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## Safety evaluation of glucosylated steviol glycosides as a food additive in different food categories

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### Abstract

The EFSA Panel on Food Additive and Flavourings (FAF) assessed the safety of glucosylated steviol glycosides proposed for use as a new food additive in different food categories. Glucosylated steviol glycosides consist of a mixture of glucosylated steviol glycosides, containing 1–20 additional glucose units bound to the parent steviol glycosides. Glucosylated steviol glycosides consist of not less than 95% (on dry, dextrin-free, basis) of total steviol glycosides, comprised of glucosylated and parent steviol glycosides. Glucosylated steviol glycosides are produced via enzymatic bioconversion using cyclomaltodextrin glucanotransferase (CGTase) (EC 2.4.1.19), derived from a non-genetically modified strain of *Anoxybacillus caldiproteolyticus*, that catalyses the transfer of glucose from starch to steviol glycosides mixtures isolated from the dried leaves of *Stevia Rebaudiana*. The Panel considered that the metabolism of glucosylated steviol glycosides is sufficiently similar to the already authorised steviol glycosides, and thus, the toxicological data previously assessed by the ANS Panel for steviol glycosides (E 960) were considered to support their safety as food additive. The existing acceptable daily intake (ADI) for steviol glycosides (E 960) of 4 mg/kg body weight (bw) per day expressed as steviol can also be applied to glucosylated steviol glycosides. The Panel concluded that there is no safety concern for the use of glucosylated steviol glycosides as a new food additive at the proposed use and use levels. The Panel recommended some modifications to the specifications proposed by the applicant for glucosylated steviol glycosides with respect to the assay, the definition of the proposed new food additive and the proposed maximum limits for arsenic.

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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 Flavourings (FAF) was asked to provide a scientific opinion on the safety of safety in use of glucosylated steviol glycosides as a food additive in different food categories, 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 either spontaneously or following request from EFSA.

The EFSA Panel on Food Additives and Nutrient Sources added to Food (EFSA ANS Panel) had previously evaluated the safety of glucosylated steviol glycosides to be used as a food additive and concluded that the submitted data were insufficient to assess its safety (EFSA ANS Panel, 2018).

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) (expressed as steviol equivalents) per day was established.

Glucosylated steviol glycosides' preparations were described by the applicant as a mixture of glucosylated steviol glycosides, containing 1–20 additional glucose units bound to the parent steviol glycoside via  $\alpha$ -(1–4) linkages. The Panel noted that the definition of the food additive in the proposed specifications does not include any value for the additional glucose units and that the mixtures on which data have been provided contain, on average, only three or four additional glucose units. The Panel made a conservative assumption that the GSG may contain up to 10 additional glucose units on average in its evaluation and considered that information on the number of additional glucose units (1–20) should be added to the proposed definition of the food additive in the specifications.

Glucosylated steviol glycosides' preparations consist of not less than 95% of the total steviol glycosides comprised of glucosylated steviol glycosides and parent steviol glycosides on the dried, dextrin-free, basis. The glucosylated fraction of the total steviol glycosides is between 80% and 92% w/w and the parent steviol glycosides between 5% and 15% w/w. Typical components of glucosylated steviol glycosides are presented in Appendix A as proposed by the applicant.

The Panel considered that rather than having a non-definite list of components, as proposed by the applicant, the assay value should cover all steviol glycosides falling under the definition of E 960a along with their glucosylated derivatives.

The proposed use and use levels for glucosylated steviol glycosides are the same as those for the already authorised steviol glycosides (E 960a–960c). Taking into account that glucosylation of the steviol glycosides results in higher molecular weights, the glucosylated steviol glycosides have a lower steviol equivalency compared to steviol glycosides E 960a. Therefore, a larger quantity of glucosylated steviol glycosides would be permitted to be added to food to give the same steviol equivalent value. Consequently, the resulting exposure to potential impurities, including toxic elements, has taken into account this higher addition levels (on a mass basis and not on a steviol equivalent basis). With the exception of arsenic, for which the lowest calculated margin of safety (MOS)/margin of exposure (MOE) was considered to be insufficient, for the other toxic elements (cadmium, mercury, lead) proposed for inclusion in the specifications the maximum limits do not give rise to safety concerns.

The Panel noted that the proposed glucosylated steviol glycosides' preparations may contain up to 20 glucose moieties per molecule of steviol glycoside and this could lead to an additional exposure to glucose from a sweetener which is proposed to have a technological function of replacing sugars in food. Based on a worst-case assumption of an average of 10 additional glucose units added to the steviol glycosides, the Panel estimated that the proposed uses and use level of the new food additive glucosylated steviol glycosides would lead to an additional glucose intake of up to 0.4 g/day for toddlers and 1.2 g/day for adults.

Glucosylated steviol glycosides are prepared in two production stages. Steviol glycosides mixtures – enriched individual steviol glycosides (e.g. high Rebaudioside A or Rebaudioside D content (> 95%)) or mixtures enriched in two or more individual steviol glycosides – are isolated from dried leaves of *S. rebaudiana* by hot water extraction and different purifications steps. The purified steviol glycosides extract undergoes enzymatic treatment to achieve glucosylation via  $\alpha$ -(1-4) linkages of the steviol glycosides. A glucose donor, such as tapioca starch (extracted from Cassava roots), is treated with the enzyme CGTase (EC 2.4.1.19), derived from a non-genetically modified strain of *Anoxybacillus caldiproteolyticus*. CGTase (EC 2.4.1.19) is also used to catalyse the intermolecular glucosylation whereby the transfer occurs of  $\alpha$ -glucosyl units from the starch onto the 4-hydroxy position of a

glucosyl moiety on the steviol glycoside (trans- $\alpha$ -1,4-glucosylation). The enzyme is inactivated by heating and removed by treatment with activated carbon. The dextrin by-product can be removed from the mixture.

The Panel noted that the safety of the enzyme CGTase (EC 2.4.1.19) derived from a non-genetically modified strain of *Anoxybacillus caldiproteolyticus* and intended to be used in the preparation of glucosylated steviol glycosides has been assessed by the EFSA CEP Panel. Based on the data provided, and the removal of food enzyme total organic solids (TOS) during the manufacture of modified steviol glycosides, the CEP Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use (EFSA CEP Panel, 2022).

Glucosylated steviol glycosides contain  $\alpha$ -oriented glycosidic bonds which are hydrolysed by digestive enzymes (i.e. salivary and/or pancreatic  $\alpha$ -amylase) resulting in the parent steviol glycosides. Therefore, the glucosylated steviol glycosides share the same metabolic fate as steviol glycosides extracted from *S. rebaudiana* Bertoni, i.e. they undergo hydrolysis forming steviol as demonstrated in an in vitro study submitted in support of the current application. The results of this study with human faecal homogenates showed an extensive microbial degradation of mono- to tetra-glucosylated steviol glycosides to steviol at similar rates as Rebaudioside A.

Overall, the Panel considered that the metabolism of glucosylated steviol glycosides is sufficiently similar to the already authorised steviol glycosides, and thus, the toxicological data previously assessed by the ANS Panel for steviol glycosides (E 960a) were considered to support their safety as food additive. Therefore, no additional toxicological data were required.

The existing ADI for steviol glycosides (E 960a) of 4 mg/kg bw per day expressed as steviol can also be applied to glucosylated steviol glycosides.

The Panel concluded that there is no safety concern for the use of glucosylated steviol glycosides as a new food additive at the proposed use and use levels.

