., 2020) were submitted within the application dossier. The Panel noted that the purpose of these studies was to investigate beneficial effects of products containing stevia (e.g. on dental caries), and efficacy of steviol glycosides on body weight and glucose handling, insulin levels, immunological parameters. In addition, the Panel noted that the substances tested in these studies were not fully characterised (e.g. purity not reported). None of the studies provided information that was considered relevant for the current assessment.

#### 3.4.3.5 | *Other studies*

The applicant provided one publication investigating the effect of stevia on glycaemia, hormones, cytokines and GALT lymphocytes in CD-1 mice (Rosales-Gomez et al., 2018). The Panel considered this study as not reliable since the test item was not characterised (stevia purity and dose in mg/kg bw per day were not reported). Therefore, the study was considered of low relevance for the safety assessment of the food additive.

Han et al. (2019) reported on effects on food intake in goats. The Panel noted that none of the endpoints reported in this publication provided information that was considered relevant for the current assessment.

## 4 | DISCUSSION

The present opinion deals with the safety evaluation of the steviol glycosides preparation composed predominantly of rebaudioside M (~90% on a dry basis), produced via a new process by fermentation of simple sugars using a genetically modified strain of *Y. lipolytica* i.e. *Y. lipolytica* VRM.

The manufacturing process of Steviol glycosides, predominantly Rebaudioside M, produced by fermentation using *Y. lipolytica* VRM begins with the *Y. lipolytica* VRM production strain being added to the fermentation medium, then, it is allowed to produce steviol glycosides under aerobic conditions. The fermentation is stopped via heat treatment to inactivate the yeast cells; subsequentially, the biomass is separated from the steviol glycosides by microfiltration. Then the steviol glycosides are purified by using filtration aids, purification resins and crystallisation. The final product is comprised mostly of rebaudioside M and contains a mixture of the following glycosides at various concentrations: rebaudiosides A, B and D, such that the total steviol glycosides' content is not less than 95%.

The Panel noted the proposal from the applicant to amend the current specification of the food additives steviol glycosides (E 960a-960c), however, it considers that separate specifications are needed for the food additive produced via the manufacturing process described in the current application, which could also contain additional parameters in the specifications related to the specific microorganism used for its production. The Panel also considered that the production process evaluated in the present assessment could generate impurities different from those that may be present in the other already authorised steviol glycosides E 960a-960d.

The Panel recommended to specify in the proposed definition the production strain, *Y. lipolytica* VRM, (DS 82603), which was deposited in the Culture Collection of the Westerdijk Fungal Biodiversity Institute (CBS, The Netherlands) with deposition number CBS 147477. The parental and recipient *Y. lipolytica* strains are considered to be safe and having QPS status. The Panel considered that the genetic modifications done to obtain the production strain do not raise a safety concern.

The Panel noted that the specifications proposed by the applicant contain parameters regarding the genetically modified microorganism used to produce the food additive (i.e. absence of viable cells and DNA of the production strain; no more than 20 mg/kg of residual protein), which are aligned with the JECFA specifications for E 960b, as laid down in Monograph 26 (2021). The Panel noted that adequate analytical data supporting the compliance with the provision for residual protein specifications were submitted by the applicant. Since no viable cells nor their DNA remained in the final product, the manufacturing process does not raise a safety concern.

Analytical data on levels of toxic elements (arsenic, lead, cadmium, mercury) in five samples of the proposed food additive were provided by the applicant, and all data were below their respective LOQs. The Panel noted that the data on toxic elements submitted by the applicant are lower than the limits in the proposed specifications. The Panel noted that based on the analytical data, the proposed maximum limits for lead, mercury, cadmium and arsenic are adequate. The potential exposure to these impurities was compared against the available health-based guidance values (HBGV) and reference points (RP) (Section 3.3.1, Tables 3, 4). The Panel performed a risk assessment on the presence of these toxic elements in the proposed food additive at the specification limits and concluded that the lower ends of the ranges of the calculated MOE values for arsenic were insufficient, i.e. below the target value of 1000. The presence of the other toxic elements does not give rise to safety concerns.

