

ADOPTED: 22 June 2021

doi: 10.2903/j.efsa.2021.6691

Safety evaluation of steviol glycoside preparations, including rebaudioside AM, obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts

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Abstract

The EFSA Panel on Food Additives and Flavourings (FAF) provides a scientific opinion on the safety of steviol glycoside preparations, including rebaudioside AM, obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts. These steviol glycoside preparations are produced via enzymatic bioconversion of highly purified stevioside and/or rebaudioside A extracts obtained from stevia plant using two UDP-glucosyltransferases and one sucrose synthase enzymes produced by the genetically modified strains of *E. coli* K-12 that facilitate the transfer of glucose to purified stevia leaf extracts via glycosidic bonds. The Panel considered that the parental strain is a derivative of *E. coli* K-12 which is well characterised and its safety has been documented; therefore, it is considered to be safe for production purposes. The Panel concluded that there is no safety concern for steviol glycoside preparations, including rebaudioside AM, obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts using UDP-glucosyltransferases and sucrose synthase enzymes produced by the genetically modified strains of *E. coli* K-12, to be used as a food additive. The Panel recommends the European Commission to consider the proposal of establishing separate specifications for steviol glycoside preparations, including rebaudioside AM, obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts in Commission Regulation (EU) No 231/2012.

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Keywords: steviol glycoside preparations, rebaudioside AM, enzymatic bioconversion, *E. coli* K-12

Requestor: European Commission

Question number: EFSA-Q-2019-00499

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Note: The full opinion will be published in accordance with Article 12(3) of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from the European Commission.

Declarations of interest: The declarations of interest of all scientific experts active in EFSA's work are available at <https://ess.efsa.europa.eu/doi/doiweb/doisearch>.

Suggested citation: EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), Younes M, Aquilina G, Castle L, Engel K-H, Fowler P, Frutos Fernandez MJ, Fürst P, Gürtler R, Gundert-Remy U, Husøy T, Manco M, Mennes W, Passamonti S, Moldeus P, Shah R, Waalkens-Berendsen I, Wölfle D, Wright M, Barat Baviera JM, Degen G, Leblanc J-C, Herman L, Giarola A, Aguilera J, Vianello G and Castle L, 2021. Scientific opinion on the safety evaluation of steviol glycoside preparations, including rebaudioside AM, obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts. *EFSA Journal* 2021;19(8):6691, 22 pp. <https://doi.org/10.2903/j.efsa.2021.6691>

ISSN: 1831-4732

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The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.



Summary

Following a request from the European Commission to the European Food Safety Authority (EFSA), the Panel on Food Additives and Flavourings (FAF) was asked to provide a scientific opinion on the safety of steviol glycoside preparations, including rebaudioside AM, obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts, in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

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

These steviol glycoside preparations ($\geq 95\%$ total steviol glycosides, determined primarily as the sum of rebaudioside A, rebaudioside D, rebaudioside M and/or stevioside and rebaudioside AM) are produced via enzymatic bioconversion of highly purified stevioside and/or rebaudioside A extracts obtained from stevia plant using two UDP-glucosyltransferases and one sucrose synthase enzymes produced by the genetically modified strains of *E. coli* K-12, that facilitate the transfer of glucose to purified stevia leaf extracts via glycosidic bonds.

The Panel considered that the proposed manufacturing process applied to the production of the steviol glycosides preparations subject of the present evaluation, involves enzymatic bioconversion steps of purified stevia leaf extracts. This process may result in impurities different from those that may be present in steviol glycosides (E 960) obtained from water extraction of the leaves of the *Stevia rebaudiana* followed by recrystallisation. In this respect, the Panel noted the proposal by the applicant to include a new entry in the applicable legislation, '*steviol glycosides produced via enzymatic bioconversion of steviol glycosides from Stevia plant*'.

The Panel considered that the parental strain is a derivative of *E. coli* K-12 which is well characterised and its safety has been documented; therefore, it is considered to be safe for production purposes. The recipient strain contains several genetic modifications which do not raise a safety concern. Since no viable cells nor their DNA remained in the final product, this manufacturing process does not pose a safety concern.

The *in vitro* studies demonstrated that human digestive enzymes are not capable of hydrolysing β -glycosidic bonds of steviol glycosides and the intestinal microflora of humans (and rats) is able to hydrolyse steviol glycosides to steviol (EFSA ANS Panel, 2010; EFSA FAF Panel, 2019). The *in vitro* anaerobic metabolism of rebaudioside AM was investigated in pooled human faecal homogenates (Documentation provided to EFSA n. 3). The Panel agreed with the authors that the metabolism of rebaudioside AM produced by enzymatic bioconversion indicated its rapid deglycosylation to steviol as final metabolite. These findings are consistent with results from similar studies conducted in the same test system and experimental conditions with a mixture of steviol glycosides, including rebaudioside AM, previously assessed by the FAF Panel for the evaluation of another proposed amendment of the specifications for steviol glycosides (E 960) (EFSA FAF Panel, 2020). The metabolic fate of steviol glycosides, including rebaudioside AM, leads to the aglycone which is absorbed. Given the similarities in metabolic fate of steviol glycosides, a read-across with regard to toxicity was considered applicable considering the availability of toxicity studies on other previously evaluated steviol glycosides (EFSA ANS Panel, 2010).

In the scientific opinion of the safety assessment of 60 steviol glycosides (EFSA FAF Panel 2020), the Panel concluded that the ADI of 4 mg/kg bw per day, expressed as steviol equivalents, can apply to all those 60 listed steviol glycosides. Considering also that no concern was identified from the manufacturing process of steviol glycoside preparations, including rebaudioside AM, obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts, no additional toxicological data were required. The Panel further confirmed that the ADI of 4 mg/kg bw per day, expressed as steviol equivalents, also applies to the steviol glycoside preparations obtained by enzymatic bioconversion described in the present application.

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1. Introduction

The present scientific opinion deals with the safety evaluation of steviol glycoside preparations, including rebaudioside AM, obtained by enzymatic reaction of highly purified stevioside and/or rebaudioside A stevia leaf extracts.

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 as in foods under the conditions of use specified therein. Moreover, food additives shall comply with the specifications as referred to in Article 14 of that Regulation and laid down in Commission Regulations (EU) No 231/2013²

Steviol glycosides (E 960) is an authorised food additive in the European Union for use in several food categories and specifications have been adopted for it. Presently, the section definition in the specifications describes two main phases of the manufacturing process, i.e. water extraction followed by preliminary purification to yield a steviol glycoside primary extraction and recrystallization of the steviol glycosides resulting in a final product containing not less than 95% of the of 11 identified steviol glycosides - stevioside, rebaudiosides A, B, C, D, E, F, and M, steviolbioside, rubusoside, and dulcoside in any combination and ratio.

The European Commission received an application requesting an amendment of the specifications in order to allow a further processing step – an enzymatic conversion of the highly purified rebaudioside A and/or stevioside from stevia leaf extract to minor glycosides that are present in the leaf, including rebaudioside AM that is not listed in the existing specifications.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to provide a scientific opinion as regards a proposed amendment of the specifications of the food additive Steviol glycosides (E 960) in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.³

1.1.3. Interpretation of 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 existing specifications for the food additive steviol glycosides (E 960), as laid down in Regulation (EC) No 231/2012, with respect to the definition of the food additive. The original proposed amendment was aimed at i) broadening the existing definition in order to capture the enzymatic bioconversion steps applied to the manufacturing of steviol glycoside preparations rich in selected steviol glycosides and ii) adding rebaudioside AM to the list of steviol glycosides detailed in the EU specifications for E 960 and contributing to the purity assay (Documentation provided to EFSA n. 1).

In the course of the assessment, the applicant however has revised its original proposal for amending the existing EU specifications for the food additive E 960, requesting the creation of a new entry in the applicable legislation, e.g. E 960c(ii), for the food additive 'steviol glycosides produced via enzymatic bioconversion of steviol glycosides from Stevia plant' (Documentation provided to EFSA n. 8). In the present scientific opinion, the Panel has evaluated the latest proposal submitted by the applicant.

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.

1.1.4. Information on existing authorisations and evaluations

Steviol glycosides (E 960) from water extraction of the leaves of the *Stevia rebaudiana* Bertoni plant and described as 'not less than 95% steviolbioside, rubusoside, dulcoside A, stevioside, rebaudiosides A, B, C, D, E, F and M on the dried basis, in any combination and ratio' is an authorised food additive in the EU according to Regulation (EC) No 1333/2008 on food additives and specifications have been defined in Commission Regulation (EU) No 231/2012.

