Adopted: 18 November 2025

DOI: 10.2903/j.efsa.2026.9779

efsa JOURNAL

SCIENTIFIC OPINION

Safety evaluation of the modification of the food additive enzymatically produced steviol glycosides (E 960c)

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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 glucosyltransferase and sucrose synthase enzymes, both produced using three newly developed genetically modified strains of *Escherichia coli* (CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO). This modification of the manufacturing process yields two distinct preparations of steviol glycosides: SBP1, composed predominantly of rebaudioside M, and SBP2, composed predominantly of rebaudioside D. The modification leads to changes in the definition of the food additive, residual protein, residual solvents, microbiological criteria and particle size. The Panel concurred with the applicant's proposal to introduce two new entries in Commission Regulation (EU) No. 231/2012 corresponding to SBP1, predominantly rebaudioside M, and SBP2, predominantly rebaudioside D. The manufacturing process does not raise a safety concern since no viable cells nor DNA of the production strains 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 via consumption of SPB1 and SPB2 can be considered negligible. The Panel considered that rebaudioside M and D produced by this new manufacturing process have the same physicochemical characteristics as the corresponding rebaudioside M and D present in E 960c(i), (ii) and (iii); therefore, the biological and toxicological data considered in previous evaluations will also apply to the safety assessment of SBP1 and SBP2. The Panel concluded that there is no safety concern with respect to the proposed modification of the food additive enzymatically produced steviol glycoside E 960c related to the use of the new genetically modified strains of *E. coli* in the production process of SBP1 and SBP2.

KEYWORDS

E 960c, enzymatic bioconversion, rebaudioside D, rebaudioside M, SBP1, SBP2, steviol glycosides

CONTENTS

1 | INTRODUCTION

The present opinion deals with the safety evaluation of the food additives rebaudioside M and rebaudioside D produced by enzyme-catalysed bioconversion from Stevia leaf extract using new genetically modified strains of *Escherichia coli* (named *E. coli* CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO, also called pCK900-CDX-044, pCK900-CDX-045, pCK900-CDX-047). The two new bioconverted products are also referred hereinafter as 'SBP1' and 'SBP2'.

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.[1] Only food additives that are included in the Union list, in particular in Annex II to that regulation, may be placed on the market and used 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]

An application has been introduced for the modification of the specifications of the food additive enzymatically produced steviol glycosides (E 960c).

The new alternative manufacturing process differs from the authorised rebaudioside M (E 960c(i), (ii)) and rebaudioside D (E 960c (iii)) on the origin and identity of the enzymes involved in the enzymatic bioconversion of purified steviol glycosides.

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 modifications of the food additive enzymatically produced steviol glycosides (E 960c) and the assessment of possible confidentiality requests in accordance with Regulation (EC) No. 1331/2008 establishing a common authorisation procedure for food additives, food enzyme and food flavourings.[3]

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* (E 960b(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) were the first steviol glycosides to be authorised as a food additive in the EU and are 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 the 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).

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,

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

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

[3]Regulation (EC) No 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.

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). Commission 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, Commission 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 'E 960c(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 Commission 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 *Stevia 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 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 Commission 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 (E 960a–d) of 4 mg/kg bw per day (expressed as steviol equivalents) (EFSA FAF Panel, 2024).

Commission 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).

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) in any of the orientations 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 May 2025 (Documentation provided to EFSA No. 2).

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 39–39e 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 to 16 October 2025.[8] No comments were received.[9]

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 the 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 Commitee , 2021) has been followed by the FAF Panel.

Provided that rebaudioside M and rebaudioside D (namely SBP1 and SBP2), produced by enzyme-catalysed bioconversion from Stevia leaf extract using the genetically modified strains of *E. coli* CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO (namely SBP1 and SBP2), have the same physicochemical characteristics as the corresponding rebaudioside M and D present in E 960c(i), E 960c(ii) or E 960c(iii), the biological and toxicological data for E 960a-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, 2015a, 2018; EFSA FAF Panel, 2019, 2020, 2021, 2022a, 2022b, 2023, 2024) will also apply to the safety assessment of SBP1 and SBP2.

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 modification of the food additive enzymatically produced steviol glycoside E 960c, resulting in two different preparations of steviol glycosides:

(i) Steviol glycosides, predominantly rebaudioside M, produced via a new enzymatic bioconversion of steviol glycosides from stevia leaf extract (named SBP1) and
(ii) Steviol glycosides, predominantly rebaudioside D, produced via a new enzymatic bioconversion of steviol glycosides from stevia leaf extract (named SBP2).

The bioconversion process involves the use of the enzymes glucosyltransferase and sucrose synthase, produced with three new genetically modified strains of *E. coli* (CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO).

In particular, the applicant requested an amendment of the specifications of steviol glycosides E 960c, in order to introduce two new entries corresponding to SBP1 and SBP2 and provided comparative information with the currently authorised specifications for E 960c (i, ii and iii).

[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, pp. 1–24.

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

[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: https://open.efsa.europa.eu/dossier/FAD-2022-11330.

[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, pp. 1–24.

[8]https://connect.efsa.europa.eu/RM/s/consultations/publicconsultation2/a0lTk000005fWlp/pc1627.

[9]https://open.efsa.europa.eu/consultations/a0cTk00000Js8tvIAB?search=efsa-q-2023-00749.

