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SCIENTIFIC OPINION

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Safety evaluation of the food additive rebaudioside M produced via enzymatic conversion of purified steviol glycosides leaf extracts

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Abstract

The EFSA Panel on Food Additives and Flavourings (FAF Panel) provides a scientific opinion on the safety of a modified manufacturing process for the food additive enzymatically produced steviol glycosides (E 960c). The new process converts purified steviol glycosides extracted from *Stevia rebaudiana* leaves through enzymatic bioconversion catalysed by UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase enzymes, produced using a newly developed genetically modified strain of *Escherichia coli* (K-12 DSM 35119). The modification leads to changes in the definition of the food additive, residual protein, residual solvents and particle size. The Panel considered that the introduction of a new entry for the proposed food additive in Commission Regulation (EU) No 231/2012 would be more appropriate than amending the existing specifications for E 960c. The food additive is composed predominantly of rebaudioside M (> 95%) with minor amounts of other steviol glycosides. The manufacturing process does not raise a safety concern since no viable cells nor DNA of the production strain remained in the final product; in addition, the food enzyme–total organic solid (TOS) are removed to at least 99%, and consequently, the exposure to the food enzyme–TOS can be considered negligible. The Panel considered that rebaudioside M produced by this new manufacturing process have the same physicochemical characteristics as the corresponding rebaudioside M present in E 960c(i) and (ii); therefore, the biological and toxicological data considered in previous evaluations will also apply to the present safety assessment. The Panel concluded that there is no safety concern with respect to rebaudioside M (Reb M) produced via enzymatic conversion of purified steviol glycosides leaf extracts (95% steviol glycosides), using UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase enzymes produced by a genetically modified strain of *Escherichia coli* (named *E. coli* K-12 DSM 35119).

KEYWORDS

enzymatic bioconversion, *Escherichia coli*, rebaudioside M, *Stevia rebaudiana*, Steviol glycosides

CONTENTS

1 | INTRODUCTION

The present opinion deals with the safety evaluation of the food additive rebaudioside M (Reb M) produced via enzymatic conversion of purified steviol glycosides leaf extracts (95% steviol glycosides), using UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase enzymes produced by a genetically modified strain of *Escherichia coli* (named *E. coli* K-12 DSM 35119). The proposed food additive is hereinafter also referred to as 'Reb M produced from *E. coli* K-12 DSM 35119'.

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

Enzymatically produced steviol glycosides (E 960c) 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 describe the enzymes used in the manufacturing process.

The European Commission received a request for an amendment of the specifications of rebaudioside M produced via enzymatic conversion of highly purified rebaudioside A Stevia leaf extracts (E960c(ii)) to include the use of enzymes for a different microbial source in the manufacturing process.

1.1.2 | Terms of Reference

The European Commission requests the European Food Safety Authority to provide a scientific opinion on the safety of the proposed amendment of the specifications of the food additive rebaudioside M produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extracts (E 960c(ii)) in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.[3]

1.1.3 | Interpretation of the Terms of Reference

The Panel noted that the original application submitted to the European Food Safety Authority (EFSA) for evaluation was in support of an amendment of the specifications of Reb M (E 960c(ii)), as laid down in Regulation (EC) No 231/2012, with respect to the definition of the food additive.

In the course of the assessment, the applicant revised its original proposal and indicated that it sought either a revision of the specifications for E 960c(ii) or, if considered more appropriate, a modification of the specifications for E 960c(i). The proposed amendment aimed to revise the definition to include the use of three enzymes (i.e. UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase enzymes) from a new genetically modified strain of *E. coli* K-12 DSM 35119 in the manufacturing process (Documentation provided to EFSA No. 1).

The Panel noted that the proposed amendments are not limited to the introduction of enzymes derived from a different microbial source. In particular, the proposed definition does not correspond to that of E 960c(ii), as the starting material consists of purified steviol glycoside leaf extracts (≥ 95% steviol glycosides), rather than highly purified steviol glycoside rebaudioside A extracts (95% steviol glycosides). Additional elements of misalignment among the proposed specification by the applicant and the specifications as reported in Commission Regulation (EU) No 231/2012 for E 960c(i) or E 960c(ii) are reported in section 3.2.1 of the present scientific opinion.

Overall, the Panel considered that the substance as defined in the proposal is not consistent with the current specifications for either E 960c(i) or E 960c(ii). Therefore, the Panel recommends the European commission to create a new entry for the proposed food additive in the applicable legislation (Commission Regulation (EU) No 231/2012).

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

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

[3]Regulation (EC) No 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.

1.2 | Information on existing evaluations and authorisations

According to Annex II to Regulation (EC) No 1333/2008, the following food additives are authorised for use in the EU, listed under the group of steviol glycosides (E 960a-d): steviol glycosides from Stevia (E 960a); rebaudioside M from fermentation produced by *Yarrowia lipolytica* (E960b(i)); enzymatically produced steviol glycosides (E 960c) and glucosylated steviol glycosides (E 960d). These food additives have combined maximum permitted levels (MPLs) for use in food, expressed as steviol equivalents and are listed in the functional group of sweeteners. Steviol glycosides from Stevia (E 960a) was the first steviol glycoside to be authorised as a food additive in the EU and is obtained by water extraction of the leaves of the *Stevia rebaudiana* (Bertoni) Bertoni plant. According to the specifications defined in Commission Regulation (EU) No 231/2012, it is described as: 'not less than 95% steviolbioside, rubusoside, dulcoside A, stevioside, rebaudiosides A, B, C, D, E, F and M on the dried basis, in any combination and ratio'.

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, 2015), rebaudioside D and M were included in the specifications for steviol glycosides (E 960).

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* 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 the specifications, there would remain a small but not insignificant fraction of the additive that was undefined and therefore could not be evaluated by the Panel. Therefore, while inclusion of the 60 steviol glycosides in the specifications for steviol glycoside (E 960) would not be of safety concern, the FAF Panel could not conclude on the safety of the proposed amendment to the specifications 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 2021, a new entry for 'enzymatically produced steviol glycosides (E 960c)' was added to Annex II to Regulation (EC) No 1333/2008. This amendment to the Regulation was based on the conclusions from EFSA on the safety of a proposed amendment of the specifications of the food additive steviol glycosides (E 960) concerning rebaudioside M produced by enzyme modification of steviol glycosides, using UDP-glucosyl transferase and sucrose synthase enzymes produced by the genetically modified yeasts *Komagaetella phaffii* UGT-A and 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 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, 2021).