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

Rebaudioside A is also authorised as an EU flavouring substance ([FL-no: 16.113]) and was assessed within Flavouring Group Evaluation 310 (FGE.310), considering the toxicological dataset available for E 960 (EFSA CEF Panel, 2011).

In 2019, the EFSA FAF Panel performed a safety evaluation of a modification of the specifications following a new production process of steviol glycosides (E 960) (EFSA FAF Panel, 2019). The FAF Panel concluded that there is no safety concern for rebaudioside M produced via enzymatic bioconversion, however recommended that the European Commission considers establishing separate specifications for rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract in Commission Regulation (EU) No 231/2012.

In 2020, the FAF Panel evaluated an application to amend the existing EU specifications for steviol glycosides to allow for the inclusion of 60 steviol glycosides identified in *S. rebaudiana* Bertoni leaves, including both 'major' and 'minor' steviol glycosides, that may comprise the assay value of not less than 95% total steviol glycosides. The Panel concluded that the overall metabolic fate of these steviol glycosides is the same and therefore it would be acceptable to use a read across approach for the safety assessment of the 60 steviol glycosides and the ADI of 4 mg/kg bw per day would apply to all those steviol glycosides. That list of 60 steviol glycosides included the three that are enriched in the current application; namely rebaudioside D and rebaudioside M (being among the 11 in the existing EU specifications) along with rebaudioside AM (being one of the additional 49 considered in the 2020 EFSA opinion). However, the Panel noted at that time that the proposed change from 11 to 60 specified steviol glycosides, while maintaining an assay value of not less than 95% as proposed by the applicant, would allow less pure preparations of the food additive onto the market. According to the proposed change in specifications, there would remain a small but not insignificant fraction of the additive that was undefined and therefore could be not evaluated by the Panel. Therefore, while inclusion of the 60 steviol glycosides in the specifications for steviol glycoside (E 960) would not be of safety concern, the FAF Panel could not conclude on the safety of the proposed amendment to the specifications of steviol glycosides (E 960) as 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).

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

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

In 2017, JECFA revised the specifications for 'Steviol Glycosides from *Stevia rebaudiana* Bertoni' that consist of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose and deoxyglucose) in any of the orientations occurring in the leaves of *S. rebaudiana* Bertoni, provided that the total percentage of steviol glycosides is not less than 95% (JECFA, 2017). These specifications have been superseded in 2019 by new tentative JECFA specifications adopted jointly with a framework approach based on the different methods of production applied to the manufacturing of steviol glycosides, i.e. water extraction, fermentation, bioconversion and glucosylation (JECFA, 2019).

The framework adopted in 2019 has been subsequently amended by JECFA at its 91st meeting in February 2021.⁴ Specifications of steviol glycosides were also revised at the same meeting and the existing assay for steviol glycosides was replaced by an HPLC–UV–MS method utilising external reference standards (JECFA, 2021).

2. Data and methodologies

2.1. Data

The present evaluation is based on the data submitted in the original application dossier (Documentation provided to EFSA N. 1) and additional information submitted by the applicant during the assessment process following requests by EFSA (Documentation provided to EFSA n. 4 and 6). In addition, in June 2020 the applicant spontaneously submitted additional information (Documentation provided to EFSA n. 5).

Following the evaluation of additional data sent to EFSA on 05 March 2021 (Documentation provided to EFSA n. 7), the applicant was invited to provide clarification during a technical hearing held at the 25th meeting of the WG Food Additives Applications.⁵

As a follow-up to the technical hearing, a further request for additional information was issued by EFSA and data in response were received on 09 June 2021 (Documentation provided to EFSA n. 8).

2.2. Methodologies

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

The current 'Guidance for submission for food additive evaluation' (EFSA ANS Panel, 2012), 'Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use' (EFSA GMO Panel, 2011) and the 'Statement on the characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) have been followed by the FAF Panel for evaluating the proposed enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts to maximise the production of selected steviol glycosides, such as rebaudioside M, rebaudioside D and rebaudioside AM.

3. Assessment

3.1. Technical data

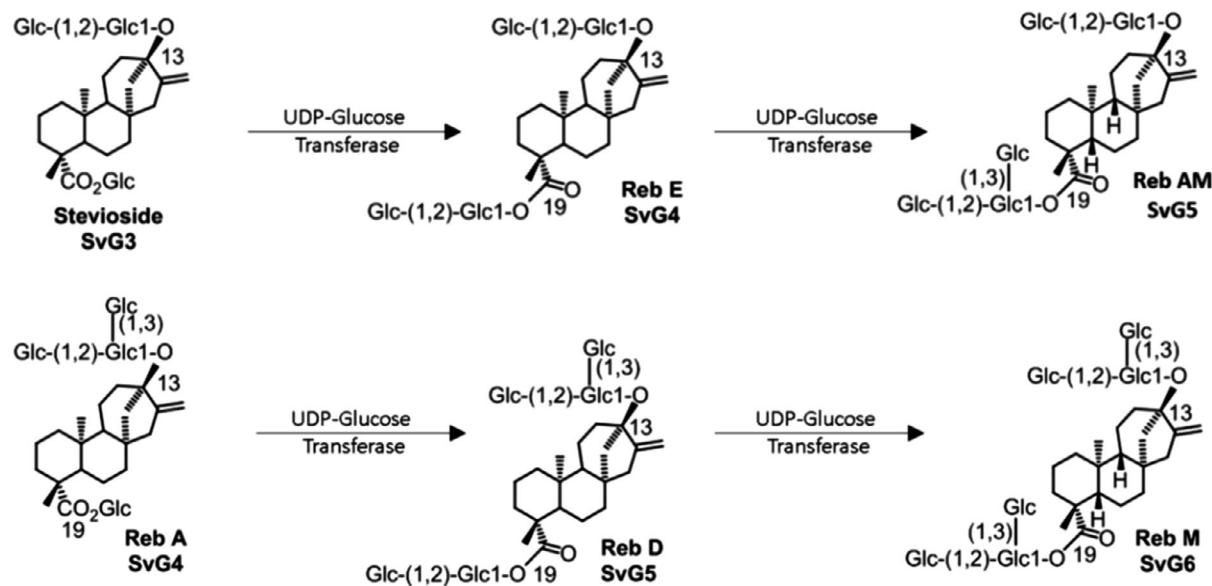
3.1.1. Identity of the substances

The applicant proposes an enzymatic bioconversion of highly purified steviol glycosides rebaudioside A and/or stevioside extracts obtained from stevia plant using UDP-glucosyltransferases (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13) enzymes, derived from genetically modified strains of *Escherichia coli* K-12. This bioconversion will be applied to maximise the production of selected steviol glycosides, such as rebaudioside M, rebaudioside D and rebaudioside AM, which are naturally present only in low concentrations in the stevia leaf extract.

The proposed enzymatic reaction is a stepwise conversion of the starting steviol glycosides extracts, as shown in Figure 1 below. When the starting material is the highly purified stevioside extract obtained from stevia plant, it is first converted to the 'intermediate' steviol glycoside rebaudioside E and then to the 'end' steviol glycoside rebaudioside AM (an isomer of rebaudioside D). Whereas, when the starting material is the highly purified rebaudioside A extract obtained from stevia plant, it is first converted to the 'intermediate' steviol glycoside rebaudioside D and then to the 'end' steviol glycoside rebaudioside M.

⁴ <http://www.fao.org/3/cb3689en/cb3689en.pdf>

⁵ https://www.efsa.europa.eu/sites/default/files/wgs/food-ingredients-and-packaging/FAA_wg.pdf



Reb = rebaudioside; UDP = uridine diphosphate.