In both cases, the manufacturing process begins with the enzymatic bioconversion of purified steviol glycosides (95%) from the *Stevia rebaudiana* plant (Documentation provided to EFSA No. 1). Other than rebaudioside M and D, the resulting mixtures contain minor amounts (< 5%) of other rebaudiosides (Tables 1 and 2) (Documentation provided to EFSA No. 1). Steviol glycosides SBP1 and SBP2 meet the ≥ 95% (assay) criterion for Steviol Glycosides as included in the current EU specifications of E 960c. The steviol glycoside mixtures were characterised using an High Performance Liquid Chromatography – Ultraviolet (HPLC-UV) HPLC-UV method (at 203 nm) developed by the applicant and adapted from the JECFA method for measuring steviol glycosides (JECFA, 2021). The High Performance Liquid Chromatography (HPLC) HPLC analysis, performed to determine the concentrations of the individual steviol glycosides, was carried out in-house and the certificates of analysis (CoAs) were provided (Documentation provided to EFSA No. 1 and 2). The applicant reported that the quantification of individual rebaudiosides was performed using individual rebaudioside calibration standards. The analysis of five non-consecutive batches showed that (i) SBP1 contains [redacted] of rebaudioside M[redacted] of rebaudioside D[redacted] of rebaudioside B [redacted] of rebaudioside E and (ii) SBP2 contains [redacted] of rebaudioside D[redacted] of rebaudioside A[redacted] of rebaudioside B, [redacted] of rebaudioside E [redacted] of rebaudioside M. For the five tested batches (dry weight basis), the sum of the steviol glycosides was for SBP1 in the range [redacted] and for SBP2 [redacted]. The corresponding chromatograms for all batches were provided (Documentation provided to EFSA No. 2).

The CAS numbers, molecular formulae and molecular weights for the individual steviol glycosides that are present in the two mixtures were provided by the applicant. According to the applicant, the chemical structures of rebaudioside M and rebaudioside D, produced by the new enzymatic process described in this application, are identical to those extracted from the leaves of *S. rebaudiana*. Based on the data provided, the Panel concurs with the applicant.

The applicant reported that SBP1 and SBP2 produced via a new enzymatic bioconversion of steviol glycosides from stevia leaves are white to off-white powders, with sweetness ≥ '*150 times higher than sucrose at 5% equivalency*' (Documentation provided to EFSA No. 1).

3.1.2 | Proposed amendment to the EU specifications

The applicant provided proposals for amending the existing specifications for the already authorised food additive E 960c, which is covering rebaudioside M produced via enzymatic conversion of steviol glycosides from Stevia (E 960c(i)) or of highly purified rebaudioside A stevia leaf extract (E 960c(ii)) and rebaudioside D produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extract (E 960c(iii)). In particular, the applicant proposed to add two new entries to E 960c i.e. E 960c(v) and E 960c(vi), proposing modifications in relation to the definition, the residual solvents, the residual protein, the particle size and the addition of microbiological parameters (Documentation provided to EFSA No. 2).

The applicant provided 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 E 960c(iii) 'Rebaudioside D produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extracts' and the changes proposed by the applicant as presented in Tables 1 and 2, for SBP1 and SBP2, respectively.

The applicant provided the product specification data and reported that SBP1 and SBP2 are manufactured within their proposed specifications (Documentation provided to EFSA No. 1–2).[10]

[10]The Panel noted that the molecular weight (MW) for rebaudioside D reported in the current EU specifications for E 960c(iii) 'Rebaudioside D produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extract' is not correct. 1291.15 g/mol should be corrected to 1129.15 g/mol.

TABLE 1 Current EU specifications as set in Commission 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 enzymatic conversion of highly purified rebaudioside A stevia leaf extracts' and as proposed by the applicant for SBP1 (Documentation provided to EFSA No. 2).

	EU specifications for E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from stevia (Commission 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 (Commission Regulation (EU) No. 231/2012)**	**Proposed specifications by the applicant SBP1**
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 *Stevia rebaudiana* Bertoni plant using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffi* (formerly known as *Pichia pastoris*) UGT-a and *K. 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 is a steviol glycoside composed predominantly of rebaudioside M with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside D, rebaudioside E, rebaudioside B and rebaudioside I Rebaudioside M is obtained via enzymatic bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides) of the *Stevia rebaudiana* Bertoni plant using glycosyltransferases and sucrose synthase enzymes produced by the genetically modified strains of *Escherichia coli* (pCK900-CDX-044, pCK900-CDX-045, pCK900-CDX-047) that facilitate the transfer of glucosyl residues from sucrose to ADP-α-D-glucose and then to steviol glycosides with the formation of glycosidic bonds After initial removal of the enzymes and other impurities by filtration, the process involves further heat treatment for remaining enzyme precipitation and further impurity removal by sequential membrane filtration steps, until the final product contains not less than 95% of rebaudioside M, followed by drying and sieving Viable cells of *Escherichia coli* (pCK900-CDX-044, pCK900-CDX-045, pCK900-CDX-047) 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*

(Continues)

TABLE 1 (Continued)

	EU specifications for E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from stevia (Commission 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 (Commission Regulation (EU) No. 231/2012)			Proposed specifications by the applicant SBP1
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*
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			Not more than 5000 mg/kg ethanol Not more than 200 mg/kg methanol
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]			Not less than 20 μm [using a mesh #635 sieve with a particle size limit of 20 μm]
Microbiological criteria	n.a			n.a			
Total bacteria	n.a			n.a			< 1000 CFU/g
Yeast and moulds	n.a			n.a			< 200 CFU/g
Escherichia coli	n.a			n.a			Negative/1 g
Salmonella	n.a			n.a			Negative/25 g

Abbreviation: n.a., not available.

TABLE 2 Current EU specifications as set in Commission Regulation (EU) No 231/2012 for 'E 960c(iii)' Rebaudioside D produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extracts' and as proposed by the applicant for SBP2 (Documentation provided to EFSA No. 2).