The Panel recommended that the specifications proposed by the applicant for glucosylated steviol glycosides would include the following modifications:

- The assay value of more than 95% for total steviol glycosides, comprised of glucosylated and parent steviol glycosides, on the dried, dextrin-free, basis should be limited to those 11 named steviol glycosides that are included in the current definition of E 960a along with their glucosylated derivatives (1–20 added glucose units).
- The description of the food enzyme used in the manufacturing process should be aligned to the description given in the CEP Panel opinion, i.e. 'Cyclomaltodextrin glucanotransferase (EC 2.4.1.19) derived from a non-GMO strain of *Anoxybacillus caldiproteolyticus* St-88'.
- The proposed maximum limit for arsenic should be lowered.

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## 1. Introduction

The present scientific opinion deals with the safety of glucosylated steviol glycosides proposed for use as a new food additive in different food categories.

### 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.<sup>1</sup> 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.

In 2017 the European Commission received a request for authorisation of the use of Glucosylated steviol glycosides as a sweetener in several food categories. Glucosylated steviol glycosides are manufactured by adding glucose units (between 1 to 10 additional units) to steviol glycosides extracted from the leaves of *Stevia rebaudiana*. This is achieved using enzymes that transfer glucose units from a starch source to steviol glycosides, and results in the production of a mixture of glucosylated (~80–92%) and non-modified parent steviol glycosides (~5–15%). The request was assessed by EFSA which concluded that the submitted data were insufficient to assess the safety of the glucosylated steviol glycoside preparations to be used as new food additives (EFSA ANS Panel, 2018).

In January 2019 the European Commission received an updated application containing additional information, which according to the applicant, should address the concerns and questions raised in the EFSA's scientific opinion (EFSA ANS Panel, 2018).

#### 1.1.2. Terms of Reference

The Commission requests the European Food Safety Authority (EFSA) to provide a scientific opinion on the safety in use of glucosylated steviol glycosides as food additive in different food categories in accordance with the Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.<sup>2</sup>

### 1.2. Information on existing authorisations and evaluations

An application for the proposed use of glucosylated steviol glycosides as a new food additive had been previously evaluated by the former EFSA ANS Panel in 2018. At that time, the ANS Panel concluded that the data submitted in support of the new application were insufficient to assess its safety (EFSA ANS Panel, 2018).

Steviol glycosides from *Stevia* (E 960a)<sup>3</sup> are an authorised food additive in the EU according to Regulation (EC) No 1333/2008 on food additives. The food additive is obtained from water extraction of the leaves of the *Stevia rebaudiana* Bertoni plant and according to the specifications defined in Commission Regulation (EU) No 231/2012<sup>4</sup>. It is described as containing not less than 95% of the identified 11 related steviol glycosides steviolbioside, rubusoside, dulcoside A, stevioside, rebaudiosides A, B, C, D, E, F and M on the dried basis, in any combination and ratio.

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

<sup>1</sup> 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.

<sup>2</sup> Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008.

<sup>3</sup> Since receipt of the present mandate, Regulation (EC) No 1333/2008 was amended by Commission Regulation (EU) 2021/1156 of 13 July 2021 replacing the entry for E 960 (Steviol glycosides) with two separate entries for 'steviol glycosides from *Stevia* (E 960a)' and 'enzymatically produced steviol glycosides (E 960c)'. Accordingly, in the present opinion, the Panel has replaced reference to E 960 with E 960a or E 960c, whenever appropriate.

<sup>4</sup> 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.



specifications for steviol glycosides (E 960a). The latest exposure assessment to steviol glycosides (E 960) was carried out by the ANS Panel in 2015 (EFSA ANS Panel, 2015b).

A new entry for 'enzymatically produced steviol glycosides (E 960c)' was added to Annex II to Regulation (EC) No 1333/2008 as amended by Commission Regulation (EU) 2021/1156 of 13 July 2021<sup>5</sup>. This amendment to the Regulation is based on the conclusions from EFSA on the safety of a proposed amendment of the specifications of the food additive steviol glycosides (E 960) concerning rebaudioside M produced by enzyme modification of steviol glycosides, using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffii* UGT-a and *K. phaffii* UGT-b (EFSA FAF Panel, 2019). Regulation (EU) No 231/2012 was also amended accordingly, with the inclusion of a new entry for 'E 960c(i) Rebaudioside M produced via enzyme modification of steviol glycosides from Stevia'.

Assessment of a new application requesting an amendment of the specifications of steviol glycosides in order to include the 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, was completed by the FAF Panel (EFSA FAF Panel, 2021).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established an ADI for steviol glycosides of 0–4 mg/kg bw per day, expressed as steviol (JECFA, 2009).

In 2016, JECFA confirmed that rebaudioside A from multiple gene donors<sup>6</sup> expressed in *Yarrowia lipolytica* is included in the ADI of 0–4 mg/kg bw, expressed as steviol. JECFA has prepared new specifications for Rebaudioside A from Multiple Gene Donors Expressed in *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 issued new specifications for 'Steviol Glycosides from *Stevia rebaudiana* Bertoni' that consist of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose and deoxyglucose) in any of the orientations occurring in the leaves of *S. rebaudiana* Bertoni, provided that the total percentage of steviol glycosides is not less than 95% (JECFA, 2017). These specifications have been superseded in 2019 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 (FAO and WHO, 2020).

The framework adopted in 2019 has been subsequently amended by JECFA at its 91st meeting in February 2021. Specifications for steviol glycosides manufacturing using four different methods have been established, including specifications for 'Enzyme modified glucosylated Steviol Glycosides' (FAO and WHO, 2021).

## 2. Data and methodologies

### 2.1. Data

The present evaluation is based on the data submitted in the application dossier ('Documentation provided to EFSA' No 1) and additional information submitted by the applicant during the assessment process either spontaneously (Documentation provided to EFSA No 2) or following request by EFSA ('Documentation provided to EFSA' No 3-5). A clarification meeting during risk assessment was held on 06 December 2019.

### 2.2. Methodologies

This opinion was formulated following the principles described in the EFSA Guidance 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 Guidance for submission for food additive evaluations (EFSA ANS Panel, 2012) was followed for the assessment.

<sup>5</sup> Commission Regulation (EU) 2021/1156 of 13 July 2021 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council and the Annex to Commission Regulation (EU) No 231/2012 as regards steviol glycosides (E 960) and rebaudioside M produced via enzyme modification of steviol glycosides from Stevia. OJ L 249, 14.7.2021, p. 87–98.