Figure 1: Enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts to selected steviol glycosides, as provided by the applicant (Documentation provided to EFSA n: 1)⁶

Upon request for clarifications on the chemical composition of the steviol glycosides preparations resulting from the enzymatic bioconversion of highly purified stevioside and/or rebaudioside A extracts obtained from stevia plant, the applicant indicated that the different duration of the enzymatic reaction on the two substrates (rebaudioside A and/or stevioside stevia leaf extracts) drives the formation to three main mixtures of selected steviol glycosides (Documentation provided to EFSA n. 1, 4 and 6):

- 1) Steviol glycosides with a high content of rebaudioside M: such a blend is produced via enzymatic bioconversion of highly purified rebaudioside A stevia leaf extract with a sufficiently long enzymatic reaction time (up to 40 h) to get most of the 'intermediate' glycoside rebaudioside D converted to the 'end' glycoside rebaudioside M. The reaction yields a steviol glycosides preparation with at least 94% of rebaudioside M and the remaining components are residuals of the starting material (e.g. unreacted rebaudioside A) and rebaudioside D.
- 2) Steviol glycosides with a high content of rebaudioside D: such a blend is produced via enzymatic bioconversion of highly purified rebaudioside A stevia leaf extract with a shorter enzymatic reaction time (up to 30 h) to get more 'intermediate' glycoside rebaudioside D by stopping its conversion to the 'end' glycoside rebaudioside M. The reaction yields a steviol glycosides preparation with at least 90% of rebaudioside D and the remaining components are residuals of the starting material (e.g. unreacted rebaudioside A) and rebaudioside M.
- 3) Steviol glycosides with a high content of rebaudioside AM: such a blend is produced via enzymatic bioconversion of highly purified stevioside stevia leaf extract with a sufficiently long enzymatic reaction time (between 24 and 48 h) to get most of the 'intermediate' glycoside rebaudioside E converted to the 'end' glycoside rebaudioside AM. The reaction yields a steviol glycosides preparation with at least 97% of rebaudioside AM and the remaining components are residuals of the starting material (e.g. unreacted stevioside) and rebaudioside E.

The applicant measured the steviol glycosides distribution in the three steviol glycosides preparations by HPLC⁷; five non-consecutive batches per each preparation were analysed. In addition, the applicant explained that such steviol glycosides preparations obtained by enzymatic bioconversion can also contain small fractions of other minor steviol glycosides that were present in the starting raw

⁶ The Panel noted that the correct names of the enzymes are UDP-glucosyltransferases.

⁷ For steviol glycosides preparations with high content of rebaudioside AM, a modified JECFA 2017 gradient HPLC method (JECFA, 2017) was used.

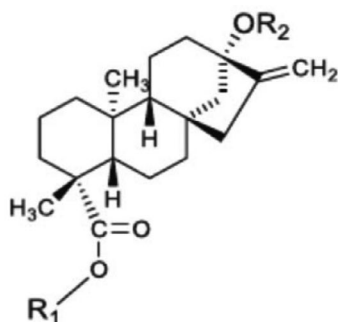
materials. For instance, the HPLC chromatogram of highly purified stevioside stevia leaf extract, used as the starting material, showed that 94.5% was accounted for stevioside and there were also small amounts of other steviol glycosides such as rebaudioside A (3.7%), steviolbioside (1.4%) and rubusoside (0.1%). Therefore, these minor steviol glycosides may be present in the final steviol glycoside preparations obtained by enzymatic bioconversion of highly purified stevia leaf extracts (Documentation provided to EFSA n. 6).

The Panel noted that rebaudioside AM is currently not listed among the 11 steviol glycosides that should comprise the 95% of the assay for steviol glycosides (E 960) as per Regulation (EU) No 231/2012.

The following information on rebaudioside AM was provided by the applicant (Documentation provided to EFSA n. 1):

Chemical name: 13-[(2-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.
 Synonyms: Rebaudioside AM, Reb AM, Stevia Reb AM
 CAS No: 2222580-26-7
 Chemical formula: C₅₀H₈₀O₂₈
 Molecular weight: 1,129.15

According to the applicant, all steviol glycosides are glycosylated derivatives of the aglycone steviol. They share the same backbone structure and differ only in terms of type and number of glycoside units in positions R₁ and R₂.



Where R₁ and R₂ are as follows (Glc = glucose):

Trivial name	R ₁	R ₂
Rebaudioside M	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside D	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside AM	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-

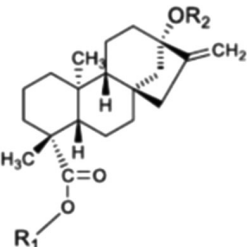
Figure 2: Structures for steviol glycosides

According to the applicant, the steviol glycosides preparations obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts are white to yellow powders and they are approximately between 150 and 350 times sweeter than sucrose (at 5% sucrose equivalency). The steviol glycosides preparations obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts are described as freely soluble (rebaudioside AM) to slightly soluble in water (rebaudioside M and D) (Documentation provided to EFSA n. 1).

3.1.2. Proposed specifications

The initial proposal of the applicant for the present application was to amend the existing definition of E 960, as laid down in EC Regulation 231/2012, in order to capture this enzymatic bioconversion for manufacturing steviol glycoside preparations rich in selected steviol glycosides and to add rebaudioside AM to the list of authorised steviol glycosides which may account for the purity assay of E 960 (Documentation provided to EFSA n. 1). However, during the assessment the applicant reconsidered its original amendment and submitted a revised proposal, as outlined in Table 1 (Documentation provided to EFSA n. 8).

Table 1: Specifications for steviol glycosides produced via enzymatic bioconversion of steviol glycosides from stevia plant as proposed by the applicant (Documentation provided to EFSA n. 8)

E 960c(ii) Steviol glycosides produced via enzymatic bioconversion of steviol glycosides from stevia plant			
Synonyms			
Definition	<p>Steviol glycosides produced via enzymatic bioconversion of steviol glycosides from stevia are composed predominately of rebaudioside M, rebaudioside D, and/or rebaudioside AM</p> <p>Steviol glycosides are obtained via enzymatic bioconversion of purified steviol glycosides leaf extracts (95% steviol glycosides) using UDP-glucosyltransferase and sucrose synthase enzymes produced by genetically modified strains of <i>E. Coli</i> (pPM294, pFAF170, and pSK401) that facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds.</p> <p>After removal of the enzymes by solid-liquid separation and heat treatment, the purification involves concentration of the steviol glycosides by resin adsorption, followed by recrystallisation of the steviol glycosides resulting in a final product containing not less than 95% of total steviol glycosides, including one or more of rebaudiosides D, M and/or AM. Viable cells of <i>E. coli</i> (pPM294, pFAH170, and pSK401) or their DNA shall not be detected in the food additive.</p>		
Chemical name	 <p>where R₁ and R₂ are as follows (Glc: glucose):</p>		
	Trivial name	R₁	R₂
	Rebaudioside M	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
	Rebaudioside D	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
	Rebaudioside AM	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-
Molecular formula	Trivial name	Formula	Conversion factor
	Rebaudioside M	C ₅₆ H ₉₀ O ₃₃	0,25
	Rebaudioside D	C ₅₀ H ₈₀ O ₂₈	0,29
	Rebaudioside AM	C ₅₀ H ₈₀ O ₂₈	0,29
Molecular weight and CAS No	Trivial name	CAS number	Molecular weight
	Rebaudioside M	1220616-44-3	1,291,30
	Rebaudioside D	63279-13-0	1,291,15
	Rebaudioside AM	2222580-26-7	1,291,15
Assay	Not less than 95% of total steviol glycosides on the dried basis, including one or more of rebaudiosides D, M, and/or AM.		
Description	White to light yellow powder, approximately between 150 and 350 times sweeter than sucrose (at 5% sucrose equivalency).		
Identification			
Solubility	Freely soluble to slightly soluble in water		
pH	Between 4,5 and 7,0 (1 in 100 solution)		
Purity			
Total ash	Not more than 1%		
Loss on drying	Not more than 6% (105°C, 2 h)		
Residual solvents	Not more than 5,000 mg/kg ethanol		
Arsenic	Not more than 0.015 mg/kg		
Lead	Not more than 0.2 mg/kg		
Cadmium	Not more than 0.015 mg/kg		
Mercury	Not more than 0.07 mg/kg		
Residual proteins	Not more than 5 mg/kg		

The Panel noted that the following definition is proposed 'steviol glycosides produced via enzymatic bioconversion of steviol glycosides from Stevia plant', i.e. making reference to the plant as the starting material. The Panel however, noted that the manufacturing process proposed by the applicant and assessed in the current application clearly specifies which are the steviol glycosides starting materials used in the enzymatic bioconversion (i.e. highly purified stevioside and/or rebaudioside A stevia leaf extracts). Therefore, the Panel was of the opinion that the starting materials rebaudioside A and stevioside stevia leaf extracts should be mentioned in the definition. The Panel noted that a more precise description of this manufacturing process could be 'steviol glycosides produced via enzymatic bioconversion of highly purified steviol glycosides rebaudioside A and/or stevioside extracts obtained from stevia plant'.

The Panel noted that the applicant provided analytical data from the analyses of 15 batches of steviol glycosides produced by enzymatic bioconversion of highly purified rebaudioside A and/or stevioside stevia leaf extracts supporting that they are consistently produced and compliant with the proposed specifications, as outlined in Table 2 (Documentation provided to EFSA n. 1).