	EU specifications for E 960c(iii) Rebaudioside D produced via enzymatic conversion of highly purified rebaudioside a stevia leaf extracts (Commission Regulation (EU) No. 231/2012)			**Proposed specifications by the applicant for SBP2**		
Definition	Rebaudioside D produced via enzymatic conversion of highly purified rebaudioside A stevia leaf extracts is a steviol glycoside composed predominantly of rebaudioside D with minor amounts of other steviol glycosides such as rebaudioside A and rebaudioside M Rebaudioside D 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 D by resin adsorption, followed by recrystallisation of the steviol glycosides resulting in a final product containing not less than 95% of rebaudioside D and rebaudioside A Viable cells of *E. coli* (pPM294, pFAF170 and pSK401) and their DNA shall not be detected in the food additive			Rebaudioside D is a steviol glycoside composed predominantly of rebaudioside D, with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside M, rebaudioside E and rebaudioside B Rebaudioside D is obtained via enzymatic bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides) of the Stevia rebaudiana Bertoni plant using glycosyltransferase and sucrose synthase enzymes, produced by the genetically modified strain *Escherichia coli* (pCK900-CDX-044 and pCK900-CDX-045), that facilitate the transfer of glucosyl residues from sucrose to ADP-α-D-glucose and then to steviol glycosides with the formation of glycosidic bonds After initial removal of the enzymes and other impurities by filtration, the process involves further heat treatment for remaining enzyme precipitation and further impurity removal by sequential membrane filtration steps, until the final product contain not less than 95% of rebaudioside D and rebaudioside A, followed by drying and sieving Viable cells of the bacteria *Escherichia coli* (pCK900-CDX-044 and pCK900-CDX-045) and their DNA shall not be detected in the food additive		
Chemical names	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-Dglucopyranosyl-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-Dglucopyranosyl ester			Rebaudioside D: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-Dglucopyranosyl-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-Dglucopyranosyl ester No proposed change		
Molecular formula	**Trivial name**	**Formula**	**Conversion factor**	**Trivial name**	**Formula**	**Conversion factor**
	Rebaudioside D	$C_{50}H_{80}O_{28}$	0.29	*No proposed changes*		
	Rebaudioside A	$C_{44}H_{70}O_{23}$	0.33			
Molecular weight and CAS No	**Trivial name**	**CAS Number**	**Molecular weight (g/mol)**	**Trivial name**	**CAS Number**	**Molecular weight (g/mol)**
	Rebaudioside D	63279-13-0	1291.15	*No proposed changes*		
	Rebaudioside A	58543-16-1	967.01			
Assay	Not less than 95% rebaudioside D and A on the dried basis			*No proposed changes*		
Description	White to light yellow powder, approximately between 150 and 350 times sweeter than sucrose (at 5% sucrose equivalency)			*No proposed changes*		
Identification						
Solubility	Freely soluble to slightly soluble in water			*No proposed changes*		
pH	Between 4.5 and 7.0 (1 in 100 solution)			*No proposed changes*		
Purity						
Total ash	Not more than 1%			*No proposed changes*		

(Continues)

TABLE 2 (Continued)

	EU specifications for E 960c(iii) Rebaudioside D produced via enzymatic conversion of highly purified rebaudioside a stevia leaf extracts (Commission Regulation (EU) No. 231/2012)	**Proposed specifications by the applicant for SBP2**
Loss on drying	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 Not more than 200 mg/kg methanol
Arsenic	Not more than 0.015 mg/kg	*No proposed changes*
Lead	Not more than 0.2 mg/kg	*No proposed changes*
Cadmium	Not more than 0.015 mg/kg	*No proposed changes*
Mercury	Not more than 0.07 mg/kg	*No proposed changes*
Residual protein	Not more than 5 mg/kg	Not more than 30 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 20 μm [using a mesh #635 sieve with a particle size limit of 20 μm]
Microbiological criteria	n.a.	
Total bacteria	n.a.	< 1000 CFU/g
Yeast and moulds	n.a.	< 200 CFU/g
Escherichia coli	n.a.	Negative/1g
Salmonella	n.a.	Negative/25 g

The applicant submitted analytical data from the analyses of five non-consecutive batches of SBP1 and SBP2 (Documentation provided to EFSA No. 1–2). Based on the data submitted, the Panel considered that SBP1 and SBP2 are consistently produced and compliant with the proposed specifications, as outlined in Tables 1 and 2.

Microorganism used in the manufacturing process

Regarding the new genetically modified *E. coli* strains used in the manufacturing process described in the present application, the Panel noted that, throughout the dossier, the following identifiers were used i.e. *E. coli* CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO. The Panel noted that the names of the new genetically modified strains in the specifications proposed by the applicant are referring to the name of the plasmids used during the genetic modification i.e. pCK900-CDX-044, pCK900-CDX-045, pCK900-CDX-047. The Panel considered this proposal acceptable.

Definition

The Panel noted that the definition in the proposed specifications for both SBP1 and SBP2 is in line with the current definition for E 960c(i). However, the Panel noted that it would be more appropriate to define:

- SBP1 as 'Steviol glycoside preparation composed predominantly of rebaudioside M with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside D, rebaudioside E, rebaudioside B and rebaudioside I'
- SBP2 as 'Steviol glycoside preparation composed predominantly of rebaudioside D, with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside M, rebaudioside E and rebaudioside B'.

Molecular weight

The Panel noted that the molecular weight for rebaudioside D in the proposed specifications of SBP2 is in line with the molecular weight reported in the current specification for E 960c(iii). However, the Panel noted that the molecular weight 1291.15 g/mol for rebaudioside D (as reported in E 960c(iii)) is not correct and should be revised to 1129.15 g/mol.

Assay

According to the applicant, SBP1 contains rebaudiosides M, D, A, B, E and I with rebaudioside M being the most abundant component, followed by rebaudioside D; SBP2 instead contains rebaudiosides D, A, B, E and M, with rebaudioside D being the most abundant compound, followed by rebaudioside A. The Panel noted that the data submitted from the analysis of SBP1 and SBP2 in five batches (see Section 3.1.1) confirmed the declared composition (Documentation provided to EFSA No. 1, 2). 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 of SBP1 as adequate. The proposed definition was considered to adequately reflect the proposed changes to the manufacturing process. For SBP2, the Panel noted that the applicant proposed in the assay entry the statement 'not less than 95% rebaudioside D and A on the dried basis'. The Panel considered this is not accurate, since rebaudioside D represents more than 95% of the composition while rebaudioside A is only a minor component. Therefore, the Panel proposed that the assay specification for SBP2 should read 'not less than 95% rebaudioside D on the dried basis' and that this should also be reflected in the definition of SBP2.