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

### 3. Assessment

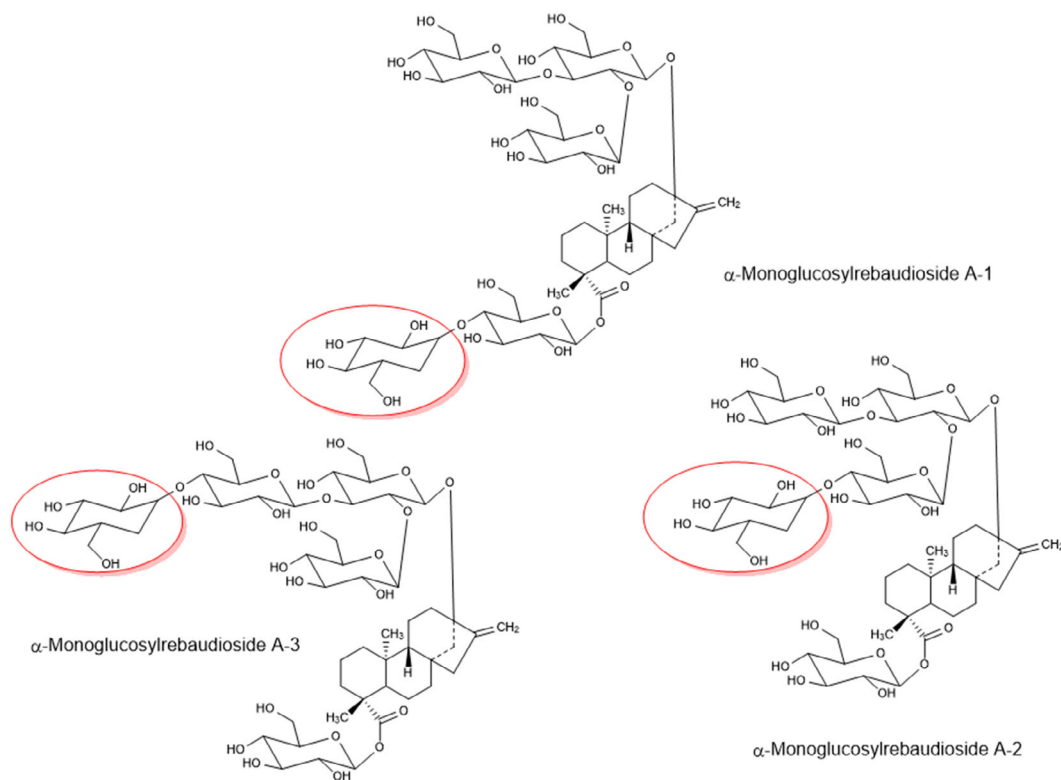
#### 3.1. Technical data

##### 3.1.1. Identity of the proposed food additive

Glucosylated steviol glycoside preparations were described by the applicant as being comprised of a mixture of glucosylated steviol glycosides, containing 1–20 additional glucose units bound to the parent steviol glycoside.

The purified steviol glycosides ( $\geq 95\%$ ), extracted from the leaves of the *Stevia rebaudiana* Bertoni plant, are enzymatically modified such that additional glucose moieties are conjugated to the parent steviol glycosides structure via  $\alpha$ -(1–4) linkages. Glucosylated steviol glycoside preparations consist of not less than 95% total steviol glycosides, made up of any combination of glucosylated steviol glycosides of different molecular weights as well as any remaining unreacted steviol glycosides (parent steviol glycosides). According to the applicant, the percentage of steviol glycosides containing additional molecules of glucose is between 80% and 92% w/w and the parent glycosides between 5% and 15% w/w (Documentation provided to EFSA n. 1, 4).

According to the applicant, all constituents of glucosylated steviol glycosides share the same steviol backbone structure. As an example, the representative chemical structures of a glucosylated steviol glycoside are depicted in Figure 1.



**Figure 1:** Representative structures of  $\alpha$ -monoglucosylated rebaudioside A

According to the applicant, glucosylated steviol glycoside preparations are white to off-white powders that have a clean taste with a mild odour.

According to the applicant, glucosylated steviol glycosides are soluble in water as determined by the standard JECFA method (JECFA, 2006).

Information on the composition of three preparations depending on the composition of the starting steviol glycosides was included in the application dossier:

- 1) from steviol glycoside starting material of  $\geq 95\%$  rebaudioside A (giving product GSG-RA);
  - 2) from steviol glycoside starting material of  $\geq 95\%$  rebaudioside D (giving product GSG-RD);
- and



- 3) from a mixed steviol glycoside starting material of  $\geq 95\%$  total steviol glycosides (giving product GSG-SG95).

The applicant stated that the parent steviol glycoside and glucosylated steviol glycoside content was characterised in accordance with the Japanese Ministry of Health, Labour and Welfare (MHLW) assay for  $\alpha$ -glucosyltransferase-treated stevia (MHLW, 2009) and the JECFA method for measuring steviol glycosides (JECFA, 2008).

Description of an adsorption chromatography and HPLC method to determine the concentration of steviol glycosides, residual dextrin and unreacted steviol glycosides was provided (Documentation provided to EFSA n. 1). Adsorption chromatography is used for separating and quantifying the dextrin and steviol glycoside fractions. The adsorption chromatographic method also helps in estimating the total amount of steviol glycosides (as the percentage of glucosylated steviol glycoside components and residual parent steviol glycosides). HPLC analysis is used for the determination of total parent steviol glycosides (by summation of the different steviol glycoside components). The total amount of glucosylated steviol glycosides is calculated by the difference between the total amount of steviol glycosides, estimated by adsorption chromatography and the total parent steviol glycosides calculated by HPLC. The Panel noted that this is the method indicated in the current JECFA specifications (FAO and WHO, 2021) to calculate total glucosylated steviol glycosides and unreacted parent steviol glycosides.

The total glucosylated steviol glycosides amount may also be quantified using a method that employs a two-stage process involving the analysis of parent steviol glycosides and glucose content separately (MHLW, 2009). This is achieved by treating the glucosylated steviol glycoside sample with glucoamylase to cleave the added  $\alpha$ -glucosyl residues from the parent steviol glycosides. The  $\alpha$ -glucosyl residues liberated are quantified through the determination of glucose using D-glucose as standard and the steviol glycosides content is also measured. The total content of glucosylated and unreacted steviol glycosides is obtained as the sum of the content of steviol glycosides and  $\alpha$ -glucosyl residues.

According to the applicant, the approach including adsorption chromatography is preferred because it estimates the total steviol equivalency of a glucosylated steviol glycoside preparation which is needed to estimate the use of glucosylated steviol glycosides in different food and beverage applications, where the additive level is defined/expressed as steviol equivalents.

The applicant provided a description of how steviol equivalence is calculated based on the compositional analysis of the glucosylated mixture (by the absorption chromatography/HPLC method) along with a correction for the molecular weight of the glucosylated steviol glycoside components in comparison with the MW of steviol itself (mw = 318). An example was provided for this compositional analysis and calculation, for a glucosylated mixture named 'GSG-RA'. This was made from a starting material of  $\geq 95\%$  rebaudioside A, showing that the average degree of glucosylation of this GSG-RA was 2.95 additional glucose units. The steviol equivalency for this GSG-RA mixture was reported as 0.222, meaning that 4.50 g of the GSG-RA mixture was equivalent to (would 'yield') 1 g of steviol (Documentation provided to EFSA n. 4).

The Panel noted that, in the absence of analytical standards for all the different glycosylated steviol glycosides (including all of the possible regioisomers), this method can only provide semiquantitative results.

Using this procedure, the five batches of GSG-RA were analysed along with one batch each of GSG-RD and GSG-SG95. The percentage of mono-, di-, tri-, tetra- and higher (i.e. 5+, the sum of n = 5 to 20 units) glucosylated steviol glycosides was reported. The total content of glucosylated steviol glycosides ranged from 91.3% to 92.6%. It was noted that the pattern of glucosylated steviol glycosides differed, depending on the starting material used and presumably the exact manufacturing conditions too. The pattern was rather similar for GSG-RA (manufactured from Reb A) and for GSG-SG95, most likely because the mixed SG95 starting material used for GSG-SG95 was predominantly Reb A. For both GSG-RA and GSG-SG95, the distribution of the glucosylated products was ca. mono- (20%), di- (19%), tri- (16%), tetra- (13%) and n = 5–20 (24%). For the product SGS-RD (manufactured from Reb D), the glucosylation was clearly much more extensive and the distribution of the glucosylated products was mono (10%), di- (11%), tri- (8%), tetra- (19%) and n = 5–20 (45%). Adding in the percentage of unmodified parent steviol glycosides (Reb A, stevioside, Reb F, Reb C and Reb D were assayed) resulted in a total content of all steviol glycosides (glucosylated steviol glycosides plus parent steviol glycosides) ranging from 98.1% to 99.3% for the three products described (Documentation provided to EFSA n. 1).