According to the applicant, the steviol glycosides preparations resulting from the enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts contain $\geq 95\%$ total steviol glycosides, determined primarily as the sum of rebaudioside A, rebaudioside D, rebaudioside M and/or stevioside and rebaudioside AM. The Panel noted that the data submitted from the analysis of steviol glycosides composition in the three different steviol glycosides preparations (see Section 3.1.1. five non-consecutive batches per each preparation were analysed) fulfil such declared purity (Documentation provided to EFSA n. 1). The Panel considered that the purity assay for steviol glycosides produced by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts can be expressed 'as not less than 95% of total steviol glycosides' referring to rebaudioside M, rebaudioside D and/or rebaudioside AM to account for the purity assay, as proposed by the applicant.

Regarding the residual solvent ethanol, the Panel noted that the highest analytical level detected in steviol glycosides prepared by enzymatic bioconversion from purified stevia leaf extracts (five batches for each of the three steviol glycoside preparations) was 3,420 mg/kg which is within the proposed maximum limit, i.e. 5,000 mg/kg (Documentation provided to EFSA n. 1).

Regarding toxic elements, the Panel noted that analytical data on the content of arsenic, lead, cadmium and mercury were provided for five batches of each of the three steviol glycosides preparations (Documentation provided to EFSA n. 1). Based on these data, the Panel considered the proposed maximum limits for toxic elements to be adequate. The anticipated impact of these proposed specifications on the potential exposure to these elements is described in Section 3.3.1 (Tables 2 and 3).

Five batches for each of the three steviol glycoside preparations were analysed for the presence of possible microbiological contaminants (Documentation provided to EFSA n. 1). Total plate count, yeast and moulds, total coliforms and individual types of microorganisms, including *E. coli* and *Salmonella*, were consistently not detectable in any tested batches.

Steviol glycosides preparations resulting from the enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts have been tested for residual proteins using the BCA assay with a limit of detection (LOD) of 5 mg/kg to confirm the absence of enzymes (UDP-glucosyltransferases and sucrose synthase) or associated impurities in the proposed food additive (Documentation provided to EFSA n. 1, 4 and 6). No protein was detected above the LOD in the tested batches (six batches of steviol glycosides rich in rebaudioside M, four batches rich in rebaudioside D and three batches rich in rebaudioside AM). The Panel noted that a provision for residual proteins is included in the proposed specifications, i.e. not more than 5 mg/kg.

Three samples of, respectively, UDP-glucosyltransferases (UGT-Sr, UGT-Sl; EC 2.4.1.17) and sucrose synthase (SuSy-At) enzymes were analysed for the presence of viable cells of the enzymes production organism (*E. coli*); each sample was tested in triplicate. No *E. coli* was detected in 1 g (further details available in Section 3.1.3.1.1) (Documentation provided to EFSA n. 1 and 4).

To confirm the absence of residual DNA from the enzyme production microorganisms, three batches of steviol glycoside preparations obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts (two industrial batches and one pilot batch) were tested by polymerase chain reaction (PCR). These tests were performed in triplicate for the presence of plasmid DNA. In addition, one industrial batch was tested in triplicate for the presence of both plasmid and chromosomal DNA. In none of the analysed batches residual DNA was detected, with a LOD between 1 and 10 ng total DNA/g (further details available in Section 3.1.3.1.1) (Documentation provided to EFSA n. 1, 4, 5, 6, 7 and 8). The Panel noted that the LOD of the method applied is adequate (EFSA CEP Panel statement, 2019).

The Panel noted that the absence of viable cell/residual DNA of the enzymes production microorganisms in the final product is captured in the proposed definition, where it is stated 'Viable cell of *E. coli* (pPM294, pFAH170, and pSK401) or their DNA shall not be detected in the food additive'.

An ad hoc meeting between EFSA and industry to talk about the possible presence of kaurenoic acid as an impurity in the food additive E 960 took place in 2018.⁸ No kaurenoic acid was detected in four batches of steviol glycosides produced by enzymatic bioconversion from purified stevia leaf extracts analysed by liquid chromatography–mass spectrometry (LC–MS) (LOD of 0.25 mg/kg) (Documentation provided to EFSA n. 4 and 7).

In view of the botanical origin of the stevia, the presence of pesticides should be considered. Steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni, used to prepare starting materials rebaudioside A and stevioside extracts, were analysed (five and six batches, respectively) for the presence of pesticide residues covering the range of commonly used pesticides. No pesticide residues were detected in the starting materials (Documentation provided to EFSA n. 1). The Panel noted that maximum residue levels (MRLs) for pesticides set under Regulation (EC) 396/2005 apply to stevia (*S. rebaudiana*), as listed in Part B of Annex I. Thus, MRLs established for this commodity code equally apply to *S. rebaudiana*. For processed products derived from stevia, the provisions of Article 20 are applicable, meaning that the changes in the levels of pesticide residues caused by processing need to be taken into account.

The Panel noted that no data on particle size and particle size distribution have been provided by the applicant; but considered that this information was not necessary given the water solubility of the proposed food additive.

3.1.3. Manufacturing process

The steviol glycosides preparations, the subject of the present assessment, are manufactured by enzymatic bioconversion of highly purified rebaudioside A and/or stevioside stevia leaf extracts. The enzymes involved, two UDP-glucosyltransferases (UGT-Sr, UGT-Sl; EC 2.4.1.17) and one sucrose synthase (SuSy-At; EC 2.4.1.13), are derived from genetically modified strains of *E. coli* K-12 and they convert rebaudioside A to rebaudioside D and rebaudioside M, or stevioside to rebaudioside E and rebaudioside AM. UDP-glucosyltransferases facilitate the transfer of glucose from an activated donor molecule (e.g. UDP-glucose) to the acceptor molecule steviol transfer glucose (Richman et al., 2005). Sucrose synthase ensures the availability of UDP-glucose by catalysing the conversion of UDP and sucrose to fructose and UDP-glucose (Wang et al., 2015).

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

Stage 1 – Production of purified steviol glycosides stevia leaf extracts (rebaudioside A and/or stevioside)

S. rebaudiana leaves are extracted with hot water and the resulting extract is filtered and treated with a flocculant to induce the precipitation of particulate impurities. The resulting solution is then filtered to separate the filtrate from the precipitate. The filtrate is then deionised by an ion exchange resin and the deionised filtrate then undergoes purification by passing it through a column packed with microporous adsorption resin that retains the glycosides. The column is then washed with deionised water to remove impurities and the glycosides are then desorbed from the resin using aqueous ethanol. The resulting glycoside solution is treated with activated carbon and the carbon is separated from the solution by filtration. Ethanol is removed from the glycoside solution by distillation and the resulting solution is deionised again by an ion exchange resin. The refined solution is then concentrated using a nanofilter and then spray dried to obtain stevia extract containing > 50% rebaudioside A. Rebaudioside powder is further purified by dissolving it in aqueous ethanol and incubated for several hours to allow for crystallisation. The resulting crystal mass containing approximately 95% rebaudioside A then undergoes centrifugation and it is further dried to obtain the highly purified rebaudioside A extract, used as a substrate for the enzymatic bioconversion to rebaudioside D and rebaudioside M.

A similar procedure is followed to get the highly purified stevioside stevia leaf extract, used as substrate for the enzymatic bioconversion to rebaudioside E and rebaudioside AM.

⁸ <https://www.efsa.europa.eu/en/events/event/180611-0>

Stage 2 – Enzymes production (UDP-glucosyltransferases (UGT-Sr, UGT-SI) and sucrose synthase (SuSy-At))

Purified rebaudioside A and/or stevioside stevia leaf extracts are converted to rebaudiosides M, D and/or AM. Three enzymes, two UDP-glucosyltransferases and one sucrose synthase, used for this conversion, are derived from three genetically modified strains of *E. coli*. The strains are derived from the same recipient strain *E. coli* LE1B109 containing each a different plasmid, respectively, pFAH170, containing the gene *UGT_Sr-0406*, pSK041, containing the gene *UGT_SI-0533*, both coding for a UDP-glucosyltransferase and pPM294 containing the gene *SuSy_At-0275*, coding for sucrose synthase.