Solubility

The proposed food additive SBP1 is described by the applicant as *'freely soluble to slightly soluble in water'*, whereas SBP2 is described as '*Freely soluble to very slightly soluble in water*' (Documentation provided to EFSA no. 1 and 2).

The applicant provided information on the water solubility for three batches of SBP1 and SBP2, determined by applying the OECD TG 105 (shake flask method) (OECD TG, 1995) (Documentation provided to EFSA No. 1). The measured solubility of SBP1, at 20–30°C and pH 7.75, ranged from 1.37 to 1.47 g/L, while values of 0.46–0.47 g/L were reported for SBP2.

The Panel noted that the performed solubility test was not fully in line with the Guidance on Particle-TR, as the recommended ultrafiltration step was not applied (EFSA Scientific Commitee, 2021). Furthermore, the Panel considered that the applicant's proposed specifications for water solubility were not supported by the experimental data submitted. Therefore, the Panel recommended SBP1 be described as 'slightly soluble' and SBP2 as 'very slightly soluble', according to the criteria established by JECFA (2006). Accordingly, the description of the water solubility in the current specifications for E 960c(i), E 960c(ii) and E 960c(iii) should be revised.

Toxic elements

Regarding the 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 additives SBP1 and SBP2 (Documentation provided to EFSA No. 1). The analyses were performed by an external laboratory using inductively coupled plasma–mass spectrometry (ICP-MS). The CoAs 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–iii) and the applicant proposed to maintain the same limits for SBP1 (Table 1) and for SBP2 (Table 2).

Residual solvents

Concerning the residual solvents, the applicant analysed the concentration of methanol and ethanol in three batches of SBP1 and SBP2 using an undescribed in-house method. Both solvents were reported to be below 100 mg/kg. The applicant proposed to introduce a specification limit of 200 mg/kg for residual methanol and to retain the current EU specifications (Commission Regulation (EU) No. 231/2012) for residual ethanol as for E 960 c(i), (ii) and (iii) (i.e. 5000 mg/kg). The applicant further clarified that although alcohol-based solvents are not used in the manufacturing processes of SBP1 and SBP2, raw materials may contain residual amounts; therefore, routine in-house monitoring is performed to prevent the transfer of these solvents into the final product during enzymatic conversion (Documentation provided to EFSA No. 1 and 2). Therefore, the Panel considered the proposal to introduce the maximum limits for these two solvents in the specifications of SBP1 and SBP2 as not necessary.

Protein content

In the proposed specifications, the applicant included limits for residual protein of 75 mg/kg for SBP1 and 30 mg/kg for SBP2 (Documentation provided to EFSA No. 2). The Panel noted that these limits are considerably higher than those established in the current EU specifications for E 960c(ii) and E 960c(iii) (i.e. 5 mg/kg).

According to the applicant, the difference is related to the analytical method employed. Whereas residual proteins in steviol glycosides are generally tested in diluted samples using the bicinchoninic acid (BCA) method with a limit of detection (LOD) of 5 mg/kg, the applicant analysed the products 'as placed on the market' to enable accurate measurement of very low levels of protein. For this purpose, a modified version of the Bradford method was validated, yielding an LOQ of 30 mg/kg and an LOD of 10 mg/kg. The applicant indicated that this approach was intended to verify that the purification steps effectively remove the enzymes used in the production process.

Three batches of SBP1 and SBP2 were analysed for residual protein using the modified Bradford method. All SBP2 samples were below the LOD, and all SBP1 batches were below the proposed specification limit of 75 mg/kg. A report containing the results and the validation of the Bradford method was provided.

The Panel considered the analytical approach appropriate and the proposed limits for residual protein acceptable.

Microbiological criteria

The applicant submitted information on the microbiological criteria analysed in five batches of SBP1 and SBP2. For both products, total aerobic plate counts ranged from [redacted] CFU/g, yeast counts ranged from [redacted] CFU/g, and mould counts were reported as ≤ 10 CFU/g. *E. coli* was not detected in 1 g samples, and *Salmonella* was absent in 25 g samples. The Panel noted that the microbiological specifications proposed by the applicant for total aerobic count (i.e. 1000 CFU/g) and for yeasts and moulds (i.e. 200 CFU/g) are set at higher levels than those reported in the batch data provided. The microbiological specification parameters and limits proposed by the applicant were consistent with those published by JECFA for 'Enzyme modified Steviol Glycosides' (JECFA, 2021), and the Panel considered the proposal from the applicant adequate.

3.1.2.1 | *Kaurenoic acid (KA)*

Analytical data on the potential impurity kaurenoic acid were submitted by the applicant in its original dossier and further requested by EFSA using a more sensitive analytical method (Documentation provided to EFSA no. 1). Therefore, the applicant provided analytical data on kaurenoic acid for five batches of SBP1 and five batches of SBP2 using Liquid chromatography with tandem mass spectrometry (LC-MS/MS) LC-MS/MS. The analytical report was submitted to EFSA along with the method validation report (Documentation provided to EFSA No. 2). The Panel noted that a very small peak assigned to kaurenoic acid was visible in the provided analytical chromatograms, and all the results were reported as [redacted]. The Panel noted that the applicant did not propose to introduce a maximum limit for kaurenoic acid in the specifications.

Particle size distribution and dissolution rate

The applicant provided the results of sieve analysis for five batches of SBP1 and SBP2, using six sieve sizes ranging from 425 µm to 20 µm.

The Panel noted that, for both SBP1 and SBP2, the amount of particles retained at or above the 20 µm sieve was approximately 99% for both SBP1 and SBP2 and that these results are supportive of the proposed specifications.

However, the Panel noted that sieve analysis is not a suitable method for investigating the presence of small particles, including nanoparticles, as it does not provide information on the size of constituent particles, in accordance with the requirements of the EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021).