In March 2023, both Regulation (EC) No 1333/2008 and Regulation (EU) No 231/2012 were again amended introducing the entry 'glucosylated steviol glycosides' (E 960d), based on the evaluation completed by the Panel (EFSA FAF Panel, 2022a). With that latest amendment introduced in the legislation, also the definition of the group of food additives named 'steviol glycosides' was changed to E 960a–960d.

In addition to the already authorised uses, the FAF Panel completed in 2022 the safety evaluation of an additional proposed amendment to the specifications of the food additive steviol glycosides (E 960). The application regarded rebaudioside D produced by enzymatic bioconversion of purified *S. rebaudiana* leaf extract, using UDP-glucosyltransferase (UGT) and sucrose synthase produced by a genetically modified strain of the yeast *K. phaffii* (EFSA FAF Panel, 2022b).

In 2024, the FAF Panel completed the safety evaluation of proposed changes to the currently permitted uses of the food additive steviol glycosides (E 960a–d) and of a proposed modification of the current acceptable daily intake (ADI). In the context of that opinion the Panel updated the dietary exposure estimate to steviol glycosides expressed as steviol equivalents, applying a methodology that has been developed and implemented by the Panel in the context of the re-evaluation of already authorised sweeteners under Regulation (EU) No 257/2010. The exposure to steviol was calculated based on the currently permitted uses and maximum permitted levels, representing the latest estimated dietary exposure to the food additive (E 960a–d) for the *regulatory maximum level exposure assessment scenario*. The Panel concluded that there was insufficient justification to change the current ADI for steviol glycosides (E960a–d) of 4 mg/kg bw per day (expressed as steviol equivalents) (EFSA FAF Panel, 2024).

Regulation (EU) No 231/2012 was further amended in April 2025 by including the entry: 'E 960b(i) rebaudioside M from fermentation produced by *Yarrowia lipolytica*', manufactured by a new fermentation process of simple sugars (EFSA FAF Panel, 2023).

In 2025, the FAF Panel provided a scientific opinion on the safety of the proposed amendment of the specifications of rebaudioside M produced via enzyme-catalysed bioconversion (E 960c(i) or E 960c(ii)), to include a different microorganism

strain in the definition i.e. *K. phaffii* CGMCC 7539. The Panel concluded that there was no safety concern with respect to the proposed amendment (EFSA FAF Panel, 2025).

In 2026, the FAF Panel provided a scientific opinion on a modified manufacturing process for enzymatically produced steviol glycosides (E 960c). The new process uses enzymatic bioconversion of purified steviol glycosides from *S. rebaudiana*, employing enzymes produced by newly developed genetically modified *E. coli* strains. This results in two specific preparations: SBP1, predominantly rebaudioside M, and SBP2, predominantly rebaudioside D, requiring corresponding updates to the additive's definition and specifications. The Panel concluded that there is no safety concern with respect to the proposed modification of the enzymatically produced steviol glycosides E 960c related to the use of the new genetically modified strains of *E. coli* in the production process of SBP1 and SBP2 (EFSA FAF Panel, 2026).

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 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 principal sugar moieties (glucose, rhamnose, xylose, fructose and deoxyglucose) at different positions and in any of the stereochemical configurations occurring in the leaves of *S. rebaudiana*, provided that the total percentage of steviol glycosides is not less than 95% (JECFA, 2017). These specifications were 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 glycosylation (FAO and WHO, 2020). The framework adopted in 2019 was 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 'Enzyme modified Steviol Glycosides' (FAO and WHO, 2021).

2 | DATA AND METHODOLOGIES

2.1 | Data

The present evaluation is based on the data submitted in the application dossier (Documentation provided to EFSA No. 1), and on additional information, following requests by EFSA, submitted by the applicant in July 2025, March 2026, May 2026 (Documentation provided to EFSA No. 2–4).

In accordance with Art. 38 of the Commission Regulation (EC) No 178/2002[4] and taking into account the protection of confidential information and of personal data in accordance with Articles 39 to 39e of the same Regulation and of the Decision of the EFSA's Executive Director laying down practical arrangements concerning transparency and confidentiality,[5] the non-confidential version of the dossier is published on Open EFSA.[6]

According to Article 32c(2) of Regulation (EC) No 178/2002[7] and to the Decision of EFSA's Executive Director laying down the practical arrangements on pre-submission phase and public consultations, EFSA carried out a public consultation on the non-confidential version of the technical dossier from 25 September 2025 to 16 October 2025.[8] No comments were received.

2.2 | Methodologies

This opinion was formulated following the principles described in the EFSA Guidance of the Scientific Committee on transparency regarding 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.

Provided that rebaudioside M produced by conversion of purified steviol glycosides leaf extracts (95% steviol glycosides) obtained from *S. rebaudiana* Bertoni plant has the same physicochemical characteristics as the corresponding

[4]Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

[5]Decision https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements.9.

[6]The non- confidential version of the dossier, following EFSA's assessment of the applicant's confidentiality requests, is published on Open. EFSA and is available at the following link: Open EFSA.

[7]Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

[8]https://connect.efsa.europa.eu/RM/s/consultations/publicconsultation2/a0lTk000005fWFZ/pc1626.

rebaudioside M present in E960c(i), E 960c(ii) the biological and toxicological data for E960(a–d) are considered by the Panel to support the safety of the food additives produced with the enzymatic bioconversion step subject of the present application. Therefore, no additional biological and toxicological data are required. The previous evaluations by the ANS and FAF Panels (EFSA ANS Panel, 2010, 2015, 2018; EFSA FAF Panel, 2019, 2020, 2021, 2022a, 2022b, 2023, 2024, 2025, 2026) will also apply to the safety assessment of rebaudioside M produced via enzymatic conversion of purified steviol glycosides leaf extracts (95% steviol glycosides).

3 | ASSESSMENT

3.1 | Technical data

3.1.1 | Identity of the proposed food additive

The present opinion deals with the safety assessment of the food additive rebaudioside M (Reb M) produced via enzymatic conversion of purified steviol glycosides leaf extracts (95% steviol glycosides) obtained from *S. rebaudiana* Bertoni plant.

In particular, the applicant has requested an amendment to the specifications for steviol glycosides E 960c(i) (rebaudioside M produced via enzymatic bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides)) or E 960c(ii) (rebaudioside M produced via enzymatic conversion of highly purified rebaudioside A extracts (95% steviol glycosides)).