The absence of kaurenoic acid was shown in five batches of the proposed food additive, in which kaurenoic acid measured though LC–MS was not detected in the tested samples (LOD of 0.3 mg/kg). Based on the available data, a genotoxic potential of kaurenoic acid could not be ruled out, and therefore the Panel considered it appropriate to apply the TTC approach for this contaminant. Therefore, the threshold value of 0.0025 μg/kg bw, considered appropriate for potential DNA-reactive mutagens and/or carcinogens, was used in this assessment. The Panel noted that the exposure calculations at the 95th percentile showed a small exceedance of the TTC. However, taking into account that the conservative exposure assessment likely resulted in an overestimation, the Panel considered that the demonstration of the absence of kaurenoic acid in the proposed food additive, using a method with an LOD of 0.3 mg/kg, is adequate to dispel the concerns for potential genotoxicity in this case. The Panel recommends introducing a specific entry for kaurenoic acid in the final product specifications.

Based on the data on particle size distribution submitted by the applicant and the criteria set in the EFSA Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles (EFSA Scientific Committee, 2021), the Panel concluded that the presence of small particles, including nanoparticles, in the pristine food additive cannot be excluded or confirmed. The Panel noted that the maximum permitted use levels for Steviol E 960a-960d for most food categories do not exceed 350 mg/L (expressed as steviol equivalents). For food categories FC 5.3, 5.2, 17.1 and 17.2 the food additive is allowed at maximum use levels in the range of 670–3300 mg/L. For table-top sweeteners the additive is allowed *quantum satis*, however, these are not intended to be consumed as such and will be largely diluted in beverages and, accordingly, particles would be expected to dissolve.

The steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM has a steviol equivalency factor of 0.25 for Reb M and 0.29 for Reb D (see Table 2), therefore MPL values expressed as steviol equivalents correspond to approximately four-fold higher concentration of the preparation. Taking into account the MPLs, the reported solubility (i.e. 1.61–1.89 g/L at 22°C) and the volume of gastric secretion (ranging from 215 mL within a single meal to 2000 mL daily; ICRP, 2002; Mudie et al., 2014), the Panel considered that full dissolution of the proposed food additive is to be expected in foods and/or in the GI tract and that ingested particles (if any) would not persist. Therefore, the Panel concluded there is no concern with regard to the potential presence of small particles, including nanoparticles, in the proposed food

additive and considered that the risk assessment can be performed following the EFSA Guidance for submission for food additive evaluations (EFSA ANS Panel, 2012).

An *in vitro* metabolic study of steviol glycosides (predominantly Reb M and Reb D) produced by fermentation performed in pooled human faecal homogenates was provided by the applicant. The authors concluded that the metabolism of the test item indicated a 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, 2022).

No toxicity study on steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM were submitted by the applicant in the dossier. However, a 90-day dietary rat study and two genotoxicity studies performed with rebaudioside A produced fermentatively by *Y. lipolytica* were considered relevant for the current assessment (Rumelhard et al., 2016). No adverse effects were reported in the 90-day study up to the highest dose of 2000 mg/kg bw per day (corresponding to approximately 700 mg steviol equivalent/kg bw per day). In addition, the results from the bacterial reverse mutation assay and the *in vitro* micronucleus assay were negative and indicated absence of genotoxicity.

The Panel considered that the metabolic fate of steviol glycosides, including steviol glycosides obtained via fermentation, leads to the aglycone which is absorbed. Given that all steviol glycosides follow the same metabolic pathways, the Panel considered that the current steviol glycosides would fall within the same group of substances (EFSA ANS Panel, 2010; EFSA FAF Panel, 2020, 2022), and the group approach would be applicable. Therefore, the Panel considered that the already existing data on rebaudioside M and structural-related steviol glycosides (EFSA ANS Panel, 2010, EFSA FAF Panel, 2019, 2020, 2021, 2022), along with new supportive toxicity data on rebaudioside A produced fermentatively by *Y. lipolytica* (same manufacturing process of the proposed food additive), are sufficient. Therefore, no additional toxicity studies are required.