The applicant provided information on the dissolution rates of SBP1 and SBP2 in water at room temperature and pH 7, following the protocol as described in the EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021) (Documentation to EFSA No. 1). The concentrations tested reflected worst-case dietary exposure to SBP1 and SBP2, based on the highest estimated steviol glycoside intake for children aged 9 years (28.5 kg of body weight) (3.9 mg steviol equivalents/kg bw per day) from the EFSA ANS Panel opinion (EFSA ANS Panel, 2015a). This corresponds to daily intakes of 445 mg/person for SBP1 and 384 mg/person for SBP2. The tested concentrations were rounded up to 500 mg and 400 mg, SBP1 and SBP2, respectively, and added to 1 L of the liquid corresponding to gastric fluid volume for children (EFSA Scientific Committee, 2021).

The concentration of dissolved substance was determined at five time points (0, 5, 10, 30 and 60 min) by HPLC following ultrafiltration using a 10 kDa cut-off centrifugal ultrafiltration tube. After 30 min, the solubilised fraction of SBP1 ranged from 94.3% to 96.0%, and for SBP2, it ranged from 93.3% to 94.4%, with no plateau observed.

The Panel considered that the results of the tests for both SBP1 and SBP2 show that, after 30 min, rapid dissolution is achieved, i.e. it exceeds the threshold value of 88% indicated in the EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021) to achieve full solubilisation in water at the concentration tested.

The Panel was of the view that there is no need to include the 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 various equipment used to manufacture the SBP1 and SBP2 was provided (Documentation provided to EFSA No. 1). The applicant indicated that all raw materials and processing aids comply with the relevant *Food Chemicals Codex* (FCC) standards or other internationally recognised standards.

3.1.3.2 | *Description of manufacturing process*

The production process of SBP1 and SBP2 consists of two main steps:

1. production of the glycosyltransferase and sucrose synthase enzymes;
2. enzymatic conversion of steviol glycosides from stevia leaf extract into SBP1 and SBP2.

The enzymes used in the manufacturing process are produced by genetically modified strains of *E. coli* (i.e. pCK900-CDX-044, pCK900-CDX-045 and pCK900-CDX-047). 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). The enzymes facilitate the transfer of glucosyl residues from sucrose to ADP-α-D-glucose, and then to steviol glycosides with the formation of glycosidic bonds. Strains of *E. coli* CDX-044, CDX-045 and CDX-047 are used for the production of SBP1, whereas strains of *E. coli* CDX-044 and CDX-045 are used for the production of SBP2. The process involves filtration steps (i.e. [redacted]) before and after inactivation of the enzymes by heat treatment, to purify the rebaudioside mixtures, and drying steps. These steps are required to obtain final products with not less than 95% of rebaudioside M (SBP1) or D (SBP2) (Documentation provided to EFSA No. 1 and No. 2).

Total organic solids (TOS) analysis

Additionally, in response to the EFSA request, the applicant provided a detailed description of the purification process to obtain SBP1 and SBP2. After the enzymatic conversion to produce steviol glycosides rebaudioside M and rebaudioside D, there is a downstream purification process. The first step consists of crystallisation of the target rebaudiosides followed by filtration to remove soluble by-products, including the food enzyme–TOS. The second step is a heat treatment, during which proteins are denatured and subsequently removed by filtration, after which the rebaudiosides are recrystallised. The third step is the same as the first one. The final product is obtained after spray-drying.

The applicant provided experimental data demonstrating the removal of [redacted] of protein for three batches of SBP1 and SBP2, respectively (Documentation provided to EFSA No. 2). In addition to these results, the applicant provided mass balances of [redacted] and [redacted], as non-protein compounds of the food enzyme–TOS, on the different purification steps of the process, and obtained more than [redacted] removal of [redacted] and [redacted].

Taking into consideration the described purification process, all the data provided and the calculation provided by the applicant, the Panel concluded that it is sufficient to remove the food enzyme–TOS to at least [redacted], and consequently, the exposure to the food enzyme–TOS via consumption of SPB1 and SPB2 can be considered negligible.

The applicant also provided bioinformatic analysis to assess the toxigenicity and allergenicity potential of the enzymes used in the manufacturing process of SBP1 and SBP2. Enzymes CDX-044, CDX-045 and CDX-047 were predicted as non-pathogenic. Regarding potential allergenicity, a search for the homology of the amino acid sequence of CDX-044, CDX-045 and CDX-047 to known allergens was made and no match was found. The Panel considered the results of the sequence homology search, the available literature search and the demonstrated removal of the food enzyme–TOS, and it is of the

view that the likelihood of the risk of allergic reactions upon dietary exposure to these enzymes through the use of the food additives SBP1 and SBP2 is low.

3.1.3.3 | *Characterisation of the production organisms*

Characteristics of the GMM production strains

The production strains of the sucrose synthase (CDX-044) and of the two glycosyltransferases (CDX-045 and CDX-047) are the genetically modified *E. coli* strains CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO, respectively. The strains are deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) with deposition numbers [redacted], respectively (Documentation provided to EFSA No. 1).

The production strains were taxonomically identified as *E. coli* K12 derivatives by Whole Genome Sequence (WGS) analysis, resulting in an average nucleotide identity (ANI) of 100% with *E. coli* W3110 (Documentation provided to EFSA No. 1).

Characteristics of the parental and recipient strains

The parental strain is *E. coli* K12. The recipient strain *E. coli* W3110-TKO was derived from the parental strain by genetic modifications aimed [redacted] (Documentation provided to EFSA No. 1).

Characteristics of the inserted sequences

The synthetic gene encoding the sucrose synthase (CDX-044) was derived from [redacted] while the synthetic genes encoding the glycosyltransferases (CDX-045 and CDX-047) were derived from [redacted] and [redacted] respectively. [redacted] (Documentation provided to EFSA No. 1).

Description of the genetic modification

The purpose of the genetic modification was to allow the production strains to express the sucrose synthase from [redacted] the glycosyltransferase from [redacted] or the glycosyltransferase from [redacted]. For this purpose, the recipient strain was transformed with the pCK900-CDX-044, pCK900-CDX-045 or pCK900-CDX-047 plasmid [redacted], and the transformants were selected by their ability to grow in the presence of [redacted]. Three transformants were selected as production strains CDX-044 W3110-TKO, CDX-045 W3110-TKO or CDX-047 W3110-TKO, depending on the plasmid inserted.