The applicant proposed to include the use of enzymes (i.e. UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase enzymes) from a different microbial source (i.e. genetically modified strain of *E. coli* K-12 DSM 35119) in the manufacturing process.

Reb M was analysed in 10 batches using an ultra-performance liquid chromatography equipped with diode array detector (UPLC–DAD) in accordance with the methods described in the JECFA monographs (JECFA, 2017, 2023). According to the applicant, quantification of Reb M was performed using a calibration curve prepared with a commercial rebaudioside M standard. Results were reported on a dry weight basis, and the corresponding certificates of analysis (CoAs) were provided (Documentation provided to EFSA No. 1 and No. 3). The Reb M content ranged from 95.1% to 98.2%.

In addition to Reb M, the resulting mixtures contained minor amounts of other steviol glycosides, together with moisture and ash (Documentation provided to EFSA No. 2–4). The steviol glycoside profile was investigated by the applicant in three batches by UPLC–DAD–MS, in accordance with the method described in the JECFA monograph (JECFA, 2017). The concentrations of the minor steviol glycosides were calculated indirectly by applying molecular weight conversion factors, as described in the same JECFA method (JECFA, 2017). Among the minor components, the Reb M isomer ranged from 0.5% to 1.5%, the Reb V isomer from 0.8% to 1.3% and the Reb V2 isomer from 0.3% to 0.5%. The Reb N isomer was present at 0.8% in all three batches. Rebaudiosides I and D isomers were detected at levels ranging from non-detected to 0.2%, while Reb N ranged from not detected to 0.5%. The sum of quantified steviol glycosides ranged from 99.9% to 100.5% on a dry weight basis. The corresponding chromatograms of the analysis of each batch were provided (Documentation provided to EFSA No. 2).

The CAS numbers, molecular formulae and molecular weight for the proposed food additive were provided by the applicant. According to the applicant, the chemical structure of Reb M produced using the new enzyme and the new genetically modified strain of *E. coli* described in this application, is identical to that of Reb M specified under E 960c(i) and E 960c(ii). Based on the data provided, the Panel concurs with the applicant.

The applicant reported that Reb M produced from *E. coli* K-12 DSM 35119 is a white to yellow powder with sweetness 'approximately between 150 and 350 times higher than sucrose at 5% sucrose equivalency' (Documentation provided to EFSA No. 1–4).

3.1.2 | Proposed specifications

The applicant provided proposals for amending the existing specifications for the already authorised food additives E 960c(ii) or E 960c(i). In particular, the applicant proposed to include the use of three enzymes (i.e. UDP-glucosyltransferase and/or UTP-glucose-1-phosphate uridylyltransferase, and/or sucrose synthase enzymes) from a genetically modified strain of *E. coli* K-12 DSM 35119 in the manufacturing process (Documentation provided to EFSA No. 1–4).

The applicant provided the product specification data and reported that the food additive is manufactured within their proposed specifications (Documentation provided to EFSA No. 4).

A comparison between the existing EU specifications for E 960c(i) 'Rebaudioside M produced via enzyme modification of steviol glycosides from Stevia', E 960c(ii) 'Rebaudioside M produced via enzyme modification of highly purified rebaudioside A stevia leaf extracts' and the changes proposed by the applicant is presented in Table 1.

TABLE 1 Current EU specifications as set in Regulation (EU) No. 231/2012 for 'E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from Stevia, 'E 960c(ii) Rebaudioside M produced via enzyme modification of highly purified rebaudioside A stevia leaf extracts' and as proposed by the applicant for Reb M produced from *E. coli* K-12 DSM 35119 (Documentation provided to EFSA No. 4).

	EU Specifications for E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from stevia (Regulation (EU) No. 231/2012)			**EU Specifications for E 960c(ii) Rebaudioside M produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extracts (Regulation (EU) No. 231/2012)**			**Proposed Specification by the applicant for Rebaudioside M produced via enzymatic conversion of purified steviol glycoside leaf extracts (95% steviol glycosides)**
Definition	Rebaudioside M is a steviol glycoside composed predominantly of rebaudioside M with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside B, rebaudioside D, rebaudioside I and stevioside. Rebaudioside M is obtained via enzymatic bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides) of the *S. rebaudiana* Bertoni plant using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffi* (formerly known as *Pichia pastoris*) UGT-a and *K. phaffii* UGT-b that facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds. After removal of the enzymes by solid–liquid separation and heat treatment, the purification involves concentration of the rebaudioside M by resin adsorption, followed by recrystallisation of rebaudioside M resulting in a final product containing not less than 95% of rebaudioside M. Viable cells of the yeasts *K. phaffii* UGT-a and *K. phaffii* UGT-b and their DNA shall not be detected in the food additive.			Rebaudioside M produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extracts is a steviol glycoside composed predominantly of rebaudioside M with minor amounts of other steviol glycosides such as rebaudioside A and rebaudioside D. Rebaudioside M is produced via enzymatic conversion of highly purified steviol glycoside rebaudioside A extracts (95% steviol glycosides) obtained from Stevia rebaudiana Bertoni plant using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified strains of *E. coli* (pPM294, pFAF170 and pSK401) that facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds. After removal of the enzymes by solid–liquid separation and heat treatment, the purification involves concentration of the rebaudioside M by resin adsorption, followed by recrystallisation of the steviol glycosides resulting in a final product containing not less than 95% of rebaudioside M. Viable cells of *E. coli* (pPM294, pFAF170 and pSK401) and their DNA shall not be detected in the food additive.			Rebaudioside M produced via enzymatic conversion of purified steviol glycoside leaf extracts is a steviol glycoside composed predominantly of rebaudioside M with minor amounts of other steviol glycosides such as rebaudioside A and rebaudioside D. Rebaudioside M is obtained via enzymatic bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides) of the Stevia rebaudiana Bertoni plant using UDP-glucosyltransferase and/or UTP-glucose-1-phosphate uridylyltransferase, and/or sucrose synthase enzymes produced by the genetically modified strains of *E. coli* (pPM294, pFAF170, pSK401 and/or MG1655) that facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds. After removal of the enzymes by solid–liquid separation and heat treatment, the purification involves concentration of the rebaudioside M by resin adsorption and/or filtration followed by recrystallisation of the steviol glycosides resulting in a final product containing not less than 95% of rebaudioside M. Viable cells of *E. coli* (pPM294, pFAF170, pSK401, genetically modified MG1655) and their DNA shall not be detected in the food additive.
Chemical names	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 **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			No proposed changes
Molecular formula	**Trivial name**	**Formula**	**Conversion factor**	**Trivial name**	**Formula**	**Conversion factor**	
	Rebaudioside M	$C_{56}H_{90}O_{33}$	0.25	Rebaudioside M	$C_{56}H_{90}O_{33}$	0.25	No proposed changes