Residual dextrin may arise from the use of starch in the manufacturing process for glucosylated steviol glycosides (see Section 3.1.3). Upon request from EFSA, the applicant declared that the use of adsorption/desorption column, foreseen in the manufacturing process, would remove dextrans (remaining less than 4%) from the reaction mixture. The applicant also indicated that in some cases for commercial and/or formulation purposes, a higher amount of dextrans may remain in the final product. In this respect, the applicant provided data from an LC-ESI-MS analysis of the dextrin fraction isolated from a glucosylated steviol glycoside sample. Using  $\alpha$ -cyclodextrin (cyclomaltohexaose),  $\beta$ -cyclodextrin (cyclomaltoheptaose) and  $\gamma$ -cyclodextrin (cyclooctapentylose) as reference materials, the analysis showed a mean content of total cyclodextrans in the analysed sample of 7.1% (BRI, 2021 in Documentation provided to EFSA n. 5). From additional data submitted by the applicant in its response to EFSA, six batches of glucosylated steviol glycosides showed residual levels of dextrin ranging from 1.72% up to 11.22% (Documentation provided to EFSA n. 5).

### 3.1.2. Specifications

The specifications for glucosylated steviol glycosides' preparations as proposed by the applicant are presented in Table 1.

**Table 1:** Specifications for glucosylated steviol glycosides' preparations as proposed by the applicant (Documentation provided to EFSA n. 5)

<b>GLUCOSYLATED STEVIOL GLYCOSIDES</b>	
<b>Synonyms</b>	
<b>Definition</b>	Glucosylated steviol glycosides (GSG) are a mixture of larger glycosides of steviol derived by glucosylation of steviol glycosides extracted from leaves of <i>Stevia rebaudiana</i> Bertoni plant. Glucosylated steviol glycosides are composed of glucosylated steviol glycosides and residual parent steviol glycosides from Stevia leaf. Glucosylated steviol glycosides are produced by treating the steviol glycosides, extracted from Stevia leaf, and starch with Cyclomalto-dextrin glucanotransferase (EC 2.4.1.19) enzyme derived from non-toxicogenic, non-pathogenic and non-GMO strain of <i>Anoxybacillus caldiproteolyticus</i> St-88. The enzyme transfers glucose units from starch to the steviol glycosides. The resulting material is heated and treated with activated carbon to remove the enzyme, then passed through adsorption/desorption resin to remove residual hydrolysed starch (dextrin), followed by purification and preparation of the product of commerce using processes that may include decolourisation, concentration and spray drying.
Chemical name, molecular formula and molecular weight	Separate table (Appendix A) for the chemical information for some example glucosylated steviol glycosides. Note: This list is not exhaustive.
Assay	Not less than 95% of total steviol glycosides, comprised of glucosylated and parent glycosides, on the dried, dextrin-free, basis.
<b>Description</b>	White to off-white powder, approximately 100–200 times sweeter than sucrose (at 5% sucrose equivalency).
Solubility	Soluble in water
pH	Between 4.5 and 7.0 (1 in 100 solution)
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 3,000 mg/kg ethanol
Arsenic	Not more than 0.1 mg/kg
Lead	Not more than 0.1 mg/kg
Cadmium	Not more than 0.1 mg/kg
Mercury	Not more than 0.1 mg/kg
<b>Microbiological criteria</b>	
Total (aerobic) plate count	Not more than 1,000 CFU/g
Yeast and moulds	Not more than 200 CFU/g

## GLUCOSYLATED STEVIOL GLYCOSIDES

<i>E. coli</i>	Negative in 1 g
<i>Salmonella</i>	Negative in 25 g

The Panel noted that according to the definition proposed by the applicant (Table 1), the solvent used for the extraction from the stevia leaves is not specified although in Section 3.1.3 the applicant describes a hot water extraction process. The Panel also noted that the primary stevia extract starting material used to manufacture glucosylated steviol glycosides is E 960a and therefore will comply with the current specifications in the EU for steviol glycosides (E 960a).

In the definition of Table 1, it is proposed by the applicant that the assay value should be not less than 95% of total steviol glycosides, comprised of glucosylated and parent steviol glycosides, on a dried, dextrin-free, basis. These components, as provided by the applicant, are illustrated in Appendix A, but no definitive or exhaustive list was proposed.

The Panel noted that the assay value of more than 95% for total steviol glycosides (combination of glucosylated steviol glycosides and unreacted steviol glycosides) should be limited to those 11 named steviol glycosides that are included in the current definition of E 960a along with their glucosylated ( $n = 1-20$ ) derivatives. The Panel noted that dextrin is a by-product of the manufacturing process, and in some cases, it is not removed from the final glucosylated steviol glycosides mixture. The Panel noted that the applicant refers to 'dextrin-free' glucosylated steviol glycosides for the purity assay of the proposed food additive (not less than 95% of total steviol glycosides, comprised of glucosylated and parent glycosides) and that the final product typically contain between 1% and 4% by weight of dextrin or even higher amounts as evidenced by the data submitted (up to 11.22%, see Section 3.1.1).

The Panel noted that according to the description provided in the application dossier, the glucosylated fraction will be approximately 80–92%; analytical data showed that the content of glucosylated steviol glycoside in the analysed samples range from 90.4% to 92.5%.

The Panel noted that the maximum levels proposed in the specifications for the solvents used in the production process (methanol and ethanol) are much higher than the analytical data provided.

Regarding toxic elements, the Panel noted that analytical data on the content of arsenic, lead, cadmium and mercury were provided for five batches of glucosylated steviol glycosides (Documentation provided to EFSA n. 1). For lead, the five batches were in the range of 0.019–0.054 mg/kg. For arsenic, cadmium and mercury, all five batches were < 0.005 mg/kg. The Panel considered the proposed maximum limits for toxic elements (Table 1) at 0.1 mg/kg for each element are consistent with the analytical data provided for lead. However, the proposals for arsenic, cadmium and mercury do not seem to be based on the lowest technologically achievable levels since the three elements in all five batches were < 0.005 mg/kg. The anticipated impact of these proposed specifications on the potential exposure to these elements is described in Section 3.3.1 (Table 3).

**Table 2:** Risk characterisation for toxic elements based on their maximum limits proposed by the applicant for the specifications of glucosylated steviol glycosides (Documentation provided to EFSA n. 5) for two different examples of glucosylated steviol glycosides' preparations differing in the average number of added glucose units

Exposure to proposed food additive expressed as steviol equivalents (mg/kg bw per day) <sup>(a)</sup>	Average number of added glucose units in GSG	Conversion factor	Corresponding exposure to GSG (mg/kg bw per day)	MOS/MOE for as at 0.1 mg/kg	MOS/MOE for Pb at 0.1 mg/kg	%age of the TWI for Cd at 0.1 mg/kg	%age of the TWI for Hg at 0.1 mg/kg
4.3	3 <sup>(b)</sup>	0.222 <sup>(b)</sup>	19.36	155–4132	258	0.54%	0.34%
	10 <sup>(c)</sup>	0.123 <sup>(c)</sup>	34.96	86–2288	143	0.98%	0.61%

bw: body weight; GSG: glucosylated steviol glycosides.

(a): Estimated exposure using MPLs and the proposed extension of use (toddlers, 95th percentile), expressed as steviol equivalents 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).

(b): Conversion factor for GSG-RA mixture as provided by the applicant (Documentation provided to EFSA n. 4).

(c): Theoretical conservative assumption by the Panel.

The Panel noted that according to the proposed specifications, starch is used as source of glucose. Despite not being stated, it is expected that starch is of a quality suitable for human consumption.