The genomes of the production strains were searched for identity with genes present in the ResFinder and NCBIs AMR Finder databases; hits with 80% identity and 70% coverage were investigated for their function and presence in the *E. coli* W3110 strain. Besides the [redacted] located on the plasmid, no other antimicrobial resistance genes of concern are present in the genome of the production strain.

Safety of the genetic modification

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

The production strains *E. coli* CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO differ from the recipient strain in their capacity to produce the sucrose synthase from [redacted], the glycosyltransferase from [redacted] or the glycosyltransferase from [redacted] respectively. They also differ in their capacity to grow in the presence of [redacted].

The presence in the production strains of a gene conferring antimicrobial resistance is considered a hazard.

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

The absence of viable cells of the production strains in the food additives (*E. coli* CDX-044, *E. coli* CDX-045 and *E. coli* CDX-047 in SBP1 and *E. coli* CDX-044 and *E. coli* CDX-045 in SBP2) was demonstrated in three independent batches of each product analysed in triplicate. In brief, 1 g of product was [redacted]. No colonies of the production strain were produced in the test of SBP1. Five colonies were produced in the test of SBP2. These colonies

were confirmed not to be the production strains *E. coli* CDX-044 or *E. coli* CDX-045 by a plasmid-specific PCR in which proper controls were included. Positive spiking controls in both food additives were included.

3.1.3.5 | *Absence of DNA in the final product*

The absence of recombinant DNA in the food additives was demonstrated by polymerase chain reaction (PCR) analysis of three batches of each product in triplicate. No DNA was detected with primers that would amplify a [redacted], with a limit of detection of 10 ng spiked DNA/g food additive (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 additives SBP1 and SBP2 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 applicant performed one study with SBP1 and SBP2 in order to evaluate their stability under conventional storage conditions for up to 24 months for SBP1 and 30 months for SBP2. The study was performed with two non-consecutive batches of SBP1 and four non-consecutive batches of SBP2. Samples were kept in sealed polyethylene bags (and stored in stability chambers) at 25°C. SBP1 was sampled at 0, 1, 6, 12, 18 and 24 months, whereas SBP2 was sampled at 0, 1, 6, 12, 18 and 30 months. The applicant measured the content of rebaudioside M in SBP1 and the content of rebaudioside D in SBP2. The two concentrations of the rebaudiosides were determined with an HPLC method, and the CoAs were provided (Documentation submitted to EFSA No. 1). There were no substantial changes in the content of rebaudioside M in SBP1 after 24 months of storage and in the content of rebaudioside D after 30 months of storage.

Based on the data obtained, the applicant concluded that SBP1 and SBP2 remained stable under the tested conventional conditions (i.e. up to 24 months for SBP1 and up to 30 months for SBP2). The Panel agreed with the applicant's 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.

SBP1 and SBP2 are 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 SBP1 and SBP2 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 SBP1 or SBP2, the exposure to rebaudiosides M and D (expressed as steviol equivalents) will not be higher than the latest EFSA estimate of exposure to steviol glycosides (E 960a–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 SBP1 and SBP2 as food additives can be calculated by assuming that the impurity is present in the food additive up to a limit value and then by calculating 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 mean and 95th percentile exposure levels to steviol glycosides 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 SBP1 and SBP2, which contain not less than 95% of rebaudioside M and not less than 95% of rebaudioside D, respectively. Since steviol itself has a molecular weight of 318.5 g/mol and rebaudiosides M and D have molecular weights of 1291.3 and 1129.2 g/mol, respectively, the steviol equivalencies of SBP1 and SBP2 are 0.25 and 0.29, respectively (conversion factors, see Tables 1 and 2). Therefore, an exposure of 1.3 and 4.8 mg/kg bw per day expressed as steviol equivalents equates to 5.2 and 19.2, and 4.5 and 16.6 mg/kg bw per day for the highest mean and highest 95th percentile of SBP1 and SBP2, respectively.

The potential level of the impurities in the food additives combined with the estimated exposure levels to SBP1 and SBP2 resulted in exposure estimates that can be compared with the reference points (RP) or health-based guidance values (HBGV) (Table 3) for the undesirable impurities potentially present in SBP1 and SBP2. It is considered that any mercury or arsenic in the SBP1 and SBP2 correspond to the element in the inorganic form rather than the organic form. Consequently, the HBGV for inorganic mercury and the RP for inorganic arsenic were used for comparison.

TABLE 3 Reference points/health-based guidance values for impurities potentially present in SBP1 and SBP2.

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)
Inorganic Mercury (iHg)/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 (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, ischaemic 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 an MOE of low concern (EFSA CONTAM Panel, 2024)

Abbreviations: BMDL, benchmark dose (lower confidence limit); bw, body weight; 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 (Section 3.3) or by estimating the contribution of the use of the food additive to the HBGV (expressed as a 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), E 960c(ii) and E 960c(iii). 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. 1–2). 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 4.

TABLE 4 Risk assessment for toxic elements from the use of SBP1 and SBP2.

	Considering the presence of toxic elements at the proposed specification limits in SBP1			
Exposure to SBP1, (mg/kg bw/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
	Considering the presence of toxic elements at the proposed specification limits in SBP2			
Exposure to SBP2 (mg/kg bw/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: 4.5[a]	556	0.1	0.02	889
95th percentile: 16.6[a]	151	0.2	0.1	241

[a]Estimated exposure converted from steviol equivalents (EFSA ANS Panel, 2015b) taking into account the concentrations of Reb M and D present in the provided batches (Documentation provided to EFSA n.1), and with the conversion factors of 0.25 and 0.29 for SPB1 and SPB2, respectively.

When considering the limits proposed for the specifications (Table 4), 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.