(Continues)

TABLE 1 (Continued)

	EU Specifications for E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from stevia (Regulation (EU) No. 231/2012)			**EU Specifications for E 960c(ii) Rebaudioside M produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extracts (Regulation (EU) No. 231/2012)**			**Proposed Specification by the applicant for Rebaudioside M produced via enzymatic conversion of purified steviol glycoside leaf extracts (95% steviol glycosides)**
Molecular weight and CAS No	**Trivial name**	**CAS Number**	**Molecular weight (g/mol)**	**Trivial name**	**CAS Number**	**Molecular weight (g/mol)**	
	Rebaudioside M	1220616-44-3	1291.29	Rebaudioside M	1220616-44-3	1291.29	No proposed changes
Assay	Not less than 95% rebaudioside M on the dried basis.			Not less than 95% rebaudioside M on the dried basis.			No proposed changes
Description	White to light yellow powder, approximately between 200 and 350 times sweeter than sucrose (at 5% sucrose equivalency).			White to light yellow powder, approximately between 150 and 350 times sweeter than sucrose (at 5% sucrose equivalency).			No proposed changes compared to E 960c(ii)
Identification							
Solubility	Freely soluble to slightly soluble in water			Freely soluble to slightly soluble in water			No proposed changes
pH	Between 4.5 and 7.0 (1 in 100 solution)			Between 4.5 and 7.0 (1 in 100 solution)			No proposed changes
Purity							
Total ash	Not more than 1%			Not more than 1%			No proposed changes
Loss on drying	Not more than 6% (105°, 2 h)			Not more than 6% (105°, 2 h)			No proposed changes
Residual solvent	Not more than 5000 mg/kg ethanol			Not more than 5000 mg/kg ethanol			No proposed changes
Arsenic	Not more than 0.015 mg/kg			Not more than 0.015 mg/kg			No proposed changes
Lead	Not more than 0.2 mg/kg			Not more than 0.2 mg/kg			No proposed changes
Cadmium	Not more than 0.015 mg/kg			Not more than 0.015 mg/kg			No proposed changes
Mercury	Not more than 0.07 mg/kg			Not more than 0.07 mg/kg			No proposed changes
Residual protein	Not more than 5 mg/kg			Not more than 5 mg/kg			Not more than 75 mg/kg
Particle size	Not less than 74 µm [using a mesh #200 sieve with a particle size limit of 74 µm]			Not less than 74 µm [using a mesh #200 sieve with a particle size limit of 74 µm]			No proposed changes

The applicant submitted analytical data from the analyses of 10 non-consecutive batches of Reb M produced from *E. coli* K-12 DSM 35119 (Documentation provided to EFSA No. 1–4). 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 1.

Definition

The Panel noted that the definition in the proposed specifications for the proposed food additive is not in line with the current definition for either E 960c(i) or E 960c(ii).

In particular the Panel noted that:

- The proposed definition differs from that of E 960c(i), as the enzymes are not produced by a genetically modified yeast (*K. phaffii*), but by a genetically modified strain of *Escherichia coli*. It also differs from the definition of E 960c(ii), as the starting material used is not purified steviol glycoside rebaudioside A extracts (95% steviol glycosides) but purified steviol glycoside leaf extracts (95% steviol glycosides).
- Rebaudioside A and rebaudioside D, listed as minor rebaudiosides in the definition, were not detected in any of the analysed samples; therefore, their inclusion is not supported by the analytical data.
- In contrast to the specifications for E 960c(i) and E 960c(ii), the proposed definition refers to filtration as a possible purification step for Reb M.
- The genetically modified strain of *E. coli* indicated in the definition (i.e. MG1655) corresponds to the parental strain. The Panel therefore considered that the definition should refer instead to the actual production strain (i.e. DSM 35119).

The Panel considered that it would be more appropriate to create a new entry for the proposed food additive and to define it as:

'Rebaudioside M is a steviol glycoside composed predominantly of rebaudioside M with minor amounts of other steviol glycosides such as rebaudioside M isomer and rebaudioside V isomer. Rebaudioside M is obtained via enzymatic bioconversion of

purified steviol glycoside leaf extracts (95% steviol glycosides) of the *S. rebaudiana* Bertoni plant using UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase enzymes produced by the genetically modified strain of *E. coli* (DSM 35119) that facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds. After removal of the enzymes by solid–liquid separation and heat treatment, the purification involves concentration of the rebaudioside M by filtration, followed by recrystallisation, resulting in a final product containing not less than 95% of rebaudioside M. Viable cells of *E. coli* (DSM 35119) and its DNA shall not be detected in the food additive'.

Molecular weight

The Panel noted that the molecular weight for Reb M produced from *E. coli* K-12 DSM 35119 in the proposed specifications is in line with the molecular weight reported in the current specification for E 960c(i) and E 960c(ii).

Assay

According to the applicant, the proposed food additive consists predominantly of Reb M and contains minor amounts of other steviol glycosides, including Reb M isomer, Reb V isomer, Reb V2 isomer, Reb N isomer, Reb I, Reb D isomer and Reb N.

The Panel noted that analytical data on rebaudioside M content were provided for 10 batches of the proposed food additive whereas data on the minor steviol glycosides were available for three batches only (see Section 3.1.1; Documentation provided to EFSA No. 1 and No. 3).

The Panel considered the statement proposed by the applicant of 'not less than 95% rebaudioside M on the dried basis' in the assay entry of the proposed specifications as adequate.

Solubility

The proposed food additive is described by the applicant as 'freely soluble to slightly soluble in water' (Documentation provided to EFSA No. 1–4). The applicant provided information on the water solubility for five batches of the proposed food additive, determined by applying the OECD Test guideline (TG) 105 (OECD, 1995) (shake flask method) (Documentation provided to EFSA No. 3). The samples were analysed by liquid chromatography with diode array detection (LC–DAD) and the measured solubility of Reb M, at 20°C under neutral condition [redacted] was determined to be approximately 1.18 g/L.

