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Safety of the proposed amendment of the specifications of the food additive steviol glycosides (E 960)

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Abstract

The EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) provides a scientific opinion on the safety of proposed amendment of the specifications of the food additive steviol glycosides (E 960). The applicant asked to amend the existing EU specifications for steviol glycosides to allow for the inclusion of all steviol glycosides identified in *Stevia rebaudiana* Bertoni leaves, including both 'major' and 'minor' glycosides, that may comprise the assay value of not less than 95% total steviol glycosides. According to the applicant, all steviol glycosides are subject to microbial metabolism in a similar manner, ultimately generating the common primary metabolite steviol. There are uncertainties on the rate and extent of the metabolism of different steviol glycosides to steviol in the evidence provided and they did not allow the Panel to endorse the applicant's argumentation that all steviol glycosides generate the common metabolite steviol when subjected to microbial metabolism under realistic conditions. The available information was not sufficient to assess the safety of the proposed amendment of the specifications for E 960 and the conclusions on the previous assessments on steviol glycosides cannot be extrapolated to any other mixture of steviol glycosides (containing not less than 95% of any steviol glycosides) extracted from *S. rebaudiana* Bertoni leaves. Therefore, the Panel concluded that the submitted data were insufficient to assess the safety of proposed amendment of the specifications of the food additive steviol glycosides (E 960).

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Summary

Following a request from the European Commission to the European Food Safety Authority (EFSA), the Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to provide a scientific opinion on the safety of 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.

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 a request by EFSA.

The applicant asked to amend the existing EU specifications for steviol glycosides to allow for the inclusion of all steviol glycosides identified in *Stevia rebaudiana* Bertoni leaves, including both 'major' and 'minor' glycosides, that may comprise the assay value of not less than 95% total steviol glycosides. This means that the food additive comprises not less than 95% of steviol glycosides conjugated with glucose, xylose, rhamnose, fructose, deoxyglucose, arabinose, galactose and/or other sugar moieties, in any combination and ratio.

The Panel noted that the proposed specifications can allow a food additive comprising any mixture of compounds extracted from *S. rebaudiana* Bertoni leaves and that the type and proportions of steviol glycosides present in the final composition can be very different from the preparations previously evaluated.

According to the applicant, all steviol glycosides are subject to microbial metabolism in a similar manner, ultimately generating the common primary metabolite steviol. Steviol is absorbed and metabolised to steviol glucuronide, which is excreted primarily via the urine in humans. Therefore, the studies conducted with rebaudioside A and stevioside and revised by the Panel in the assessment of steviol glycosides as a food additive can extend to other steviol glycosides due to the shared metabolic fate.

It would seem plausible that the metabolic fate of any steviol glycoside identified in the leaves of *S. rebaudiana* Bertoni would be comparable with the metabolic fate of those included in the current EU specifications for E 960. However, there are uncertainties on the rate and extent of the metabolism of different steviol glycosides to steviol in the evidence provided and these did not allow the Panel to endorse the applicant's argumentation that all steviol glycosides generate the common metabolite steviol when subjected to microbial metabolism under realistic conditions (Documentation provided to EFSA n. 1).

The available information was not sufficient to assess the safety of the proposed amendment of the specifications of the food additive steviol glycosides (E 960) and the conclusions on the previous EFSA assessments on steviol glycosides cannot be extrapolated to any other mixture of steviol glycosides extracted from *S. rebaudiana* Bertoni leaves and complying with the proposed specification of containing not less than 95% of any steviol glycosides.

The Panel concluded that the submitted data were insufficient to assess the safety of proposed amendment of the specifications of the food additive steviol glycosides (E 960).

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1. Introduction

The present scientific opinion deals with the evaluation of the safety of proposed amendment of the specifications of the food additive steviol glycosides (E 960).

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 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 Regulation (EU) No 231/2012².

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, those specifications stipulate steviol glycosides (E 960) as a final product containing not less than 95% 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 a request vis-à-vis an amendment of the present EU specifications of Steviol glycosides (E 960) to include all steviol glycosides identified in *Stevia rebaudiana* Bertoni leaf extracts, including both 'major' and 'minor' glycosides, that may contribute to the assay value of not less than 95% total steviol glycosides.