3.3.1.2 | *Kaurenoic acid*

The applicant submitted results for the analysis of kaurenoic acid in five batches of SBP1 and SBP2. In all batches, kaurenoic acid was reported as [redacted]. The Panel noted that the 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 (EFSA Scientific Committee, 2019). Based on the data provided, the Panel calculated the potential exposure to kaurenoic acid as if it was present in SBP1 and SBP2 at the concentration of [redacted]. The outcome of this calculation is presented in Table 5.

TABLE 5 Risk assessment for kaurenoic acid.

Exposure to enzymatically produced steviol glycosides (E 960c), (mg/kg bw/day)	**Exposure to kaurenoic acid if at [redacted] in SBP1**
Mean: 5.2[a]	[redacted] μg/kg bw per day
95th percentile: 19.2[a]	[redacted] μg/kg bw per day
Exposure to enzymatically produced steviol glycosides (E 960c) (mg/kg bw/day)	Exposure to kaurenoic acid if at [redacted] in SBP2
Mean: 4.5[a]	[redacted] μg/kg bw per day
95th percentile: 16.6[a]	[redacted] μg/kg bw per day

[a]Estimated exposure converted from steviol equivalents (EFSA ANS Panel, 2015b) taking into account the concentrations of Reb M and D present in the provided batches (Documentation provided to EFSA n.1), and with the conversion factors of 0.25 and 0.29 for SPB1 and SPB2, respectively.

The calculated mean potential exposure to kaurenoic acid is [redacted] μg/kg bw per day and [redacted] μg/kg bw per day for SBP1 and SBP2, respectively, and at the 95th percentile is [redacted] μg/kg bw per day and [redacted] μg/kg bw per day. The Panel noted that these calculations indicate a potential for exposure at up to one and a half 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, (ii) the conservative assumption that the concentration of kaurenoic acid is present in the food additive at the level of [redacted] mg/kg and (iii) the uncertainties in the genotoxicity data. Taking these aspects into account, the Panel considered that a genotoxicity concern derived from the potential presence of kaurenoic acid in SBP1 and SBP2 could be ruled out.

The Panel recommends introducing a specific entry for kaurenoic acid as a potential impurity in the EU specifications for these proposed food additives.

4 | DISCUSSION

The present opinion deals with the safety assessment of the modification of the food additive enzymatically produced steviol glycoside E 960c, resulting in two different preparations of steviol glycosides, referred to by the applicant as SBP1 and SBP2.

The bioconversion process involves the use of the enzymes glucosyltransferase and sucrose synthase, produced with three new genetically modified strains of *Escherichia coli* (*E. coli* CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO, also called pCK900-CDX-044, pCK900-CDX-045 and pCK900-CDX-047). The applicant requested an amendment of the EU specifications for steviol glycosides (E 960c) by introducing two new entries in Commission Regulation (EU) 231/2012 corresponding to SBP1, predominantly rebaudioside M, and SBP2, predominantly rebaudioside D. The Panel concurs with this proposal.

In detail, in the current application dossier, the applicant applies a modification of the manufacturing process that leads to changes in the definition of the food additive, limits for residual protein, limits for residual solvents, microbiological criteria and particle size.

The Panel considered that the definition of the proposed food additives should be revised as recommended in Section 3.1.2.

Analytical data from five non-consecutive batches of both SBP1 and SBP2 confirmed consistent composition. SBP1 contained [redacted] rebaudioside M, with minor amounts of rebaudiosides D, B and E, while SBP2 contained [redacted] rebaudioside D with minor amounts of rebaudiosides A, B, E and M. The Panel noted that the products meet the ≥ 95% assay requirement for steviol glycosides and that the chemical structures of rebaudiosides M and D produced via this process are identical to those extracted from the leaves of *Stevia rebaudiana*.

The Panel noted that adequate analytical data supporting the compliance with the provision for residual protein specifications were submitted by the applicant. No viable cells nor DNA of the production strain remained in the final product; in addition, the Panel considered that the food enzyme–TOS are removed to at least [redacted], and consequently, the exposure to the food enzyme–TOS via intake of SPB1 and SPB2 can be considered negligible. The Panel considered that the manufacturing process does not raise a safety concern.

For SBP1, the Panel considered adequate the statement 'not less than 95% rebaudioside M on the dried basis' in the assay entry of the proposed specifications. However, for SBP2, the Panel noted that the statement 'not less than 95% rebaudioside D and A on the dried basis' is not accurate since rebaudioside D represents more than 95% of the composition; therefore, the Panel proposed that the assay entry in the proposed specifications for SBP2 should be 'not less than 95% rebaudioside D on the dried basis', being rebaudioside A only a minor component.

Regarding solubility, the applicant described SBP1 as 'freely soluble to slightly soluble in water' and SBP2 as 'freely soluble to very slightly soluble in water'. The Panel considered that the applicant's proposed descriptions were not supported by the data and recommended instead describing SBP1 as 'slightly soluble' and SBP2 as 'very slightly soluble', according to the JECFA criteria (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 SBP1 and SBP2. The Panel noted that these results were below the existing limits for toxic elements in the EU specification for E 960c (iii). The potential exposure to these impurities was compared against the available HBGVs and RPs (Section 3.3.1, Tables 4 and 5). The Panel performed a risk assessment considering the presence of these toxic elements in the SBP1 and SBP2 at the proposed specification limits and concluded that the presence of the toxic elements does not give rise to safety concerns.

For residual solvents, methanol and ethanol were detected at levels below 100 mg/kg in all batches tested. The applicant proposed to introduce a specification limit of 200 mg/kg for methanol and 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 SBP1 and SBP2. In addition, possible residues of these solvents in the raw materials are expected to be removed during the purification process. Therefore, the Panel considered that introducing maximum limits for these two solvents in the specifications of SBP1 and SBP2 was not necessary.

Concerning residual protein, the Panel agreed with the applicant's proposal to introduce in the specifications a limit of 75 mg/kg for SBP1 and of 30 mg/kg for SBP2. These limits were supported by data generated with a validated modified Bradford method (LOQ 30 mg/kg, LOD 10 mg/kg).