The Panel considered that the applicant's proposed specification for water solubility was not supported by the experimental data submitted. Therefore, the Panel recommended the proposed food additive to be described as 'slightly soluble in water', according to the criteria established by JECFA (2006). Accordingly, the description of the water solubility in the current specifications for E 960c(i) and E 960c(ii) should be revised.

Toxic elements

The applicant provided analytical data on the content of arsenic (As), lead (Pb), cadmium (Cd) and mercury (Hg) in five batches of the proposed food additive (Documentation provided to EFSA No. 3). The analyses were performed by an external laboratory using inductively coupled plasma–mass spectrometry (ICP–MS) in accordance with AOAC 2015.01 (modified) methodology. The certificates of analysis were provided. Results for the analysis of the toxic elements in all batches were reported as below the limit of quantification (LOQ), which were 0.01 mg/kg for As and 0.005 mg/kg for Pb, Cd and Hg. The Panel noted that the analytical data were below the EU specification limits for toxic elements in the specifications for E 960c(i–ii) and the applicant proposed to maintain the same limits for the proposed food additive (Table 1).

Residual solvents

The applicant analysed the concentration of methanol and ethanol in 10 batches of the proposed food additive (Documentation provided to EFSA No. 1 and No. 3). For three batches of the proposed food additive, methanol and ethanol were reported to be below the LOD of 33.9 mg/kg for methanol and below the LOD of 12.5 mg/kg for ethanol. For the remaining seven batches the results were reported to be below 5000 mg/kg for ethanol and below 200 mg/kg for methanol, which are the existing EU specification for these solvents. The Panel noted that the two solvents are not used in the manufacturing process of the proposed food additive. Therefore, the Panel considered that introducing maximum limits for ethanol in the specifications of the proposed food additive was not necessary.

Protein content

Five batches of the proposed food additive were analysed for residual protein using the modified Bradford method (Documentation provided to EFSA No. 4). A report containing the results and the validation of the Bradford method was provided and an overestimation of the residual protein content is expected. Results ranged from 31.6 to 68.8 mg/kg, which complies with the specification proposed (i.e. < 75 mg/Kg). Additionally, the applicant demonstrated that more than 99% of the food enzyme-TOS used in the enzymatic conversion is removed during the purification of the Reb M.

The applicant also provided bioinformatic analysis to assess the allergenicity potential of the enzymes used in the manufacturing process (Documentation provided to EFSA No. 1). A search for the homology of the amino acid sequence of UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase to known allergens was made and no match was found. The Panel considered that taking into account the results of the sequence homology search, the removal of more than 99% of the food enzyme–TOS and the available literature search, the likelihood of the risk of allergic reactions upon dietary exposure to these enzymes through the use of the proposed food additive is low.

The Panel noted that the proposed limit for residual protein is considerably higher than those established in the current EU specifications for E 960c(i) and E 960c(ii) (i.e. 5 mg/kg), however, taking into account the information provided, the Panel considered the proposed specification limit for residual protein to be acceptable.

Microbiological criteria

The applicant did not propose the inclusion of microbiological parameters in the specifications. Data on microbiological criteria analysed in 10 batches of the proposed food additive supported this proposal.

Total aerobic plate counts ranged from 50 to 500 CFU/g in five batches, were reported as < 1000 CFU/g in two batches and were not detected in two batches. Yeasts and moulds were not detected in eight batches and were reported at < 100 CFU/g in the remaining two batches. Coliforms and *E. coli* were < 3 MPN/g in all 10 batches, and *Salmonella* was absent in 25 g samples in all batches (Documentation provided to EFSA No. 1 and No. 3).

Taking into account the various steps of the manufacturing process (see Section 3.1.3) and the data submitted by the applicant, the Panel considered that microbiological contamination is unlikely. In addition, the Panel noted that according to Commission Regulation (EU) No 231/2012, no microbiological specifications are currently set for E 960c(i) and E 960c(ii). The Panel did not consider necessary to recommend the inclusion of microbiological criteria in the EU specifications.

Kaurenoic acid (KA)

Analytical data on the potential impurity kaurenoic acid were submitted by the applicant and further requested by EFSA using a more sensitive analytical method (Documentation provided to EFSA no. 4).

Therefore, the applicant provided analytical data on kaurenoic acid for five batches of the proposed food additive using liquid chromatography mass spectrometry (LC–MS). The analytical report was submitted to EFSA along with the method validation report (Documentation provided to EFSA No. 4). The Panel noted that all the results were reported as below the LOD of 0.1 mg/kg (w/w) in Reb M. The Panel noted that the applicant did not propose to introduce a maximum limit for kaurenoic acid in the specifications.

Pesticides

The applicant provided analytical data, obtained by GC–MS/MS, on two batches of the proposed food additive and no pesticides residues were detected (Documentation provided to EFSA No. 1), with all analytes reported below the LOD of 0.01 mg/kg.

Particle size

The applicant proposed to retain the particle size limit established in the current specifications of E 960c(i) and E 960c(ii) (i.e. Not less than 74 μm (using mesh #200 sieve with particle size limit of 74 μm)). The Panel is of the view that this description of the particles size is not relevant in the context of the characterisation of the small particles including nanoparticles (EFSA Scientific Committee, 2021). Additionally, based on the considerations on solubility, there is no need to include particle size parameter in the EU specifications.

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 Reb M produced from *E. coli* K-12 DSM 35119 were provided by the applicant. Applicant informed that all raw materials, nutrient media and processing aids comply with the relevant Food Chemicals Codex (FCC) standards and European Regulations.

3.1.3.2 | *Description of manufacturing process*

The production process of the proposed food additive consists of two main steps:

1. production of enzyme preparations containing UDP-glucosyltransferases, UTP uridylyltransferase and sucrose synthase, using a genetically modified strain of *Escherichia coli* K-12, designated *E. coli* K-12 strain DSM 35119; and

2. enzymatic conversion of steviol glycosides from stevia leaf extract into rebaudioside M (Reb M), using the enzyme preparations produced in step (i).

A comprehensive description of the genetic modifications and the steps required to obtain the *E. coli* production strains was provided by the applicant (Documentation provided to EFSA No. 1).