Stage 3 – Production of selected steviol glycosides (rebaudioside D, M and AM)

A) Bioconversion of purified stevia extracts to rebaudioside D, E, M and AM

The purified stevia leaf extracts (with high content of rebaudioside A and/or stevioside) from stage 1 and sucrose⁹ are dissolved in reverse-osmosis water, followed by the addition of the enzymes (UDP-glucosyltransferase (UGT-Sr, UGT-SI) and sucrose synthase (SuSy-At)) and UDP disodium salt (5'-UDP-Na₂) to formulate the reaction mixture for enzymatic conversion. Depending on the enzymatic reaction time, steviol glycoside mixtures with different ratios of starting glycosides rebaudioside A and/or stevioside (and also possibly other minor steviol glycosides present in the starting stevia extracts), intermediate glycosides rebaudioside D and/or rebaudioside E, and the primary final glycosides rebaudioside M and/or rebaudioside AM can be produced (see Section 3.1.1). The resulting reaction mixture is heated to inactivate the enzymes. The mixture is then treated with a flocculant to remove impurities and filtered. The obtained filtrate is deionised using an ion-exchange resin.

B) Rebaudioside D, M and AM purification

The deionised filtrate is fed to a column packed with macroporous adsorption resin that retains rebaudioside M, rebaudioside D and rebaudioside AM and other steviol glycosides. The column is washed with deionised water to remove impurities that did not adsorb to the resin and the glycosides are eluted using aqueous ethanol. The filtrate is maintained at low temperature for several hours to allow the steviol glycosides to crystallise. Steviol glycoside crystals are separated by conventional centrifugation and dried under vacuum.

3.1.3.1. Information on the enzymes used in the manufacturing process

The enzymes used for the enzymatic conversion are two UDP-glucosyltransferases (UGT-Sr and UGT-SI, EC 2.4.1.17) and one sucrose synthase (SuSy-At, EC 2.4.1.13). The production strains of these enzymes are genetically modified and have been deposited in the DSMZ culture collection (Germany) with the accession number DSM [REDACTED] (LEB109_pPM294), DSM [REDACTED] (LEB109_pPAH170) and DSM [REDACTED] (LEB109_pSK401) (Documentation provided to EFSA n. 1).

3.1.3.1.1. Characterisation of the production organism

The production strains are the genetically modified bacterial strains *E. coli* LE1B109_pFAH170, *E. coli* LE1B109_pPM294 and *E. coli* LE1B109_pSK041, which are deposited in the Leibniz Institute German collection of microorganisms and cell cultures (DSMZ, Germany) with deposition numbers DSM [REDACTED], DSM [REDACTED] and DSM [REDACTED], respectively. The production strains were identified as *E. coli* K-12 derivatives by whole genome sequence (WGS) analysis. They are derived from the same recipient strain (*E. coli* LE1B109), and only differ in the plasmids they contain, each encoding for a different enzyme: pFAH170 for UGT-Sr, pSK041 for UGT-SI and pPM294 for SuSy-At (Documentation provided to EFSA n. 1).

Characteristics of the recipient strain

The recipient strain LE1B109 is confirmed to be an *E. coli* K-12 derivative by whole genome sequence analysis. The alignment-based calculation of average nucleotide identity (ANI) gave a value of > 99,99% identity with several *E. coli* K-12 strains present in the database and the typical *ilvG* mutation was identified in the recipient strain.

Whole genome sequence analysis identified in the recipient strain several *bla* genes [REDACTED]

[REDACTED] also present in the genomes of *E. coli* K-12 strains [REDACTED]

⁹ Sucrose and UDP disodium salt (5'-UDP-Na₂) are added to the reaction mixture as source of glucose units.

██████████ The genes ██████████ were not considered of concern.

The recipient strain *E. coli* LE1B109 ██████████

Characteristics of the donor sequences

The plasmid pFAH170 ██████████

contains the *UGT_Sr-0406* gene coding for a UDP glucosyltransferase

The plasmid pPM294 ██████████ and contains the *SuSy_At-0275* gene coding for a sucrose synthase, derived from ██████████

The plasmid pSK041 ██████████ contains the *UGT_Sl-0533* gene, coding for UDP-glucosyltransferase ██████████ derived from ██████████. The Panel noted that none of the plasmid contains an antimicrobial resistance gene.

Description of the genetic modification process

The production strains LE1B109_pFAH170 (DSM ██████████), LE1B109_pPM294 (DSM ██████████) and LE1B109_pSK041 (DSM ██████████) contain the multiple copy plasmids pFAH170, pPM294 and pSK041, respectively.

Safety aspects of the production strains

The parental strain is a derivative of *E. coli* K-12. *E. coli* K-12 is well characterised and its safety has been documented (Gorbach, 1978). *E. coli* K-12 was shown to be ineffective in colonising the human gut and its genome has been fully sequenced (Hayashi et al., 2006). The recipient strain contains several genetic modifications which do not raise a safety concern. Therefore, the Panel concluded that the production strains are considered to be safe.

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

A total of three samples of, respectively, UGT-Sr, UGT-Sl and SuSy enzymes were analysed for the presence of *E. coli*, each tested in triplicate. No *E. coli* was detected in 1g after a non-selective enrichment of 24–72 h at 37°C followed by a plating on selective glucuronide containing medium and incubation for 24–48 h at 44°C (Documentation provided to EFSA n. 1 and 4).

Absence of DNA of the production strain in the end product

Three samples of steviol glycoside product samples (two industrial batches and one pilot batch) were tested by conventional PCR, each in triplicate, for the absence of plasmid and chromosomal DNA with three primer pairs, each targeting one of the three genes coding for the three enzymes (two different UDP-glucosyltransferases genes *UGT_Sl-0553*, *UGT_Sr-0406* and one sucrose synthase gene *SuSy_At-0275*) ██████████. In none of the samples DNA was detected, with a LOD between 1 and 10 ng/g (Documentation provided to EFSA n. 1 and 7). In a second experiment, one sample of steviol glycoside was tested, using primer pairs targeting a ██████████ fragment corresponding to *UGT_Sr-0406*, a ██████████ fragment corresponding to the ██████████ origin of replication and a ██████████ fragment corresponding to the *E. coli* chromosomal *pcnB* gene. No DNA was detected with LOD of 10 ng/g (Documentation provided to EFSA n. 8).

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

Data from three independent batches of each food enzyme show that the mean fraction of total organic solids (TOS) are 6.9% (for the UGT-Sr), 6.5% (for UGT-SI) and 5.6% (for SuSy-At) (Documentation provided to EFSA n. 1). Taking into account the amounts of enzymes used in the manufacturing process and the purification process applied (Documentation provided to EFSA n. 6), the Panel concluded that no significant amount of TOS will remain in the final product.

The manufacturing process is similar for each of the three enzymes. In each case, the production strain is grown as a pure culture using a typical industrial medium in a submerged fermentation system with conventional process controls in place. [REDACTED]

[REDACTED] After completion of the fermentation, the solid biomass is removed from the fermentation broth, homogenised to release the intracellular enzyme, and treated with nucleases to destroy nucleic acids. The cell debris are separated from the liquid fraction containing the enzyme, which is then filtered.

The applicant demonstrated the absence of [REDACTED] in the final product (LOD 0.16 µg/g) (Documentation provided to EFSA n. 5).

3.1.4. Methods of analysis in food

While no information on a method of analysis for steviol glycosides obtained by enzymatic bioconversion of highly purified rebaudioside A and/or stevioside stevia leaf extracts in food was provided, the Panel assumed that methods of analysis available for other steviol glycosides preparations would also be applicable.

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

Storage and pH stability of steviol glycosides obtained by enzymatic bioconversion of highly purified rebaudioside A and/or stevioside stevia leaf extracts was assessed by the applicant (Documentation provided to EFSA n. 1, 4 and 6).

Regarding the storage stability, steviol glycosides with a high rebaudioside M and/or rebaudioside D content (one batch) and steviol glycosides with a high rebaudioside AM content (one batch) were tested. Such steviol glycosides preparations were stored up to 3 months (the one with a high rebaudioside M and/or D content) and up to 6 months (the one with a high rebaudioside AM content) at 25°C at a relative humidity of 60% and at 40°C at a relative humidity of 75%, respectively. The two samples were analysed by HPLC at various time points in order to determine the individual steviol glycosides within the mixtures (rebaudioside D, M and A for steviol glycosides with a high rebaudioside M and/or rebaudioside D content and rebaudioside AM and steviolbioside for steviol glycosides with a high rebaudioside AM content) and the total content of steviol glycosides.

The steviol glycosides preparations were reported to be stable both in terms of individual steviol glycosides distribution and the total steviol glycosides content under the tested conditions and the respective time span.