Regarding microbiological parameters, the applicant proposed limits to be introduced in the specifications for total aerobic count (i.e. 1000 CFU/g) and for yeasts and moulds (i.e. 200 CFU/g). The Panel noted that the proposal is in line with JECFA specifications for 'Enzyme modified Steviol Glycosides' (JECFA, 2021) and considered it adequate.

Analytical data on kaurenoic acid, using a validated LC–MS/MS method, for five batches of SBP1 and SBP2, were reported as below [redacted] Based on the available data and the calculated potential exposure to kaurenoic acid applying a TTC approach for this contaminant (TTC value for potential DNA-reactive mutagen and/or carcinogen), a genotoxicity concern derived from the potential presence of kaurenoic acid in the final proposed food additives could be ruled out. However, the Panel recommends introducing a specific entry for kaurenoic acid in the EU specifications.

The applicant provided dissolution rate tests performed according to EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021) showing that more than 94% of both SBP1 and SBP2 dissolved within 30 min at pH 7, exceeding the threshold of 88% established in the EFSA Guidance on Particle-TR. These results demonstrated that SBP1 and SBP2, when used as proposed food additives up to the concentrations tested (see Section 3.1.1), will be fully dissolved in the

gastrointestinal tract and that consumers will not be exposed to small particles, including nanoparticles. Hence, the Panel considered that SBP1 and SBP2 can be assessed following the conventional risk assessment, as outlined in EFSA Guidance for submission for food additive evaluations (EFSA ANS Panel, 2012). In addition, the Panel does not see the need to include the particle size parameter in the EU specifications.

Based on the data provided, the Panel considered that rebaudioside M and rebaudioside D produced by enzyme-catalysed bioconversion from Stevia leaf extract (namely SBP1 and SBP2) using the genetically modified strains of *E. coli* CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO have the same physicochemical characteristics as the corresponding rebaudioside M and D present in E 960c(i), (ii) and (iii); therefore, the available biological and toxicological data considered in the previous evaluations by the ANS and FAF Panel (EFSA ANS Panel, 2010, 2015a, 2018; EFSA FAF Panel, 2019, 2020, 2021, 2022a, 2022b, 2023, 2024) will also apply to the safety assessment of SBP1 and SBP2.

5 | CONCLUSIONS

The Panel concluded that there is no safety concern with respect to the proposed modification of the food additive enzymatically produced steviol glycoside E 960c related to the use of the new genetically modified strains *E. coli* CDX-044 W3110-TKO, CDX-045 W3110-TKO and CDX-047 W3110-TKO in the manufacturing process of:

(i) Steviol glycosides, predominantly rebaudioside M, produced via a new enzymatic bioconversion of steviol glycosides from stevia leaf extract (named SBP1) and
(ii) Steviol glycosides, predominantly rebaudioside D, produced via a new enzymatic bioconversion of steviol glycosides from stevia leaf extract (named SBP2).

6 | DOCUMENTATION AS PROVIDED TO EFSA

1. Application to modify the specifications of the already authorised food additive enzymatically produced steviol glycoside E 960c. Technical dossier. July 2024. Submitted by Tate & Lyle Ingredients France S.A.S.
2. Additional information submitted by Tate & Lyle Ingredients France S.A.S. following a request from EFSA. May 2025.

ABBREVIATIONS

ADI	acceptable daily intake
ANI	average nucleotide identity
ANS Panel	Panel on Food Additives and Nutrient Sources added to Food
As	arsenic
BCA	bicinchoninic acid
BMDL	benchmark dose (lower confidence limit)
bw	body weight
CAS	Chemical Abstract Service
Cd	cadmium
CEP Panel	Panel on Food Contact Materials. Enzymes and Processing Aids
CFU	colony forming units
CGMCC	China General Microbiological Culture Collection Cent
CONTAM Panel	Panel on Contaminants in the Food Chain
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EFSA	European Food Safety Authority
FAF Panel	Panel on Food Additives and Flavourings
FAO/WHO	Food and Agriculture Organisation/World Health Organisation
FCC	Food Chemical Codex
FOB	Functional Observational Battery
GLP	Good Laboratory Practice
GMO	Genetically Modified Organisms
HBGV	health-based guidance value
Hg	mercury
HPLC	High Performance Liquid Chromatography
HPLC-UV	High Performance Liquid Chromatography – Ultraviolet
ICP-MS	Inductively Coupled Plasma – Mass Spectrometry
[redacted]	[redacted]
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC–MS/MS	Liquid chromatography with tandem mass spectrometry
LOD	limit of detection

LOQ	limit of quantification
MOE	margin of exposure
MPLs	maximum permitted levels
MW	molecular weight
NOAEL	no observed adverse effect level
OECD	Organization for Economic Co-operation and Development
Pb	lead
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
ppb	parts per billion
ppm	parts per million
Reb D	rebaudioside D
Reb M	rebaudioside M
RP	reference point
SC	Scientific Commission
[redacted]	[redacted]
TG	test guideline
TOS	total organic solid
TTC	threshold of toxicological concern
TWI	Tolerable Weekly Intake
UDP	Uridine diphosphate
UGT	UDP glucosyltransferase
WGS	Whole-genome sequencing

ACKNOWLEDGEMENTS
The Panel wishes to thank the following for the support provided to this scientific output: Concepción Medrano-Padial.

REQUESTOR
European Commission

QUESTION NUMBER
EFSA-Q-2023-00749

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How to cite this article: EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), Castle, L., Andreassen, M., Aquilina, G., Bastos, M. L., Boon, P., Fallico, B., FitzGerald, R., Frutos Fernandez, M. J., Grasl-Kraupp, B., Gundert-Remy, U., Gürtler, R., Houdeau, E., Kurek, M., Louro, H., Morales, P., Passamonti, S., Barat Baviera, J. M., Degen, G., … Ruggeri, L. (2026). Safety evaluation of the modification of the food additive enzymatically produced steviol glycosides (E 960c). *EFSA Journal*, *24*(1), e9779. https://doi.org/10.2903/j.efsa.2026.9779