According to the applicant, UDP-glucosyltransferases facilitate the transfer of glucose from an activated donor molecule (e.g. UDP-glucose) to steviol glycosides with the formation of glycosidic bonds. The enzymes UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase ensure the in-situ availability of the activated donor molecule, UDP-glucose.

Following the enzymatic glycosylation of steviol glycosides to form Reb M, the reaction broth is centrifuged to separate the *E. coli* biomass from the aqueous phase. Reb M is then recovered from the aqueous phase and purified through filtration, crystallisation and centrifugation. The resulting Reb M crystals are dried and milled to obtain the final product, containing Reb M at a purity of ≥ 95% on a dry-matter basis (Documentation provided to EFSA No. 1 and No. 3).

3.1.3.3 | *Characterisation of the production organism*

Characteristic of the production strain

The production strain of the steviol glycosides is the genetically modified bacterium *E. coli* DSM 35119, which was deposited at the [redacted], with the deposition number DSM 35119 (Documentation provided to EFSA 1). The production strain was identified as *E.coli* K-12 by WGS analysis, showing an [redacted] with the parental strain *E.coli* MG1655 and confirmed by the alignment between the production and the parental strains (Documentation provided to EFSA No. 1–3).

AMR genes were searched in the WGS of the production strain with [redacted] and [redacted] All hits were also found in the genome of the parental strain *E. coli* K12 MG1655 and were not considered a concern.

Characteristic of the parental strain

The parental strain *E. coli* MG1655 is a K-12 derivative that was obtained from the *E. coli* Genetic Stock Center (listed as CGSC 8003). The parental strain is a non-pathogenic and non-toxicogenic organism (Documentation provided to EFSA No. 1).

Description of the genetic modification

The purpose of the genetic modification was to facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds. [redacted]

Safety aspects of the genetic modification

The technical dossier contains all necessary information on the parental microorganism, the donor organisms and the genetic modification process.

The absence of the antimicrobial resistance genes used during the genetic modification in the genome of the production strain was confirmed by WGS analysis. The Panel concluded that the genetic modification of the production strain does not give rise to safety concerns.

Absence of viable cells of the production strain in the final product

The absence of viable cells of the production strain in the food additive was demonstrated in three batches, each tested in triplicate. One gram of sample was plated on non-selective medium and incubated at 37°C for 2 days. No colonies were detected. A positive control was included (Documentation provided to EFSA No. 1).

Absence of DNA in the final product

The absence of DNA of the production strain in the food additive was demonstrated in five batches, each tested in triplicate. No DNA was detected with primers that would amplify a 617 bp fragment present in the production strain, with a limit of detection of 0.1 ng spiked DNA/g product (Documentation provided to EFSA No. 1).

3.1.4 | Method(s) of analysis in food

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

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

The stability of the proposed food additive was tested in a single batch stored under accelerated conditions at 50°C for 6 months. The samples were stored in polypropylene jars with screw top lids and heat sealed to mimic commercial packaging. The applicant measured the content of Reb M and total steviol glycosides (expressed as % on a dry basis) at time point 0, 3 and 6 months. The concentrations were determined with an high performance liquid chromatography–diode array detector (HPLC–DAD) method, and the certificates of analysis were provided (Documentation submitted to EFSA No. 3). There were no substantial changes in the content of Reb M in the proposed food additive after 6 months of storage.

Based on the data obtained, the applicant concluded that the proposed food additive remained stable, under the tested conventional conditions. The Panel agreed with the applicant conclusions.

3.2 | Proposed uses and use levels

Maximum permitted levels (MPLs) of steviol glycosides (E 960a–d) expressed as steviol equivalents are defined in Annex II to Regulation (EC) No 1333/2008. Reb M produced from *E. coli* K-12 DSM 35119 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 Reb M produced from *E. coli* K-12 DSM 35119 are the same as the already authorised food additive steviol glycosides (E 960a–d), the applicant did not provide a dietary exposure assessment. The Panel considers that if steviol glycosides would be replaced by Reb M produced from *E. coli* K-12 DSM 35119, the exposure to rebaudioside M (expressed as steviol equivalents) will not be higher than the latest EFSA estimate of exposure to steviol glycosides (E960a–d) (EFSA FAF Panel, 2024). At the time, based on the MPLs, the FAF Panel concluded that the conservative estimates of the exposure (mean, 95th percentile) to steviol glycosides (E 960a–d) were below the ADI of 4 mg/kg bw per day in all population groups, except for infants and toddlers at the upper range of the exposure estimates in one country (4.1 and 4.8 mg steviol equivalents/kg bw per day, respectively).

3.3.1 | Anticipated exposure to impurities

The potential exposure to impurities from the use of the proposed 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, the latest exposure estimates by the FAF Panel (EFSA FAF Panel, 2024) were considered. The highest exposure levels to steviol glycosides for the mean and 95th percentile among the different population groups were considered, i.e. 1.3 and 4.8 mg/kg bw per day respectively, for toddlers.

The current application concerns Reb M produced from *E. coli* K-12 DSM 35119, which contain not less than 95% of Reb M. Since steviol itself has a molecular weight of 318.5 g/mol and rebaudioside M has molecular weight of 1291.3 g/mol, the steviol equivalencies of rebaudioside M is 0.25 (conversion factor, see Table 1). Therefore, an exposure of 1.3 and 4.8 mg/kg bw/day expressed as steviol equivalents equates to 5.2 and 19.2 mg/kg bw/day for the highest mean and highest 95th percentile.

The potential level of the impurities in the food additive combined with the estimated exposure levels to the proposed food additive, results in exposure estimates that can be compared with the reference points (RP) or health-based guidance values (HBGV) (Table 2) for the undesirable impurities potentially present in Reb M produced from *E. coli* K-12 DSM 35119. It is considered that any mercury or arsenic in the proposed food additive corresponds to the element in the inorganic form rather than organic form. Consequently, the HBGV for inorganic mercury and the RP for inorganic arsenic were used for comparison.

TABLE 2 Reference points/health-based guidance value for impurities present in the proposed food additive.