The existing ADI of 4 mg/kg bw per day (expressed as steviol equivalents) can also be applied to steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM as described in the present opinion.

## 5 | CONCLUSIONS

The Panel concluded that there is no safety concern for steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM to be used as a food additive at the proposed uses and use levels, taking into account the existing ADI of 4 mg/kg bw per day (expressed as steviol equivalents). Separate specifications for steviol glycosides, predominantly rebaudioside M, produced by fermentation using *Y. lipolytica* VRM should be considered in Commission Regulation (EU) No 231/2012, since the manufacturing process may lead to impurities different from those that may be present in the other, already authorised, steviol glycosides (E 960a-960d).

## 6 | RECOMMENDATIONS

## 7 | DOCUMENTATION AS PROVIDED TO EFSA

1. Application for the approval of steviol glycosides produced by *Yarrowia lipolytica* pursuant to European Parliament and Council Directive (EC) 1333/2008, 16 December 2008 on Food Additives. Technical Dossier. Avansya V.O.F September 2021
2. Additional information submitted by the applicant Avansya V.O.F. following a request from EFSA. May 2022.
3. Additional information submitted by the applicant Avansya V.O.F. following a request from EFSA. September 2022.
4. Additional information submitted by the applicant Avansya V.O.F following a request from EFSA. June 2023.

### ABBREVIATIONS

| | |
|---|---|
| ADI | acceptable daily intake |
| ADME | Absorption, distribution, metabolism and excretion |
| ANS Panel | Panel on Food Additives and Nutrient Sources added to Food |
| AOAC | Association of Official Agricultural Chemists |
| ATCC | American Type Culture Collection. |
| BMDL | benchmark dose (lower confidence limit) |
| bw | body weight |
| CAS | Chemical Abstract Service |
| CEP Panel | Panel on Food Contact Materials, Enzymes and Processing Aids |
| CONTAM Panel | Panel on Contaminants in the Food Chain |
| DNA | deoxyribonucleic acid |
| FAF Panel | Panel on Food Additives and Flavourings |
| FAO/WHO | Food and Agriculture Organisation/World Health Organisation |
| FCC | Food Chemical Codex |
| FDA | Food and Drug Administration |
| FOB | functional observational battery |

| | |
|---|---|
| FSANZ | Food Standards Australia New Zealand |
| GLP | Good Laboratory Practice |
| GMO | Genetically Modified Organisms |
| GMP | Good Manufacturing Practice |
| GRAS | Generally Recognised as Safe |
| HBGV | health-based guidance value |
| HPLC | high performance liquid chromatography |
| HPLC-UV | high performance liquid chromatography–ultraviolet |
| ICP-MS | Inductively Coupled Plasma–Mass Spectrometry |
| ICRP | International Commission on Radiological Protection |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| LC/MS | liquid chromatography–mass spectrometry |
| LOD | limit of detection |
| LOQ | limit of quantification |
| MA | motor activity |
| MOE | margin of exposure |
| MPLs | maximum permitted levels |
| MS | mass spectrometry |
| NOAEL | no observed adverse effect level |
| OECD | Organisation for Economic Co-operation and Development |
| pH | potential of hydrogen |
| ppb | parts per billion |
| ppm | parts per million |
| PSD | particle size distribution |
| QPS | qualified presumption of safety |
| RP | reference point |
| SC | Scientific Commission |
| SEM | scanning electron microscopy |
| SIR | selective ion recording |
| TDI | tolerable daily intake |
| TTC | threshold of toxicological concern |
| TG | test guideline |
| TWI | tolerable weekly intake |
| UDP | uridine diphosphate |
| UGT | UDP glucosyltransferase |
| WGS | whole-genome sequencing |

## CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

## REQUESTOR

European Commission

## QUESTION NUMBER

EFSA-Q-2021-00356

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