The same steviol glycosides preparations were assessed over a pH range (from 2 to 8) up to 3 months at 4°C, 25°C, 37°C and 56°C (steviol glycosides with high rebaudioside D and/or M content) and up to 6 months at 4°C, 25°C and 37°C (steviol glycosides with high rebaudioside AM content). The two mixtures were analysed by HPLC likewise to what was done for the storage stability testing. Significant losses in the two tested mixtures were observed at pH 2 and 3 and at temperatures of 37°C and 56°C. Following HPLC analysis of the two tested batches (steviol glycosides with high rebaudioside M and/or rebaudioside D and another with high rebaudioside AM content) to study the degradation compounds at week 0 and after 8 weeks at pH 2 at 37°C, the found compounds were (i) for steviol glycosides with high rebaudioside M content; the isomers *iso* rebaudioside M and B, rebaudioside A and rebaudioside B; (ii) for steviol glycosides with high rebaudioside AM content; the isomers *iso* rebaudioside AM and *iso*-steviolbioside, and steviolbioside. Therefore, the degradation products are considered to be 'primarily related steviol glycosides' compounds.

3.2. Proposed use and use levels

Maximum levels of steviol glycosides (E 960) expressed as steviol equivalents are defined in Annex II to Regulation (EC) No 1333/2008.¹

In this application, with a proposed new production process and including rebaudioside AM in the definition/specification of the food additive (see above), it is proposed to use the high-intensity

sweetener in food and beverages under the same conditions as those already approved for steviol glycosides (E 960) in EU (Regulation (EC) No 1333/2008) (Documentation provided to EFSA n. 1).

3.3. Exposure data

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

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

3.3.1. Anticipated exposure to toxic elements from proposed specifications

The applicant provided analytical data on the content of arsenic (As, < 0.005 mg/kg), lead (Pb, 0.038 mg/kg), cadmium (Cd, < 0.005 mg/kg) and mercury (Hg, < 0.005 mg/kg) and proposed maximum limits for these elements to be included in the specifications for the food additive (see Table 1). The potential exposure to the toxic elements from the use of the proposed food additive can be calculated by assuming contamination of the additive may be up to (i) the highest reported analytical data or (ii) the specifications limit values, and then by calculation pro-rata to the estimate of exposure to the proposed food additive itself (EFSA ANS Panel, 2015b). The outcome of such an exercise illustrates the health impact that would result if the maximum limits for toxic elements were based on the analytical data provided or the limits as proposed by the applicant (Tables 2 and 3, respectively).

Table 2: Risk characterisation for toxic elements using the highest reported analytical data (Documentation provided to EFSA n. 1)

Exposure to proposed additive (mg/kg bw per day)	MOS/MOE for As at 0.005 mg/kg	MOS/MOE for Pb at 0.038 mg/kg	%age of the TWI for Cd at 0.005 mg/kg	%age of the TWI for Hg at 0.005 mg/kg
4.3 ^(a)	14,000–372,000	3,060	0.006%	0.004%

bw: body weight.

(a): Estimated exposure using MPLs and the proposed extension of use (toddlers, 95th percentile). Data from EFSA ANS Panel scientific opinion on the safety of the extension of use of steviol glycosides (E 960) as a food additive (EFSA ANS Panel, 2015b).

Table 3: Risk characterisation for toxic elements using the maximum limits as proposed by the applicant (Documentation provided to EFSA n. 1)

Exposure to proposed additive (mg/kg bw per day)	MOS/MOE for As at 0.015 mg/kg	MOS/MOE for Pb at 0.2 mg/kg	%age of the TWI for Cd at 0.015 mg/kg	%age of the TWI for Hg at 0.07 mg/kg
4.3 ^(a)	4,650–124,000	580	0.02%	0.05%

bw: body weight.

(a): Estimated exposure using MPLs and the proposed extension of use (toddlers, 95th percentile). Data from EFSA ANS Panel scientific opinion on the safety of the extension of use of steviol glycosides (E 960) as a food additive (EFSA ANS Panel, 2015b).

The resulting figures show that the exposure to toxic elements from the proposed uses of steviol glycosides produced by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts would not give rise to an anticipated level of exposure that would be of concern using either the highest reported analytical data or the maximum limit values proposed by the applicant for these elements.

For arsenic, the reference points are BMDL₀₁ values (LB and UB) of 0.3 and 8 µg/kg bw per day from human epidemiological studies (EFSA CONTAM Panel, 2009a). The reference points are based on carcinogenicity and so the MOS/MOE should be at least 10,000 (EFSA Scientific Committee, 2005). Considering that the human studies were the basis to derive the BMDL, an interspecies extrapolation factor may not be needed. Hence, the Panel considered that the lowest calculated MOS/MOE of 4,650 (Table 3) is sufficient.

For lead, the reference point is a BMDL₀₁ of 0.5 µg/kg bw per day (EFSA CONTAM Panel, 2010). 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. In the opinion on lead (EFSA CONTAM Panel, 2010) it is mentioned that a 1 point reduction in IQ is related to a 4.5% increase in the risk of failure to graduate from high school and that a 1 point reduction in IQ in children can be associated with a decrease of later productivity of about 2%. A risk cannot be excluded if the exposure exceeds the BMDL₀₁ (MOS/MOE lower than 1). The MOS/MOE is well above 1 (Tables 2 and 3).

For cadmium, a TWI of 2.5 µg/kg bw has been established (EFSA CONTAM Panel, 2009b) and the exposure to Cd would be only a very minor fraction of the TWI value (Tables 2 and 3).

For mercury, a TWI of 4 µg/kg bw has been established (EFSA CONTAM Panel, 2012) and the exposure to Hg would be only a very minor fraction of the TWI value (Tables 2 and 3).

3.4. Biological and toxicological data

Within the application dossier, scientific publications considered by the applicant relevant to the safety of steviol glycosides were submitted together with data from *in vitro* metabolic studies.

3.4.1. Absorption, distribution, metabolism and excretion

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

The microbial hydrolysis of different steviol glycosides, in particular rebaudioside A, B, C, D, E, F, M, steviolbioside and stevioside (with different purity levels or purity not specified) has been investigated *in vitro* with human faecal incubations.

In vitro metabolic studies in human faecal homogenate samples incubated with different steviol glycosides preparations, including rebaudioside D, M and AM, have been previously assessed by the Panel for the evaluation of other proposed amendments to the specifications of the food additive steviol glycosides (E 960) (EFSA FAF Panel, 2019, 2020).

***In vitro* studies submitted by the applicant**

In support of the current application, data from *in vitro* metabolic studies in human faecal homogenate samples performed with rebaudioside D and rebaudioside M derived from bioconversion (Documentation provided to EFSA n. 2) and with a mixture of minor steviol glycosides, including rebaudioside AM prepared by enzymatic bioconversion (Documentation provided to EFSA n. 3) have been submitted for evaluation. The two studies have been included in a publication by Purkayastha and Kwok (2020).

In the first study, an equal mixture of bioconversion rebaudioside D and bioconversion rebaudioside M was incubated with adult male and adult female pooled faecal homogenate samples (6 individuals each, age 22–60) at a concentration of 0.2 mg/mL under anaerobic conditions at 37°C for 0 to 72 h (Documentation provided to EFSA n. 2).

In the second study, the steviol glycoside mixture prepared with minor steviol glycosides (rebaudioside AM, W2, Y, U2, V, N and O) was incubated with adult male and adult female pooled faecal homogenate samples (6 individuals each, age 22–60) at concentrations of 0.2 and 0.4 mg/mL under anaerobic conditions at 37°C for 0 to 48 h (Documentation provided to EFSA n. 3). In addition, the stability of a mixture of Reb AM, Reb W2, Reb Y, Reb U2, Reb V, Reb N and Reb O in brain–heart infusion was evaluated to demonstrate that the formation of steviol metabolite in pooled human faecal homogenate was mediated by a metabolism-based process.

In both studies, rebaudioside A was used as a metabolic activity positive control in parallel to ensure that the experimental incubation conditions were satisfactory. LC–MS analysis was used to provide metabolic mass balance on the molar equivalent formation of the steviol metabolite over the time-course.

Data from the first study indicated rapid deglycosylation of rebaudioside D and rebaudioside M within the first 12 h of metabolic incubation. The authors stated there was no apparent difference in the extent of deglycosylation of rebaudioside D and rebaudioside M between incubates with adult male or adult female faecal samples (Documentation provided to EFSA n. 2).

Individual minor steviol glycosides (Reb AM, W2, Y, U2, V, N and O) showed a deglycosylation to steviol within 12 h of incubation. Among the minor steviol glycosides rebaudiosides AM and O had the quickest rate of degradation with a detected concentration of ~ 50% detected at the 4-h timepoint and almost complete deglycosylation at the 12-h. According to the authors, no apparent difference in the extent of deglycosylation was noted in the pooled faecal homogenates between adult male and adult female donors. The absence of steviol metabolite formation in the negative control confirmed that the conversion observed in the pooled faecal homogenates was mediated by a metabolism-based process (Documentation provided to EFSA n. 3).