1.1.2. Terms of Reference

The European Commission (EC) requests the European Food Safety Authority (EFSA) to provide a scientific opinion as regards a proposed amendment of the specifications of the food additive steviol glycosides (E 960), in accordance with Regulation (EC) No 1331/2008³ establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

1.1.3. Information on existing evaluations and authorisations

The safety of steviol glycosides as a food additive was evaluated by EFSA in 2010 (EFSA ANS Panel, 2010). Following the EFSA assessment in 2015 (EFSA ANS Panel, 2015a), rebaudioside M was included in the specifications for the food additive steviol glycosides (E 960) according to the Commission Regulation (EU) No 231/2012⁴.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) recently issued new specifications for 'Steviol Glycosides from *Stevia rebaudiana* Bertoni' that consist of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose and deoxyglucose) in any of the orientations occurring in the leaves of *S. rebaudiana* Bertoni, provided that the total percentage of steviol glycosides is not less than 95% (JECFA, 2017).

2. Data and methodologies

2.1. Data

The present evaluation is based on the data on steviol glycosides in a newly submitted dossier by the applicant (Documentation provided to EFSA n.1) and additional information submitted by the applicant during the assessment process in response to a request by EFSA (Documentation provided to EFSA n.5).

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, p. 16–33.

² 231/2012.

³ OJ L 354, 31.12.2008, p. 16.

⁴ 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, p. 1–295.

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA Scientific Committee, 2009) and following the relevant existing Guidance from the EFSA Scientific Committee.

The current 'Guidance for submission for food additive evaluations' (EFSA ANS Panel, 2012) has been followed by the ANS Panel for the evaluation of the application dossier.

3. Assessment

3.1. Technical data

3.1.1. Identity of the substance

Specific purity criteria for steviol glycosides (E 960) have been defined in the Commission Regulation (EU) No 231/2012. The current EU specifications for steviol glycosides (E 960) stipulate that a steviol glycoside preparation must contain not less than 95% of 11 named steviol glycosides: stevioside, rebaudiosides A, B, C, D, E, F and M, steviolbioside, rubusoside and dulcoside A.

The applicant asked to amend the existing EU specifications for steviol glycosides to allow for the inclusion of all steviol glycosides identified in *S. rebaudiana* Bertoni leaves, including both 'major' and 'minor' glycosides, that may comprise the assay value of not less than 95% total steviol glycosides.

The applicant stated that slight modifications can be applied at certain stages of the steviol glycoside production processes (e.g. extraction, purification), while still adhering to the same general manufacturing principles (hot-water extraction of *Stevia* leaves followed by isolation and step-wise purification using ion exchange resins and alcohol solvents). Therefore, it is possible to obtain steviol glycoside preparations with higher levels of 'minor' steviol glycosides than the levels present in currently available steviol glycoside products.

According to the applicant, all steviol glycoside constituents are glycosylated derivatives of the aglycone steviol and as such, all share the same backbone structure, differing only with respect to the type and number of glycoside units (glucose, rhamnose, xylose, fructose, deoxyglucose, arabinose, galactose and/or other sugar moieties) at positions R1 and R2 (Figure 1).

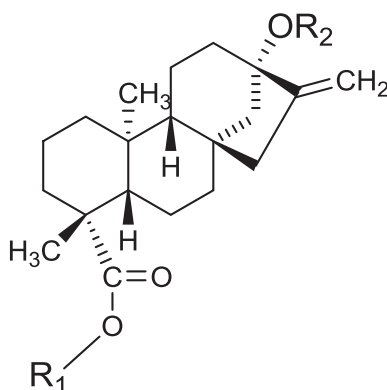


Figure 1: Structure for aglycone steviol

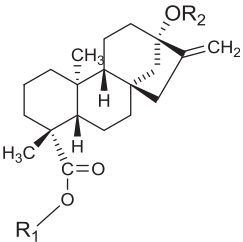
The results of an analytical study to isolate and characterise minor steviol glycosides in a *Stevia* leaf extract was provided; over forty steviol glycosides were elucidated by liquid chromatography–mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR) (Documentation provided to EFSA n. 1).

According to the applicant, steviol glycoside mixtures are slightly soluble in water.