### 3.1.3. Manufacturing process

Glucosylated steviol glycosides' preparations are prepared in two production stages.

1. Extraction and purification of steviol glycosides from the dried leaves of *S. rebaudiana* Bertoni

Steviol glycosides are isolated from dried leaves of *S. rebaudiana* by hot water extraction (50–60°C for 1–2 h). The filtered extract is treated with a flocculant (calcium hydroxide) to remove co-extracted materials (i.e. proteins, polysaccharides, coloured components). The resulting precipitate is filtered out and the filtrate, containing the steviol glycosides, is deionised by ion exchange resins, and then, it undergoes a series of purification and concentration steps (macroporous adsorption resins with different sections, activated carbon, ion-exchange resins). Finally, the concentrate is spray-dried to yield a stevia extract in powder form with a total steviol glycoside content of not less than 95%. Steviol glycoside blends may also be subjected to additional crystallisation steps (in ethanol), in order to obtain enriched individual steviol glycosides (e.g. high Reb A or Reb D content (> 95%)) or mixtures enriched in two or more individual steviol glycosides. According to the proposed specifications (Table 1), the purified extract should meet the specifications of E 960a.

2. Glucosylation of the purified steviol glycosides via enzymatic treatment in the presence of a glucose source

The purified extract undergoes enzymatic treatment to achieve glucosylation via  $\alpha$ -(1-4) linkages of the steviol glycosides. In order to generate glucose units, a glucose donor such as tapioca starch (extracted from Cassava roots) is treated with CGTase (EC 2.4.1.19), derived from a non-genetically modified strain of *Anoxybacillus caldiproteolyticus*, to hydrolyse and transfer glucose units from starch to the steviol glycoside molecule (EFSA CEP Panel, 2022).

The purified steviol glycosides' extract is mixed with the liquified tapioca starch along with the CGTase. The reaction mixture is incubated at 60°C for 48 h to generate glucosylated steviol glycosides and dextrin (residual hydrolysed starch). The enzyme is then inactivated by heating at 100°C for 15 min and removed in the subsequent purification steps.

The residual dextrin is mainly removed from the reaction mixture by the use of an adsorption/desorption column that can remove all dextrin to a typical lower level of up to 4% by weight (Documentation provided to EFSA n.5).

The mixture then undergoes a series of purification and concentration steps using the same ion exchange and adsorption resins that are employed at stage 1. The refined solution is spray-dried and the dry product is powdered, sifted and packed.

Ethanol is used as a crystallisation solvent (stage 1) and as a column desorption solvent (both stage 1 and stage 2).

The Panel noted that the safety of the enzyme CGTase (EC 2.4.1.19) derived from a non-genetically modified strain of *Anoxybacillus caldiproteolyticus* and intended to be used in the preparation of glucosylated steviol glycosides has been assessed by the EFSA Panel on Food Contact Materials, Enzymes and Processing Aids Panel Members (CEP Panel). Based on the data provided, and the removal of food enzyme TOS during the manufacture of modified steviol glycosides, the CEP Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use (EFSA CEP Panel, 2022).

### 3.1.4. Methods of analysis in food

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

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

A storage stability test with three samples of glucosylated steviol glycoside preparations (GSG-RA, GSG-RD and GSG-SG95) was submitted. The three samples were stored under accelerated storage conditions (40°C and 75% relative humidity) for up to 4 weeks (GSG-RA) or 8 weeks (the other two samples). Overall, the total steviol glycoside content did not change by more than 1% although the precise distribution of glucosylated and parent steviol glycosides did vary slightly over time, depending on the product type. Therefore, the total steviol glycoside content was considered to remain stable for at least 8 weeks at 40°C and 75% RH (PureCircle Sdn Bhd, 2015a in Documentation provided to EFSA n. 1).

One sample of GSG-RA was tested for hydrolytic stability at 50 g/L over a pH range of 2.0–8.0 and held for up to 7 weeks at 5°C, 25°C, 37°C and 56°C. The reported data shown that degradation of GSG-RA is pH, temperature and time dependent. The samples were generally stable over 7 weeks at pH 4.0–8.0 at all four temperatures tested. Significant degradation was observed at pH 2 and 3 stored at 56°C for 7 weeks, with ca. 30% and 22% loss, respectively. No analysis of the resulting degradation products has been provided (PureCircle Sdn Bhd, 2015b in Documentation provided to EFSA 1).

The applicant provided the following statement in the dossier:

*'One may infer that the degradation of steviol glycosides and glucosylated steviol glycosides at low pH and high temperature follows the same mechanism. At low pH and high temperature, rebaudioside A undergoes hydrolysis and generates different steviol glycosides and glucose (Prakash et al., 2012). Wölwer-Rieck et al. (2010) showed that stevioside (SvG3) and rebaudioside A (SvG4) in soft drinks (approximately pH 3) stored at 80°C for 72 h, generate smaller steviol glycosides by successive elimination of glucose units. Rebaudioside A or stevioside spiked in a soft drink and stored at 80°C generated rebaudioside B (SvG3), steviolbioside (SvG2), rubusosides (SvG2), steviol monoside (SvG1), but no steviol. The glucosylated steviol glycoside molecules are expected to generate smaller steviol glycosides with fewer glucose units'.*

Although not conclusive, the Panel found this explanation to be reasonable. The Panel noted, however, that the relevance of this last study to the stability of glucosylated steviol glycosides was limited due to the short heating period and temperature range employed (e.g. the latter not being representative of baking processes).

According to the applicant, it is expected that the stability of glucosylated steviol glycosides would be similar to that of individual steviol glycosides given their similarities in structure. The Panel concurred with this view.

### 3.2. Proposed uses and use levels

According to the applicant, the glucosylated steviol glycoside preparations are proposed for use as a high-intensity sweetener in the same manner as currently authorised for steviol glycosides (E 960) as defined in Annex II to Regulation (EC) No 1333/2008 (Documentation provided to EFSA n. 1).

The proposed permitted levels for glucosylated steviol glycosides are expressed on a steviol equivalent basis in the same way as for the already authorised food additive steviol glycosides (E 960).

The procedure and the factors used for estimation of the steviol equivalents were described by the applicant. As an example, a factor of 0.222 for converting a preparation of GSG-RA into steviol equivalent was reported. The applicant stated that using this approach, the content of steviol equivalent in each type of commercial preparation of glucosylated steviol glycosides is calculated (Documentation provided to EFSA n.4).

The Panel noted that this estimation requires full quantification of the different components of the glucosylated steviol glycosides preparations.

### 3.3. Exposure data

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

The Panel considers that if steviol glycosides would be replaced by glucosylated steviol glycosides, exposure to steviol glycosides (expressed as steviol equivalent) will not be higher than the last EFSA estimate of exposure to steviol glycosides (E 960a) (EFSA ANS Panel, 2015b).

The Panel considered that exposure to glucosylated steviol glycosides should be calculated using the following formula:

$$\text{Exposure to glucosylated steviol glycosides} = \frac{\text{exposure to steviol equivalents}}{\text{factor dependent on starting material and degree of glucosylation}}$$

Using the factor of 0.222 indicated by the applicant for the calculation of steviol equivalents for the glucosylated steviol glycosides GSG-RA mixture, a maximum intake of steviol equivalent of 4.3 mg/kg



bw per day (derived earlier, EFSA ANS Panel, 2015b), the Panel estimated that the maximum exposure to this GSG-RA mixture would be 19.4 mg/kg bw per day.