Impurity/constituent/HBGV/RP	Basis/reference
Lead (Pb)/0.5 μg/kg bw per day ($BMDL_{01}$)	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 μg/kg bw per week (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 μg/kg bw per week (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 (2009)
Inorganic arsenic (iAs)/0.06 μg/kg bw per day ($BMDL_{05}$)	The reference point is based on a benchmark dose lower confidence limit ($BMDL_{05}$) of 0.06 μg/kg bw per day identified for skin cancer. The reference point is considered to cover lung cancer, bladder cancer, skin lesions, ischemic heart disease, chronic kidney disease, respiratory disease, spontaneous abortion, stillbirth, infant mortality and neurodevelopmental effects. An MOE of 1 would correspond to the exposure level that is associated with a 5% increase relative to the background incidence for skin cancer, based on the available data. **An MOE of 1** raises a health concern. Because there are no precedents in EFSA for identification of a MOE of low concern, when using a BMDL derived from human cancer data the CONTAM Panel decided not to determine a value for a MOE of low concern. EFSA CONTAM Panel (2024)

Abbreviations: $BMDL_{01}$, benchmark dose (lower confidence limit); bw, body weight; HBGV, health-based guidance value; MOE, margin of exposure; RP, reference point; TDI, tolerable daily intake; 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 were 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 2) by the exposure estimate (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 proposed specifications limits for toxic elements are in line with the already existing specifications for toxic elements for E 960c(i) and E 960c(ii).

The Panel noted that the analytical data on toxic elements submitted by the applicant are lower than the limits in the proposed specifications (Documentation provided to EFSA No. 3). The Panel noted that the proposed limits for As, Hg and Cd are close to LOQ values reported by the applicant for these toxic elements, with exception for Pb, for which the LOQ is 40 times lower than the proposed limit in the specifications. 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 of the Panel is illustrated in Table 3.

TABLE 3 Risk assessment for toxic elements from the use of the proposed food additive.

	Considering the presence of toxic elements at the proposed specifications limits in the proposed food additive			
Exposure to Reb M (mg/kg bw per day)	**MOE for Pb at 0.2 mg/kg**	**% of the TWI for Hg at 0.07 mg/kg**	**% of the TWI for Cd at 0.015 mg/kg**	**MOE for As at 0.015 mg/kg**
Mean: 5.2[a]	481	0.06	0.02	769
95th percentile: 19.2[a]	130	0.2	0.08	208

[a]Estimated exposure converted from steviol equivalents (EFSA FAF Panel, 2024) taking into account Reb M at 95% and a conversion factor of 0.25.

When considering the limits proposed for the specifications (Table 3), the Panel concluded that the presence of the toxic elements in the proposed food additive would not give rise to concern.

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

The applicant submitted results for the analysis of kaurenoic acid in five batches of the proposed food additive. In all batches, kaurenoic acid was reported as below the LOD of 0.1 mg/kg. The Panel noted that applicant did not propose to introduce a maximum limit for kaurenoic acid in the specifications.

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 when assessing the safety of this impurity if present in the proposed food additive (EFSA Scientific Committee, 2019). Based on the data provided, the Panel calculated the potential exposure to kaurenoic acid as if it was present in the proposed food additive at the concentration of 0.1 mg/kg. The outcome of this calculation is presented in Table 4.

TABLE 4 Risk assessment for kaurenoic acid.

Exposure to Reb M (mg/kg bw per day)	Exposure to kaurenoic acid if at 0.1 mg/kg in in the proposed food additive (μg/kg bw per day)
Mean: 5.2[a]	0.00052
95th percentile: 19.2[a]	0.00192

[a]Estimated exposure converted from steviol equivalents (EFSA FAF Panel, 2024) taking into account Reb M at 95% and a conversion factor of 0.25.

The calculated mean potential exposure to kaurenoic acid is 0.00052 μg/kg bw per day and at the 95th percentile is 0.00192 μg/kg bw per day. The Panel noted that these calculations indicate a potential for exposure at levels below the TTC value of 0.0025 μg/kg bw per day. The Panel considered that kaurenoic acid was not detected in the proposed food additive using a method with an LOD of 0.1 mg/kg; therefore, it dispels the uncertainties and concerns for potential genotoxicity. The Panel recommends introducing a specific entry for kaurenoic acid in the proposed specifications.

4 | DISCUSSION

The present opinion deals with the safety assessment of the food additive rebaudioside M (Reb M) produced via enzymatic conversion of purified steviol glycosides leaf extracts (95% steviol glycosides) obtained from *S. rebaudiana* Bertoni plant.

The bioconversion process involves the use of the enzymes UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase, produced with a genetically modified strain of *E. coli* (*E. coli*. K-12 DSM 35119). The applicant requested an amendment of the EU specifications for steviol glycosides E 960c(i) or E 960c(ii). The proposed amendment aimed to revise the definition to include the use of the three enzymes from a genetically modified strain of *E. coli* K-12 DSM 35119 in the manufacturing process.

The Panel noted that the definition in the proposed specifications for the proposed food additive is not in line with the current definition for either E 960c(i) or E 960c(ii). In particular, it differs from the definition of E 960c(i), as the enzymes are not produced by genetically modified yeasts (*K. phaffii*), but by genetically modified strains of *E. coli*. It also differs from the definition of E 960c(ii), as the starting material used is not purified steviol glycoside rebaudioside A extracts (95% steviol glycosides) but purified steviol glycoside leaf extracts (95% steviol glycosides). In addition, the Panel noted that the proposed food additive is characterised by a specific distribution of minor steviol glycosides, including Reb V isomer, Reb V2 isomer, Reb N isomer, Reb I, Reb D isomer and Reb N. The Panel considered that the definition of the proposed food additive should be revised as recommended in the relevant section above. In view of the identified differences with the current specifications for E 960c(i) and E 960c(ii), relating to the production microorganism, the starting material and the profile of minor steviol glycosides, the Panel recommends the European commission to create a new entry for the proposed food additive in the applicable legislation (Commission Regulation (EU) No 231/2012).

Analytical data from 10 non-consecutive batches of Steviol glycosides produced by bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides) confirmed consistent composition. The proposed food additive contained 95.1%–98.2% rebaudioside M, with minor amounts of Reb V isomer, Reb V2 isomer, Reb N isomer, Reb I, Reb D isomer and Reb N. The Panel noted that the products meet the ≥ 95% assay requirement for Reb M and that the chemical structures of rebaudiosides M produced via this process are identical to those from E 960c(i) and E 960c(ii).

The Panel noted that adequate analytical data supporting the compliance with the provision for residual protein specifications were submitted by the applicant. In addition, the Panel considered the proposed specification limit for residual

protein (i.e. 75 mg/kg) to be acceptable. No viable cells nor DNA of the production strain remained in the final product. The Panel considered that the manufacturing process does not raise a safety concern. For the proposed food additive, the Panel considered adequate the statement 'not less than 95% rebaudioside M on the dried basis' in the assay entry of the proposed specifications.