The authors concluded that the metabolism of 'bioconversion rebaudioside AM' in pooled male and female human faecal homogenate indicated deglycosylation of the steviol glycosides to a final steviol metabolite over the first 12 h of metabolic incubation.

3.4.2. Toxicological data

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

3.4.3. Other studies

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

3.5. Discussion

The subject of the present application under evaluation concerns a new enzymatic bioconversion process to produce high purity steviol glycosides preparations. Highly purified steviol glycosides rebaudioside A and/or stevioside extracts obtained from stevia plant are submitted to enzymatic bioconversion using two UDP-glucosyltransferases (EC 2.4.1.17) and one sucrose synthase (EC 2.4.1.13), derived from genetically modified strains of *E. coli* K-12. The purpose of this bioconversion is maximisation of the production of selected steviol glycosides, such as rebaudioside M, rebaudioside D and rebaudioside AM, which are naturally present in low concentrations in the leaves of *Stevia rebaudiana* plant. When the starting material is the highly purified rebaudioside A stevia leaf extract, it is first converted to the 'intermediate' glycoside rebaudioside D and then to the 'end' glycoside rebaudioside M. Whereas, when the starting material is the highly purified stevioside stevia leaf extract, it is first converted to the 'intermediate' glycoside rebaudioside E and then to the 'end' glycoside rebaudioside AM (an isomer of rebaudioside D). According to the different duration of the enzymatic reaction on the two substrates (rebaudioside A and/or stevioside stevia leaf extracts) three main mixtures of selected steviol glycosides can be obtained:

1. *Steviol glycosides with a high content of rebaudioside M*: reaction time up to 40 h to get most of the 'intermediate' glycoside rebaudioside D converted to the 'end' glycoside rebaudioside M. The reaction yields a steviol glycosides preparation with at least 94% of rebaudioside M.
2. *Steviol glycosides with a high content of rebaudioside D*: reaction time up to 30 h to get more 'intermediate' glycoside rebaudioside D by stopping its conversion to the 'end' glycoside rebaudioside M. The reaction yields a steviol glycosides preparation with at least 90% of rebaudioside D.

3. Steviol glycosides with a high content of rebaudioside AM: reaction time between 24 and 48 h to get most of the 'intermediate' glycoside rebaudioside E converted to the 'end' glycoside rebaudioside AM. The reaction yields a steviol glycosides preparation with at least 97% of rebaudioside AM.

Such steviol glycosides preparations obtained by enzymatic bioconversion can also contain residuals of the starting materials, e.g. unreacted stevioside and/or rebaudioside A and other minor steviol glycosides that were present in the starting materials (steviolbioside, rubusoside).

The Panel considered that the proposed manufacturing process applied to the production of the steviol glycosides preparations subject of the present evaluation, involves enzymatic bioconversion steps of purified stevia leaf extracts. This process may result in impurities different from those that may be present in steviol glycosides (E 960) obtained from water extraction of the leaves of the *Stevia rebaudiana* followed by recrystallisation. In this respect, the Panel noted the proposal by the applicant to include a new entry in the applicable legislation, 'steviol glycosides produced via enzymatic bioconversion of steviol glycosides from Stevia plant'. The Panel recommends the European Commission to consider this proposal.

The Panel noted that the proposed specifications also contain additional parameters related to the specific genetically modified microorganisms used to produce the enzymes involved in the bioconversion steps (i.e. absence of viable cells of *E. coli* (pPM294, pFAH170 and pSK401) or their DNA; not more than 5 mg/kg of residual protein), which are aligned with the recommendations issued for the specifications of rebaudioside M produced via enzyme-catalysed bioconversion of purified stevia leaf extract in the case it would be authorised as food additive (EFSA FAF Panel, 2019). The Panel noted that adequate analytical data supporting the compliance with these above mentioned proposed specifications were provided by the applicant. Since no viable cells nor their DNA remained in the final product, this manufacturing process does not raise a safety concern.

The Panel noted that the following definition is proposed 'steviol glycosides produced via enzymatic bioconversion of steviol glycosides from Stevia plant'. The Panel pointed out that the manufacturing process proposed by the applicant clearly specifies which are the steviol glycosides starting materials used in the enzymatic bioconversion (i.e. highly purified stevioside and/or rebaudioside A stevia leaf extracts). Therefore, the starting materials rebaudioside A and stevioside stevia leaf extracts should be mentioned in the definition. A more precise description of this manufacturing process could be 'steviol glycosides produced via enzymatic bioconversion of highly purified steviol glycosides rebaudioside A and/or stevioside extracts obtained from stevia plant'.

According to the applicant, the steviol glycosides preparations resulting from the enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts contain $\geq 95\%$ total steviol glycosides, determined primarily as the sum of rebaudioside A, rebaudioside D, rebaudioside M and/or stevioside and rebaudioside AM. Based on the analytical data provided, the Panel considered that the purity assay for steviol glycosides produced by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts can be expressed as 'not less than 95% total steviol glycosides' referring to rebaudioside M, rebaudioside D and/or rebaudioside AM to account for the proposed purity assay.

Regarding toxic elements, the Panel noted that based on the analytical data provided the proposed maximum limits for toxic elements are adequate. The anticipated impact of these proposed specifications on the potential exposure to these elements is described in Section 3.3.1 (Tables 2 and 3).

The absence of kaurenoic acid in four batches of steviol glycosides produced by enzymatic bioconversion from purified stevia leaf extracts has been demonstrated using a LC-MS method with an adequate detection limit.

The Panel noted that *in vitro* studies demonstrated that human digestive enzymes are not capable of hydrolysing β -glycosidic bonds of steviol glycosides and the intestinal microflora of humans (and rats) is able to hydrolyse steviol glycosides to steviol (EFSA ANS Panel, 2010, EFSA FAF Panel, 2019).

The *in vitro* anaerobic metabolism of rebaudioside AM was investigated in pooled human faecal homogenates (Documentation provided to EFSA n. 3). The Panel agreed with the authors that the metabolism of rebaudioside AM produced by enzymatic bioconversion indicated its rapid deglycosylation to steviol as final metabolite. These findings are consistent with results from similar studies conducted in the same test system and experimental conditions with a mixture of steviol glycosides including rebaudioside AM, previously assessed by the FAF Panel for the evaluation of another proposed amendment of the specifications for steviol glycosides (E 960) (EFSA FAF Panel, 2020).

The metabolic fate of steviol glycosides, including rebaudioside AM, leads to the aglycone which is absorbed. Given the similarities in metabolic fate of steviol glycosides, a read-across with regard to toxicity was considered applicable considering the availability of toxicity studies on other previously evaluated steviol glycosides (EFSA ANS Panel, 2010).

In the scientific opinion of the safety assessment of 60 steviol glycosides (EFSA FAF Panel 2020), the Panel concluded that the ADI of 4 mg/kg bw per day, expressed as steviol equivalents, can apply to all those 60 listed steviol glycosides (EFSA FAF Panel 2020, Appendix A). Considering also that no concern was identified from the manufacturing process of steviol glycoside preparations, including rebaudioside AM, obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts, no additional toxicological data were required. The Panel further confirmed that the ADI of 4 mg/kg bw per day, expressed as steviol equivalents, also applies to the steviol glycoside preparations obtained by enzymatic bioconversion.

4. Conclusions

The Panel concluded that there is no safety concern for the three steviol glycosides preparations subject of this opinion to be used as a food additive. These preparations, including rebaudioside AM, are obtained by enzymatic bioconversion of highly purified stevioside and/or rebaudioside A stevia leaf extracts using UDP-glucosyltransferases (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13) enzymes that are derived from genetically modified strains of *E. coli* K-12.

Documentation provided to EFSA

- 1) Dossier 'Application for a change in the steviol glycoside specification in the European Union to include a new manufacturing method for selected steviol glycosides from stevia leaf extracts with highly purified stevioside and rebaudioside'. February 2019. Submitted by PureCircle Limited.
- 2) BRI Report no. PUR-2018-001, December 2018. In vitro anaerobic metabolism of bioconversion rebaudioside D (PCS-3021) and bioconversion rebaudioside M (PCS-3018) in pooled human fecal homogenates from healthy male and female adult subjects. Unpublished report. Submitted within the application dossier.
- 3) BRI Report no. RPT-PUR-2018-001, January 2019. In vitro anaerobic metabolism of leaf extract steviol glycosides minor components in pooled human fecal homogenates from healthy male and female adult subjects. Unpublished report. Submitted within the application dossier.
- 4) Additional information on May 2020. Submitted by PureCircle Limited in response to request from EFSA.
- 5) Additional information on June 2020. Spontaneous submission by PureCircle Limited.
- 6) Additional information on December 2020. Submitted by PureCircle Limited in response to request from EFSA.
- 7) Additional information on March 2021. Submitted by PureCircle Limited in response to request from EFSA.
- 8) Additional information on June 2021. Submitted by PureCircle Limited in response to request from EFSA.