### 3.3.1. Anticipated exposure to toxic elements from the use of the proposed food additive

The applicant proposed maximum limits for arsenic, lead, cadmium and mercury at less than 0.1 mg/kg for each, to be included in the EU specifications for the proposed food additive (see Table 1). The potential exposure to these toxic elements from the use of the proposed food additive can be calculated by assuming contamination of the additive may be up to the proposed specification limit values, and then by calculation pro-rata to the estimate of exposure to the proposed food additive itself.

As noted above, if steviol glycosides would be replaced by glucosylated steviol glycosides, exposure to the proposed food additive (expressed as steviol equivalent) will not be higher than the last EFSA estimate of exposure to steviol glycosides (E 960a). The scenario calculated by the ANS Panel using MPLs and the proposed extension of use (toddlers, 95th percentile) resulted in a highest estimated exposure of 4.3 mg/kg bw per day expressed as steviol equivalents (EFSA ANS Panel, 2015b).

Since, in the current application, the proposed food additive may have up to 20 additional glucose units added to the parent steviol glycoside structure, it is appropriate to calculate the actual mass of additive that would correspond to the steviol equivalents (see Table 3). However, because the conversion factor is based on an average number of added glucose units, the Panel considered that an average of 20 glucose units would be extremely unlikely and therefore considered a value of 10 as a conservative assumption.

The outcome of such an exercise illustrates the health impact that would result from the maximum limits for toxic elements as proposed by the applicant for the proposed specifications of glucosylated steviol glycosides, assuming different scenarios with respect to the number of added glucose units in different preparations (Table 3).

For arsenic, the reference points are BMDL<sub>01</sub> 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, 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 86 and 155 (Table 3) are insufficient. As noted above, the specification value proposed by the applicant for arsenic, and used for this illustration (Table 3), was 0.1 mg/kg whereas for the five batches of GSG analysed, all five results for arsenic were < 0.005 mg/kg. The Panel considers that the maximum limits in the EU specifications for toxic elements should be established based on actual levels measured in the proposed food additive.

For lead, the reference point is a BMDL<sub>01</sub> 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 BMDL<sub>01</sub> (MOS/MOE lower than 1). The MOS/MOE is well above 1 (Table 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 minor fraction of the TWI value (Table 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 minor fraction of the TWI value (Table 3).

### 3.3.2. Potential exposure to glucose resulting from the use of the proposed food additive

The Panel noted that the proposed food additive may have up to 20 additional glucose units incorporated by the enzymatic glucosylation process, in addition to the sugar units already present in the steviol glycoside starting material.

As an example, in the case of the GSG-RA mixture described earlier (see Section 3.1.1), the starting material was ≥ 95% rebaudioside A, which includes four glucose units (see Figure 1) before enzymatic addition of additional glucose units.

Since the glucosylated steviol glycosides are expected to readily undergo stepwise hydrolysis in the GI tract forming steviol, the release of glucose can be estimated as follows.



For the GSG-RA mixture, by considering that it is composed only of Rebaudioside A (four glucose units/molecule) and that enzymatic glucosylation resulted in the addition of 2.95 glucose units/molecule, a maximum exposure of 4.3 mg/kg bw per day (expressed as steviol equivalents) corresponds to a maximum exposure to steviol (MW 318) of 13.52  $\mu$ moles/kg bw per day. Since each mole of steviol equivalents yields 6.95 moles of glucose (MW 180), the maximum exposure to glucosylated steviol glycoside-derived glucose would be 16.9 mg/kg bw per day.

Applying this approach to the conservative assumption of a glucosylated steviol glycosides having an average number of 10 added glucose units per molecule, the Panel calculated a potential resulting intake of glucose of 34.1 mg/kg bw per day. The Panel noted that this would result in a maximum additional intake of glucose of up to 0.4 g/day for a 12-kg toddler.

For the adult population, the resulting maximum additional intake of glucose was calculated by the Panel to be 1.2 g/day for a 70-kg person, based on the maximum P95 intake of 2.2 mg/kg bw per day estimated in the previous ANS Panel opinion (EFSA ANS Panel, 2015b) and the conservative assumption of 10 added glucose units per steviol equivalent in the glucosylated steviol glycosides preparation.

For the general population, the contribution of the GSG to the overall glucose intake as estimated by the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA Panel) in its draft scientific opinion on the Tolerable Upper Intake Level for Dietary Sugars<sup>7</sup> would not be of major significance.

### 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.

The present evaluation will focus on existing and new data provided by the applicant on the rates of *in vitro* hydrolysis of glucosylated steviol glycosides by pooled faecal homogenates from human volunteers.

#### 3.4.1. Absorption, distribution, metabolism and excretion

Data on the metabolism of glucosylated steviol glycosides have been considered and summarised in a previous EFSA opinion (EFSA ANS Panel, 2018). The assessment was based on an *in vitro* investigation of the microbial metabolism of the glucosylated steviol glycosides  $\alpha$ -monoglucosylated rebaudioside A and  $\alpha$ -monoglucosylated stevioside (Koyama et al., 2003b). The substrates (at 0.2 and 10 mg/mL) were incubated with human faecal homogenates under anaerobic conditions, and metabolites generated were analysed and identified by liquid chromatography–mass spectrometry. The glucosylated steviol glycosides were largely metabolised to steviol after 24 h. A mixture of enzymatically modified stevia, containing the primary components  $\alpha$ -glucosylated rebaudioside A,  $\alpha$ -glucosylated stevioside,  $\alpha$ -glucosylated rebaudioside C and  $\alpha$ -glucosylated dulcoside A was also hydrolysed to steviol after incubation with human faecal homogenates. The authors proposed that the metabolic degradation of  $\alpha$ -glucosylated steviol glycosides starts with  $\alpha$ -deglycosylation to the parent steviol glycoside, followed by further hydrolysis to steviol. The ANS Panel noted that a considerable proportion (20%) of enzymatically modified stevia remained intact or partially degraded after 24-h incubation with faecal homogenates (EFSA ANS Panel, 2018). As hydrolysis of a tested mixture was only determined after 24 h, the ANS Panel concluded that the publication from Koyama et al. (2003b) did not allow a determination of the extent of hydrolysis of glucosylated steviol glycosides in the human colon in relation to that of steviol glycosides.

#### Considerations of published studies submitted by the applicant

Metabolic studies with steviol glycosides report that human digestive enzymes are not capable of hydrolysing  $\beta$ -glycosidic bonds (Hutapea et al., 1997; Geuns et al., 2007), and, thus, steviol glycosides are not digested in the upper gastrointestinal tract (Koyama et al., 2003a). For glucosylated steviol glycosides which also contain  $\alpha$ -oriented glycosidic bonds, it is envisaged that these bonds are hydrolysed by digestive enzymes (i.e. salivary and/or pancreatic  $\alpha$ -amylase). This would allow for the parent steviol glycosides to be degraded by the established metabolic pathway within the colon by the gut microbiota to steviol, and would release the  $\alpha$ -glycosidic bonded sugar moieties to be absorbed in the intestine and metabolised via normal carbohydrate metabolism pathways.

<sup>7</sup> See <https://open.efsa.europa.eu/questions/EFSA-Q-2016-00414>

## Evaluation of a new unpublished *in vitro* study submitted by the applicant

A mixture of steviol glycosides and glucosylated steviol glycosides has been tested in human faecal homogenate samples (BRI, 2018 in Documentation provided to EFSA n. 1). The composition of the GSG-RA mixture used was established using an HPLC-UV method, using calibration standards of Rebaudioside A, stevioside, Rebaudioside F and Rebaudioside C and making an assumption that all steviol glycosides have the same molar extinction coefficient at the UV wavelength employed for detection. The chemical composition of the test material was very similar to the composition of the batches of GSG-RA described in Section 3.1.1. The unmodified (parent) steviol glycosides were Reb. A (8%) with a lower amount of stevioside (ca. 0.1%) along with traces of Reb F and Reb C (< 0.05% of each). Correspondingly, the dominant glucosylated steviol glycosides were the series of mono- to deca-glucosylated Rebaudioside A (ca. 20, 18, 14, 11, 5.9, 3.9, 3.8, 1.8, 2.1 and 1.3%, respectively, summing to ca. 82%) along with smaller amounts of mono- to hexa-glucosylated stevioside (0.2, 2.6, 1.5, 2.4, 0.7 and 2.2%, respectively, summing to ca. 10%).