Regarding solubility, the applicant described the proposed food additive as 'freely soluble to slightly soluble in water'. The Panel considered that the applicant's proposed descriptions were not supported by the data and recommended instead describing the proposed food additive as 'slightly soluble in water', according to the classification by JECFA criteria (JECFA, 2006).

With respect to toxic elements (As, Pb, Cd and Hg), analytical data were reported as below the LOQs (0.01 mg/kg for As; 0.005 mg/kg for Pb, Cd and Hg) in all analysed batches of the proposed food additive. The Panel noted that these results were below the existing limits for toxic elements in the EU specification for E 960c(i) and E 960c(ii). The potential exposure to these impurities was compared against the available HBGVs and RPs (Section 3.3.1, Table 2). The Panel performed a risk assessment considering the presence of these toxic elements in the proposed food additive at the proposed specification limits and concluded that the presence of the toxic elements does not give rise to safety concerns.

For solvents, methanol and ethanol were found to be below the LOD in three batches tested (i.e. 33.9 ug/g for methanol and 12.5 ug/g for ethanol). For the remaining seven batches the results were reported to be below 5000 mg/kg for ethanol and below 200 mg/kg for methanol. The applicant proposed to retain the existing EU specification for ethanol (5000 mg/kg). The Panel noted that the two solvents are not used in the manufacturing process of the proposed food additive. Therefore, the Panel considered that introducing maximum limits for ethanol in the specifications of the proposed food additive is not necessary.

Regarding microbiological parameters, taking into account the various steps of the manufacturing process (see Section 3.1.3) and the data submitted by the applicant, the Panel considered that a microbiological contamination is unlikely. In addition, the Panel noted that according to Commission Regulation (EU) No 231/2012, no microbiological specifications are currently set for E 960c(i) and E 960c(ii) and the Panel did not consider necessary to recommend the inclusion of microbiological criteria in the EU specifications for the proposed additive.

Analytical data on kaurenoic acid, using a validated high performance liquid chromatography–mass spectrometry (HPLC–MS) method, for five batches of the proposed food additive were reported as below the LOD of 0.1 mg/kg. Based on the available data and the calculated potential exposure to kaurenoic acid applying a TTC approach for this impurity (TTC value for a potential DNA-reactive mutagen and/or carcinogen), a genotoxicity concern derived from the potential presence of kaurenoic acid in the final proposed food additive could be ruled out. The Panel recommends introducing a specific entry for kaurenoic acid in the EU specifications.

The applicant provided data on the water solubility of the proposed food additive, measured at approximately 1.18 g/L in line with previous assessments (EFSA FAF Panel, 2023, 2025, 2026). Taking into account the MPLs, the reported solubility and the volume of gastric-intestinal secretion, the Panel considered that full dissolution of the food additive is to be expected in foods and/or in the gastrointestinal (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), without nanospecific considerations. In addition, the Panel does not see the need to include the particle size parameter in the specifications for the proposed food additive.

Based on the data provided, the Panel considered that rebaudioside M produced by enzymatic conversion of purified steviol glycosides leaf extracts (95% steviol glycosides) obtained from *S. rebaudiana* Bertoni plant using UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase enzymes produced by the genetically modified strain of *E. coli* K-12 DSM 35119 have the same physicochemical characteristics of E 960c(i) and E 960c(ii); therefore, the available biological and toxicological data considered in the previous evaluations by the ANS and FAF Panel (EFSA ANS Panel, 2010, 2015, 2018; EFSA FAF Panel, 2019, 2020, 2021, 2022a, 2022b, 2023, 2024, 2025, 2026) will also apply to the safety assessment of the proposed food additive.

5 | CONCLUSIONS

The Panel concluded that there is no safety concern with respect to rebaudioside M produced by enzymatic conversion of purified steviol glycosides leaf extracts (95% steviol glycosides) obtained from *S. rebaudiana* Bertoni plant using UDP-glucosyltransferase, UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase enzymes produced by the genetically modified strain of *E. coli (E. coli*. K-12 DSM 35119).

DOCUMENTATION AS PROVIDED TO EFSA

1. Application to modify the specifications of the already authorised food additive enzymatically produced steviol-glycoside E 960c(ii). Technical dossier. November 2024. Submitted by Manus Bio
2. Additional information submitted by Manus Bio following a request from EFSA. July 2025.
3. Additional information submitted by Manus Bio following a request from EFSA. March 2026.
4. Additional information submitted by Manus Bio following a request from EFSA. May 2026.

ABBREVIATIONS

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
AMR	antimicrobial resistance
ANS Panel	Panel on Food Additives and Nutrient Sources added to Food
AOAC	Association of Analytical Communities
BMDL	benchmark dose (lower confidence limit)
bw	body weight
CAS	Chemical Abstract Service
CEP Panel	Panel on Food Contact Materials, Enzymes and Processing Aids
CFU	colony forming units
CoAs	certificates of analysis
CONTAM Panel	Panel on Contaminants in the Food Chain
ESI	electrospray ionisation
FAF Panel	Panel on Food Additives and Flavourings
FAO/WHO	Food and Agriculture Organisation/World Health Organisation
FCC	Food Chemical Codex
GI	gastrointestinal
GLP	Good Laboratory Practice
GMO	genetically modified organisms
GRAS	generally recognised as safe
HBGV	health-based guidance value
HPLC	high performance liquid chromatography
HPLC/MS	high performance liquid chromatography/mass spectrometry
HPLC–DAD	high performance liquid chromatography–diode array detector
HPLC–UV	high performance liquid chromatography–ultraviolet
ICP–MS	inductively coupled plasma–mass spectrometry
IPA	isopropanol
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC/MS	liquid chromatography–mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MOE	margin of exposure
MPLs	maximum permitted levels
MS	mass spectrometry
NOAEL	no observed adverse effect level
OECD	Organization for Economic Co-operation and Development
pH	potential of hydrogen
RP	reference point
SC	Scientific Commission
TG	test guideline
TWI	tolerable weekly intake
UDP	uridine diphosphate
UGT	UDP-glucosyltransferase
UPLC–DAD	ultra-performance liquid chromatography equipped with diode array detector
WGS	whole-genome sequencing

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REQUESTOR

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