References

- EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources), 2010. Scientific Opinion on safety of steviol glycosides for the proposed uses as a food additive. EFSA Journal 2010;8(4):1537, 85 pp. <https://doi.org/10.2903/j.efsa.2010.1537>
- EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources), 2012. Guidance for submission for food additive evaluations. EFSA Journal 2012;10(7):2760, 60 pp. <https://doi.org/10.2903/j.efsa.2012.2760>
- EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources), 2015a. Scientific opinion on the safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive. EFSA Journal 2015;13(12):4316, 29 pp. <https://doi.org/10.2903/j.efsa.2015.4316>
- EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food), 2015b. Scientific Opinion on the extension of use of steviol glycosides (E 960) as a food additive. EFSA Journal 2015;13(6):4146, 20 pp. <https://doi.org/10.2903/j.efsa.2015.4146>
- EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2011. Scientific Opinion on Flavouring Group Evaluation 310 (FGE.310): Rebaudioside A from chemical group 30. EFSA Journal 2011;9(5):2181, 37 pp. <https://doi.org/10.2903/j.efsa.2011.2181>. Available online: www.efsa.europa.eu/efsajournal.html

- EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), 2019. Statement on the characterisation of microorganisms used for the production of food enzymes. *EFSA Journal* 2019;17(6):5741, 13 pp. <https://doi.org/10.2903/j.efsa.2019.5741>
- EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2009a. Scientific Opinion on arsenic in food. *EFSA Journal* 2009;7(10):1351, 199 pp. <https://doi.org/10.2903/j.efsa.2009.1351>
- EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2009b. Scientific opinion on cadmium in food. *EFSA Journal* 2009;7(10):980, 139 pp. <https://doi.org/10.2903/j.efsa.2009.980>
- EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2010. Scientific Opinion on lead in food. *EFSA Journal* 2010;8(4):1570, 151 pp. <https://doi.org/10.2903/j.efsa.2010.1570>
- EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2012. Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. *EFSA Journal* 2012;10(12):2985, 241 pp. <https://doi.org/10.2903/j.efsa.2012.2985>
- EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2019. Scientific Opinion on the safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive: Rebaudioside M produced via enzyme-catalysed bioconversion of purified stevia leaf extract. *EFSA Journal* 2019;17(10):5867, 19 pp. <https://doi.org/10.2903/j.efsa.2019.5867>
- EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2020. Scientific Opinion on the safety of a proposed amendment of the specifications for steviol glycosides (E 960) as a food additive: to expand the list of steviol glycosides to all those identified in the leaves of *Stevia Rebaudiana* Bertoni. *EFSA Journal* 2020;18(4):6106, 32 pp. <https://doi.org/10.2903/j.efsa.2020.6106>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organism), 2011. Guidance for the risk assessment of genetically modified microorganism and their products intended for food and feed use. *EFSA Journal* 2011;9(6):2103, 54 pp. <https://doi.org/10.2903/j.efsa.2011.2193>
- EFSA Scientific Committee, 2005. Opinion of the Scientific Committee on a request from EFSA related to A Harmonised Approach for Risk Assessment of Substances Which are both Genotoxic and Carcinogenic. *EFSA Journal* 2005;282, 31 pp. <https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2005.282>
- EFSA Scientific Committee, 2009. Guidance of the Scientific Committee on Transparency in the Scientific Aspects of Risk Assessments carried out by EFSA. Part 2: general Principles. *EFSA Journal* 2009;7(7):1051, 22 pp. <https://doi.org/10.2903/j.efsa.2009.1051>
- El-Mesallamy AMD, Mahmoud SA, Elazab KM, Hussein SAM and Hussein AM, 2018. Attenuation of metabolic dysfunctions in the skeletal muscles of type 1 diabetic rats by *Stevia rebaudiana* extracts, via AMPK upregulation and antioxidant activities. *Acta Scientiarum Polonorum. Technologia Alimentaria*, 17, 289–297.
- Gorbach SL, 1978. Risk assessment of recombinant DNA experimentation with *Escherichia coli* K-12. Proceedings from a workshop at Falomuth Massachusetts. *Journal of Infectious Diseases*, 137, 613–714.
- Han X, Chen C, Zhang X, Wei Y, Tang S, Wang J, Tan Z and Xu L, 2019. Effects of dietary stevioside supplementation on feed intake, digestion, ruminal fermentation, and blood metabolites of goats. *Animals*, 9, 32. <https://doi.org/10.3390/ani9020032>
- Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, Ohtsubo E, Baba T, Wanner BL, Mori H and Horiuchi T, 2006. Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. *Molecular Systems Biology*, 2, 0007. <https://doi.org/10.1038/msb4100049>
- JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2009. Steviol glycosides (addendum). In: Toxicological evaluation of certain food additives. Sixty-ninth report of JECFA, June 17-26-29, 2008, Rome. WHO Food Additives series, No. 60, 183–219.
- JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2016. Rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica* [Prepared at the 82nd JECFA, 2016]. In: Combined compendium of food additive specifications. 82nd Meeting, June 7-16, Geneva, Switz. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy/World Health Organization (WHO), Geneva, Switz. FAO JECFA Monographs 19, 91–96.
- JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2017. Monograph 20. Combined compendium of food additive specifications. Residue Monograph prepared by the meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), 84th meeting 2017. Steviol Glycosides from *Stevia rebaudiana* Bertoni. Available online: <http://www.fao.org/ag/agn/jecfa-additives/search.html>.
- JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2019. Joint FAO/WHO Expert Committee on Food Additives, Eighty-seventh meeting, 4–13 June 2019. (Framework for) STEVIOL GLYCOSIDES. Available online: <http://www.fao.org/3/cb0731en/cb0731en.pdf>
- JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2021. Joint FAO/WHO Expert Committee on Food Additives, Ninety-first meeting, 1–12 February 2021. Summary and conclusions. Issued on 5 March 2021. Available online: <http://www.fao.org/3/cb3689en/cb3689en.pdf>
- Purkayastha S and Kwok D, 2020. Metabolic fate in adult and pediatric population of steviol glycosides produced from stevia leaf extract by different production technologies. *Regulatory toxicology and pharmacology: RTP*, 104727.
- Ramos-Tovar E, Flores-Beltrán RE, Galindo-Gómez S, Vera-Aguilar E, Diaz-Ruiz A, Montes S, Camacho J, Tsutsumi V and Muriel P, 2018. *Stevia rebaudiana* tea prevents experimental cirrhosis via regulation of NF- κ B, Nrf2, transforming growth factor beta, Smad7, and hepatic stellate cell activation. 32, 2568–2576.

- Richman A, Swanson A, Humphrey T, Chapman R, McGarvey B, Pocs R and Brandle J, 2005. Functional genomics uncovers three glucosyltransferases involved in the synthesis of the major sweet glucosides of *Stevia rebaudiana*. *The Plant Journal: For Cell and Molecular Biology*, 41, 56–67.
- Wang M, Li J, Qi H, Lu J and Xu Y, 2015. Mechanism-based evaluation of interaction potentials between steviol and selected drugs. *Drug Metabolism Reviews*, 47 (Suppl. 1), 109 (abstract P150).
- Zhao L, Wang X, Xu M, Lian Y, Wang C, Yang H and Mehmood A, 2018. Dietary intervention with Stevia residue extracts alleviates impaired glucose regulation in mice. *Journal of Food Biochemistry*, 42, e12651, 12 pp. <https://doi.org/10.1111/jfbc.12651>

Abbreviations

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
ANI	average nucleotide identity
ANS	EFSA Panel on Food Additives and Nutrient Sources added to Food
BCA	bicinchoninic acid
bw	body weight
CAS	Chemical Abstracts Service
CFU	colony forming units
FAF	EFSA Panel on Food Additives and Flavourings
FAO	Food and Agriculture Organisation
GMO	EFSA Panel on Genetically Modified Organism
HPLC	high-performance liquid chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC–MS	liquid chromatography–mass spectrometry
MRL	maximum residue level
PCR	polymerase chain reaction
TOS	total organic solids
UPD	uridine diphosphate
WHO	World Health Organization