3.1.2. Proposed specifications

The new proposed specifications for steviol glycosides from *S. rebaudiana* Bertoni are presented in Table 1.

Table 1: Specifications for steviol glycosides from *S. rebaudiana* Bertoni as proposed by the applicant (Documentation provided to EFSA n. 1)

E 960 STEVIOL GLYCOSIDES	
Synonyms	
Definition	The manufacturing process comprises two main phases: the first involving water extraction of the leaves of the <i>Stevia rebaudiana</i> Bertoni plant and preliminary purification of the extract by employing ion exchange chromatography to yield a steviol glycoside primary extract, and the second involving recrystallisation of the steviol glycosides from menthol or aqueous ethanol resulting in a final product comprising not less than 95% of steviol glycosides conjugated with glucose, xylose, rhamnose, fructose, deoxyglucose, arabinose, galactose and/or other sugar moieties, in any combination and ratio. The additive may contain residues of ion exchange resins used in the manufacturing process. Several other related steviol glycosides that may be generated as a result of the production process, but do not occur naturally in the <i>S. rebaudiana</i> plant have been identified in small amounts (0,10–0,37% w/w).
Structural formula	 <p>Where R1 and R2 can be one or more sugar moieties, including, but not limited to glucose, rhamnose, xylose, fructose, deoxyglucose, arabinose and galactose.</p>
Assay	Not less than 95% of total steviol glycosides on the dried basis
Description	White to light yellow powder, approximately between 200 and 350 times sweeter than sucrose (at 5% sucrose equivalency)
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 200 mg/kg methanol Not more than 5,000 mg/kg ethanol
Arsenic	Not more than 1 mg/kg
Lead	Not more than 1 mg/kg

The applicant has provided certificate of analysis of three non-consecutive batches for two different steviol glycosides mixtures – one containing > 50% rebaudioside D and the other > 50% rebaudioside A and < 10% rebaudioside D (Documentation provided to EFSA n.1).

No information was provided by the applicant on particle size, shape and distribution.

The Panel noted that neither microbiological parameters have been included nor limits for environmental contaminants as pesticides in the proposed specifications. The maximum levels proposed in the specifications for the solvents (methanol and ethanol) are much higher than the analytical data provided. Regarding toxic elements, only maximum limits are proposed for lead and arsenic while cadmium and mercury were also reported in the analysis provided. The maximum limits proposed for toxic elements are much higher compared to the values obtained in the five batches analysed. The Panel noted that these parameters should be added to the proposed specifications and that, based on the analytical results provided by the applicant, lower levels of the maximum limits for the heavy metals present as impurity, should be considered in the proposed specifications.

3.1.3. Manufacturing process

The crushed *Stevia* leaves are extracted with hot-water and the resulting extract is subjected to isolation and purification steps (by use of ion exchange chromatography, adsorption resins and alcohol

solvents). It is recognised that slight modifications can be applied at certain stages of the production process (e.g. extraction, purification), while still adhering to the same general principles, to obtain steviol glycoside preparations with specific distributions of individual steviol glycosides ('major' and 'minor'). In particular, the adsorption column system contains different sections that adsorb different proportions of steviol glycosides from a mixture. Because these different sections are desorbed separately with solvent, steviol glycoside mixtures with different ratios of individual steviol glycosides may be produced. This initial stage is followed by additional purification steps, including further and repeated recrystallisation and separation steps. This process results in a preparation that contains 95% total steviol glycosides consisting of one or more steviol glycosides conjugated with glucose, xylose, rhamnose, fructose, deoxyglucose, arabinose, galactose or other sugar moieties in any combination and ratio.

3.1.4. Methods of analysis in food

No method of analysis of steviol glycosides in foods was provided.

Information on a validated high-performance liquid chromatography (HPLC)/ultraviolet (UV)/MS assay method for quantitation of total steviol glycosides in *S. rebaudiana* Bertoni extracts containing a mixture of major and minor steviol glycosides was submitted (Documentation provided to EFSA n.1).

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

The submitted data demonstrated that the stability of steviol glycoside mixture is temperature-, time- and pH dependent.