This glucosylated steviol glycoside GSG-RA preparation was incubated, in triplicate, at a concentration of 0.2 mg/mL with adult male and female pooled faecal homogenate samples diluted 50-fold overall and under anaerobic conditions at 37°C for up to 48 h. Samples were taken at 4, 12, 24 and 48 h and analysed by liquid chromatography-mass spectrometry (LC/MS) to measure the levels of the major glucosylated steviol glycosides. Since Rebaudioside A and stevioside themselves differ by just one sugar moiety, the LC/MS analysis was for the major pairs of substances that share a common molecular weight. These were mono-glucosylated Rebaudioside A plus di-glucosylated stevioside (MW 1129, called SvG5), di-glucosylated Rebaudioside A plus tri-glucosylated stevioside (MW 1291, called SvG6) and tri-glucosylated Rebaudioside A plus tetra-glucosylated stevioside (MW 1453, called SvG7). The level of steviol itself was also monitored since it is the final metabolite of complete deglycosylation. The LC/MS analysis was not calibrated using authentic standards of the glucosylated steviol glycosides. Rather, the concentrations were calculated from the initial composition of the mixture multiplied by the peak areas at time=*t* normalised to peak areas at the start (time=zero). Steviol itself was calibrated against an authentic standard.

SvG5, SvG6 and SvG7 were extensively degraded (> 96% loss, mean of triplicates) at the 4-hour time point. However, the percentage of steviol formed after 4 h of incubation was only 15% and 27% of the expected yield, in male and female samples, respectively. At the 12-h time point, the percentage of steviol formed was much higher, at 83.4% and 83.9% in male and female samples, respectively. This lag phase in the formation of the ultimate metabolite steviol was also seen in a control experiment using Rebaudioside A. It suggests that microbial degradation occurs in a stepwise manner involving a number of partially deglycosylated intermediates (which were not analysed here) until hydrolysis is nearly completed and yields the final metabolite steviol.

The glucosylated steviol glycosides contain  $\alpha$ -glycosidic bonds which may be hydrolysed more easily (more rapidly) than the  $\beta$ -glycosidic bonds in the parent steviol glycosides (Hutapea et al., 1997; Geuns et al., 2007), but a stepwise degradation would imply a longer lag phase in the appearance of steviol formed from glucosylated steviol glycosides compared to formation from the parent (unglycosylated) glycosides. Since only mono- to tetra-glucosylated forms were monitored in these tests (BRI, 2018 in Documentation provided to EFSA n. 1), the lag phase in the appearance of steviol may be expected to be longer still for the higher glucosylated forms, since up to *n* = 10 glucose units have been quantified in the starting GSG-RA mixture and up to *n* = 20 glucose units are mentioned in the mixture described in Section 3.1.1. On the other hand, this slightly longer lag may be due in part or in full, to an artefact of the *in vitro* experiment used. If  $\alpha$ -oriented glycosidic bonds are hydrolysed by digestive enzymes (i.e. salivary and/or pancreatic  $\alpha$ -amylase), this earlier hydrolysis is not simulated by the human faecal homogenate experiments submitted by the applicant.

The Panel noted that the study submitted by the applicant in support of the current application has since been published in a peer-reviewed journal (Purkayastha and Kwok, 2020).

Overall, the Panel considered that glucosylated steviol glycosides readily undergo stepwise hydrolysis forming steviol which is the same metabolite that is formed from the already authorised steviol glycosides.

### 3.4.2. Acute toxicity

No acute toxicity studies were provided.

### 3.4.3. Short-term and subchronic toxicity

No short-term or subchronic toxicity studies were provided.

### 3.4.4. Genotoxicity

No studies were provided.

### 3.4.5. Chronic toxicity and carcinogenicity

No studies were available.

### 3.4.6. Reproductive and developmental toxicity

No studies were available.

### 3.4.7. Hypersensitivity, allergenicity and food intolerance

No studies were available.

## 3.5. Discussion

The current assessment by the Panel is based on the information submitted in the application dossier (Documentation provided to EFSA n. 1) and the additional information provided by the applicant either spontaneously or following requests from EFSA (Documentation provided to EFSA n. 2–5).

Glucosylated steviol glycosides' preparations were described by the applicant as a mixture of glucosylated steviol glycosides, containing 1–20 additional glucose units bound to the parent steviol glycoside via  $\alpha$ -(1–4) linkages. The Panel noted that the definition of the food additive in the proposed specifications does not include any value for the additional glucose units and that the mixtures on which data have been provided contain, on average, only three or four additional glucose units. The Panel made a conservative assumption that the GSG may contain up to 10 additional glucose units on average in its evaluation and considered that information on the number of additional glucose units (1–20) should be added to the proposed definition of the food additive in the specifications.

Glucosylated steviol glycosides' preparations consist of not less than 95% of the total steviol glycosides comprised of glucosylated steviol glycosides and parent steviol glycosides on the dried, dextrin-free, basis. The glucosylated fraction of the total steviol glycosides is between 80% and 92% w/w and the parent steviol glycosides between 5% and 15% w/w. Typical components of glucosylated steviol glycosides are presented in Appendix A as proposed by the applicant.

The Panel considered that rather than having a non-definite list of components, as proposed by the applicant and reported in Appendix A, the assay value should cover all steviol glycosides falling under the definition of E 960a along with their glucosylated derivatives.

The proposed use and use levels for glucosylated steviol glycosides are the same as those for the already authorised steviol glycosides (E 960a–960c). Taking into account that glucosylation of the steviol glycosides results in higher molecular weights, the glucosylated steviol glycosides have a lower steviol equivalency compared to steviol glycosides E 960a. Therefore, a larger quantity of glucosylated steviol glycosides would be permitted to be added to food to give the same steviol equivalent value. Consequently, the resulting exposure to potential impurities, including toxic elements, has taken into account this higher addition levels (on a mass basis and not on a steviol equivalent basis). With the exception of arsenic, for which the lowest calculated MOS/MOE was considered to be insufficient, for the other toxic elements (cadmium, mercury, lead) proposed for inclusion in the specifications, the maximum limits do not give rise to safety concerns.

The Panel noted that the proposed glucosylated steviol glycosides' preparations may contain up to 20 glucose moieties per molecule of steviol glycoside and this could lead to an additional exposure to glucose from a sweetener which is proposed to have a technological function of replacing sugars in food. Based on a worst-case assumption of an average of 10 additional glucose units added to the steviol glycosides, the Panel estimated that the proposed uses and use level of the new food additive glucosylated steviol glycosides would lead to an additional glucose intake of up to 0.4 g/day for toddlers and 1.2 g/day for adults.