According to applicant: 'stability data for rebaudioside A and stevioside in food matrices can be extrapolated to steviol glycosides in general, and the conclusions regarding the stability of steviol glycosides made by EFSA and other scientific bodies (that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions) can be extended to include the "major" and "minor" steviol glycosides present in the steviol glycoside mixtures that are the subject of this safety assessment'.

3.2. Proposed uses and use levels

Maximum level of steviol glycosides (E 960) are defined in Annex II to Regulation (EC) No 1333/2008⁵.

Steviol glycoside mixtures, containing both 'major' and 'minor' steviol glycosides, are proposed for use as high-intensity sweeteners in food and beverages under the same conditions as those already approved for steviol glycosides (E 960) in the EU. As the maximum permitted levels are expressed as steviol equivalents, the applicant proposed to utilise a single conversion factor of 0.33 for steviol glycoside mixtures since rebaudioside A is generally the predominant glycoside present in commercial steviol glycoside preparations; the value of 0.33 represents the median conversion factor of all 'major' steviol glycosides present, with the 'minor' glycosides typically representing much smaller amounts of the final preparation.

3.3. Exposure estimate

Because the proposed uses and use levels are the same as the already authorised food additive steviol glycosides (E 960), the applicant did not provide an exposure estimate for the purpose of this food additive amendment but made reference to the latest estimated exposure to E 960 (EFSA ANS Panel, 2015b).

3.4. Biological and toxicological data

3.4.1. Absorption, distribution, metabolism and excretion

In vitro incubation assays with pooled human fecal homogenates, using steviol glycosides already authorised as E 960 – rebaudiosides A, B, C, D, E, F and M, steviolbioside and dulcoside A – at two concentrations over 24–48 h was submitted within the application dossier (Purkayastha et al., 2016 (Documentation provided to EFSA n.3)). The authors concluded that the steviol glycosides tested in

⁵ 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, p. 16–33.

the different assays are hydrolysed to steviol at generally similar rates, with minor differences accounted for by the number of sugar moieties present.

A pilot study investigating the *in vitro* metabolism and hydrolysis of RAF, a mixture of steviol glycosides containing predominantly fructosylated steviol glycoside, in male and female pooled human fecal homogenate preparations at 37°C under anaerobic conditions was provided (BRI Biopharmaceutical Research Inc., 2015 (Documentation provided to EFSA n.2)). RAF was metabolised to steviol in each of the pooled human fecal homogenates (n = 3) with 100% molar equivalent formation of steviol as an end metabolite. Mass balance on the molar equivalent formation of steviol at 24 and 48 h was calculated based on the chemical composition of RAF containing 7% w/w of rebaudioside A and 93% fructosylated rebaudioside A. The Panel noted that it was not possible to assess the rate and extent of breakdown under realistic conditions from these data. Therefore, it was not possible to ascertain whether reliance on data on steviol were sufficient for assessing the risks of these steviol glycosides.

The toxicokinetic profiles of steviol and steviol glucuronide following administration of high-dose stevioside were determined in two groups of male and female Sprague–Dawley (CrI:CD(SD)) rats (72 animals/sex per dose group) (Roberts et al., 2016 (Documentation provided to EFSA n.4)). Animals were administered by gavage stevioside ($\geq 95\%$ purity) single oral doses of 40 or 1,000 mg/kg body weight (bw) (equivalent to 16 or 396 mg steviol equivalents/kg bw). Additionally, the pharmacokinetics of steviol and steviol glucuronide following consumption of stevioside were examined in an open-label, single dose trial in 10 healthy adult males between the ages of 20 and 45 years. The subjects were provided 40 mg/kg bw of stevioside (equivalent to 16 mg steviol equivalents/kg bw) in an aqueous solution. The Panel noted that these studies would only be relevant to the assessment if complete breakdown to steviol had been established under realistic conditions.

3.4.2. Toxicological data

No toxicity studies were submitted as part of this application (Documentation provided to EFSA n.1 and 2).

3.5. Discussion

The present evaluation is based on the data on steviol glycosides in a newly submitted dossier by the applicant (Documentation provided to EFSA n.1) and additional information submitted by the applicant during the assessment process in response to a request by EFSA (Documentation provided to EFSA n.5).