Glucosylated steviol glycosides are prepared in two production stages. Steviol glycosides mixtures – enriched individual steviol glycosides (e.g. high Rebaudioside A or Rebaudioside D content (> 95%)) or

mixtures enriched in two or more individual steviol glycosides – are isolated from dried leaves of *S. rebaudiana* by hot water extraction and different purifications steps. The purified steviol glycosides extract undergoes enzymatic treatment to achieve glucosylation via  $\alpha$ -(1-4) linkages of the steviol glycosides. A glucose donor, such as tapioca starch (extracted from Cassava roots), is treated with the enzyme CGTase (EC 2.4.1.19), derived from a non-genetically modified strain of *Anoxybacillus caldiproteolyticus*. CGTase (EC 2.4.1.19) catalyses the intermolecular glucosylation whereby  $\alpha$ -glucosyl units are transferred from the starch onto the 4-hydroxy position of a glucosyl moiety on the steviol glycoside (trans- $\alpha$ -1,4-glucosylation). The enzyme is inactivated by heating and removed by treatment with activated carbon. The dextrin by-product can be removed from the mixture.

The Panel noted that the safety of the enzyme CGTase (EC 2.4.1.19) derived from a non-genetically modified strain of *Anoxybacillus caldiproteolyticus* and intended to be used in the preparation of glucosylated steviol glycosides has been assessed by the EFSA CEP Panel. Based on the data provided, and the removal of food enzyme TOS during the manufacture of modified steviol glycosides, the CEP Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use (EFSA CEP Panel, 2022).

The Panel considered that the specifications proposed by the applicant for glucosylated steviol glycosides (Table 1) should be taken into account for the EU specifications for glucosylated steviol glycosides as a new food additive with the following modifications:

- The assay value of more than 95% for total steviol glycosides, comprised of glucosylated and parent steviol glycosides, on the dried, dextrin-free, basis should be limited to those 11 named steviol glycosides that are included in the current definition of E 960a along with their glucosylated derivatives (1–20 added glucose units)
- The description of the food enzyme used in the manufacturing process should be aligned to the description given in the CEP Panel opinion, i.e. 'Cyclomaltodextrin glucanotransferase (EC 2.4.1.19) derived from a non-GMO strain of *Anoxybacillus caldiproteolyticus* St-88'.

Glucosylated steviol glycosides contain  $\alpha$ -oriented glycosidic bonds which are hydrolysed by digestive enzymes (i.e. salivary and/or pancreatic  $\alpha$ -amylase) resulting in the parent steviol glycosides. Therefore, the glucosylated steviol glycosides share the same metabolic fate as steviol glycosides extracted from *S. rebaudiana* Bertoni, i.e. they undergo hydrolysis forming steviol as demonstrated in an *in vitro* study submitted in support of the current application. The results of this study with human faecal homogenates showed an extensive microbial degradation of mono- to tetra-glucosylated steviol glycosides to steviol at similar rates as Rebaudioside A.

Overall, the Panel considered that the metabolism of glucosylated steviol glycosides is sufficiently similar to the already authorised steviol glycosides, and thus, the toxicological data previously assessed by the ANS Panel for steviol glycosides (E 960a) were considered to support their safety as food additive. Therefore, no additional toxicological data were required.

The existing ADI for steviol glycosides (E 960a) of 4 mg/kg bw per day expressed as steviol can also be applied to glucosylated steviol glycosides.

## 4. Conclusions

The Panel concluded that there is no safety concern for the use of glucosylated steviol glycosides as a new food additive at the proposed use and use levels.

## 5. Recommendations

The Panel recommend that the specifications proposed by applicant for glucosylated steviol glycosides would include the following modifications:

- The assay value of more than 95% for total steviol glycosides, comprised of glucosylated and parent steviol glycosides, on the dried, dextrin-free, basis should be limited to those 11 named steviol glycosides that are included in the current definition of E 960a along with their glucosylated derivatives (1–20 added glucose units)
- The description of the food enzyme used in the manufacturing process should be aligned to the description given in the CEP Panel opinion, i.e. 'Cyclomaltodextrin glucanotransferase (EC 2.4.1.19) derived from a non-GMO strain of *Anoxybacillus caldiproteolyticus* St-88'.

The proposed maximum limit for arsenic should be lowered.



## Documentation provided to EFSA

- 1) Application for the Authorisation of Glucosylated Steviol Glycosides as a Food Additive in the European Union pursuant to Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on Food Additives Technical Dossier. PureCircle Limited. December 2018. Including:
  - PureCircle Sdn Bhd, 2015a. Storage stability of GSG-RA. Unpublished report.
  - PureCircle Sdn Bhd, 2015b. pH stability of GSG-RA. Unpublished report.
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- 1) Spontaneous submission by the applicant. April 2019.
- 2) Additional information submitted by the applicant following a request from EFSA. July 2019.
- 3) Additional information submitted by the applicant following a request from EFSA. January 2020.
- 4) Additional information submitted by the applicant following a request from EFSA. March 2021. Including:
  - BRI (Biopharmaceutical Research Inc.), 2021. LC/MS Assay of Cyclodextrins in a Glucosylated Steviol Glycoside Material. March 2021. Unpublished report.

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## Abbreviations

ADI	acceptable daily intake
bw	Body weight
CGTase	cyclomaltodextrin glucanotransferase
GSG	Glucosylated Steviol Glycosides
MOE	Margin of Exposure
MOS	Margin of Safety
NOAEL	no observed adverse effect level
TOS	Total Organic Solids

## Appendix A – Typical components of glucosylated steviol glycoside preparations (Documentation provided to EFSA n. 1)

Molecules	Molecular formula	Molecular weight (Da)	CAS number
<b>Example Glucosylated Steviol Glycosides (~ 80 to 92% final steviol glycoside content)</b>			
n-Glucosylated stevioside	$C_{(38+n \cdot 6)}H_{(60+n \cdot 10)}O_{(18+n \cdot 5)}$	$804.87 + n \cdot 162.15$	Not available
n-Glucosylated rebaudioside C	$C_{(44+n \cdot 6)}H_{(70+n \cdot 10)}O_{(22+n \cdot 5)}$	$951.01 + n \cdot 162.15$	Not available
n-Glucosylated rebaudioside A	$C_{(44+n \cdot 6)}H_{(70+n \cdot 10)}O_{(23+n \cdot 5)}$	$967.01 + n \cdot 162.15$	Not available
n-Glucosylated rebaudioside D	$C_{(50+n \cdot 6)}H_{(80+n \cdot 10)}O_{(28+n \cdot 5)}$	$1,129.2 + n \cdot 162.15$	Not available
n-Glucosylated rebaudioside M	$C_{(56+n \cdot 6)}H_{(90+n \cdot 10)}O_{(33+n \cdot 5)}$	$1,291.3 + n \cdot 162.15$	Not available
<b>Example Parent Steviol Glycosides (~ 5–15% final steviol glycoside content)</b>			
Rubusoside	$C_{32}H_{50}O_{13}$	642.73	64849-39-4
Steviolbioside	$C_{32}H_{50}O_{13}$	642.73	41093-60-1
Dulcoside A	$C_{38}H_{60}O_{17}$	788.87	64432-06-0
Stevioside	$C_{38}H_{60}O_{18}$	804.87	57817-89-7
Rebaudioside B	$C_{38}H_{60}O_{18}$	804.87	58543-17-2
Rebaudioside F	$C_{43}H_{68}O_{22}$	936.99	438045-89-7
Rebaudioside C	$C_{44}H_{70}O_{22}$	951.01	63550-99-2
Rebaudioside A	$C_{44}H_{70}O_{23}$	967.01	58543-16-1
Rebaudioside E	$C_{44}H_{70}O_{23}$	967.01	63279-14-1
Rebaudioside D	$C_{50}H_{80}O_{28}$	1,129.2	63279-13-0
Rebaudioside M	$C_{56}H_{90}O_{33}$	1,291.3	1220616-44-3

CAS: Chemical Abstracts Service; n: the number of glucose units enzymatically added to the parent steviol glycoside;  
n\*: n multiplied by.