The applicant asked to amend the existing EU specifications for steviol glycosides to allow for the inclusion of all steviol glycosides identified in *S. rebaudiana* Bertoni leaves, including both 'major' and 'minor' glycosides, that may comprise the assay value of not less than 95% total steviol glycosides. This means that the food additive comprises not less than 95% of steviol glycosides conjugated with glucose, xylose, rhamnose, fructose, deoxyglucose, arabinose, galactose and/or other sugar moieties, in any combination and ratio.

The Panel noted that the proposed specifications can allow a food additive comprising any distribution of individual steviol glycosides extracted from *S. rebaudiana* Bertoni leaves and these specific preparations can be very different from the steviol glycoside preparations previously evaluated and authorised.

According to the applicant, all steviol glycosides are subject to microbial metabolism in a similar manner, ultimately generating the common primary metabolite steviol. Steviol is absorbed and metabolised to steviol glucuronide, which is excreted primarily via the urine in humans. Therefore, the studies conducted with rebaudioside A and stevioside and revised by the Panel in the assessment of steviol glycosides as a food additive (EFSA ANS Panel, 2010) can extend to other steviol glycosides due to the shared metabolic fate.

It would seem plausible that the metabolic fate of any steviol glycoside identified in the leaves of *S. rebaudiana* Bertoni would be comparable with the metabolic fate of those included in the current EU specifications for E 960. However, there are uncertainties on the rate and extent of the metabolism of different steviol glycosides to steviol in the evidence provided (Purkayastha et al. (2016) and BRI Biopharmaceutical Research Inc. (2015) (Documentation provided to EFSA n. 3 and 2)) and these did not allow the Panel to endorse the applicant's argumentation that all steviol glycosides generate the common metabolite steviol when subjected to microbial metabolism under realistic conditions (Documentation provided to EFSA n.1).

The available information was not sufficient to assess the safety of the proposed amendment of the specifications of the food additive steviol glycosides (E 960) and the conclusions on the previous assessments on steviol glycosides (EFSA ANS Panel, 2010, 2015a) cannot be extrapolated to any other mixture of steviol glycosides extracted from *S. rebaudiana* Bertoni leaves and complying with the proposed specification of containing not less than 95% of any steviol glycosides.

4. Conclusions

The Panel concluded that the submitted data were insufficient to assess the safety of proposed amendment of the specifications of the food additive steviol glycosides (E 960).

Documentation provided to EFSA

- 1) Dossier 'Application for a Change in the Steviol Glycoside Specification to Expand the List of Steviol Glycosides in the European Union to all Those Identified in the Leaves of *Stevia rebaudiana* Bertoni'. Submission on 27 March 2017. Missing information submitted on 14 June 2017.
- 2) BRI Biopharmaceutical Research Inc., 2015. Pilot *In vitro* metabolism of RAF in male and female pooled human intestinal fecal homogenate under physiological anaerobic conditions [Unpublished&Confidential]. (BRI Study No.: PUR-2015-002). Submitted within documentation provided to EFSA n.1.
- 3) Purkayastha S, Markosyan A, Prakash I, Bhusari S, Pugh G Jr, Lynch B and Roberts A, 2016. Steviol glycosides in purified stevia leaf extract sharing the same metabolic fate. *Regulatory Toxicology and Pharmacology: RTP*, 77, 125–133. Submitted within documentation provided to EFSA n.1.
- 4) Roberts A, Lynch B, Rogerson R, Renwick A, Kern H, Coffee M, Cuellar-Kingston N, Eapen A, Crincoli C, Pugh G, Bhusari S, Purkayastha S and Carakostas M, 2016. Chemical-specific adjustment factors (inter-species toxicokinetics) to establish the ADI for steviol glycosides. *Regulatory Toxicology and Pharmacology: RTP*, 79, 91–102. Submitted within documentation provided to EFSA n.1.
- 5) Additional information. 12 December 2017. Submitted by PureCircle in response to a request from EFSA.

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Abbreviations

ANS	EFSA Panel on Food Additives and Nutrient Sources added to Food
bw	body weight
HPLC	high-performance liquid chromatography
FAO	Food and Agriculture Organization of the United Nations

JECFA Joint FAO/WHO Expert Committee on Food Additives
LC-MS liquid chromatography–mass spectrometry
NMR nuclear magnetic resonance
UV ultraviolet
WHO World Health Organization