REGULATED PRODUCTS SAFETY ASSESSMENT

Safety Assessment on a Modification of Specifications of Rebaudioside D (E960c) to Include Manufacture by Enzymatic Conversion (RP1245)

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FSA Research and Evidence

The FSA (Food Standards Agency) and FSS (Food Standards Scotland) received an application for the modification of specifications of rebaudioside D (E960c) to include manufacture via enzymatic conversion of a genetically modified production strain of *Komagataella phaffi,* using the enzymes sucrose synthase and uridine 5'-diphospho(UDP)-glucosyltransferase. This request for authorisation was assessed by the European Food Safety Authority (EFSA) in the European Union (EU) which was published in 2022; however, concern was raised over the possible presence of residual DNA within the final product. Upon receipt of additional information provided by the Applicant, the FSA/FSS reviewed the information available, including the EFSA Opinion. The FSA and FSS confirmed that RP1245, as described in the application, was unlikely to have any adverse effect on human health with its intended uses in GB, on the condition that the Applicant could show that the residual DNA did not remain. Concerns were initially raised over the possibility of residual kanamycin DNA encoding for the resistance gene being present within the final product; however, PCR data supplied by the Applicant demonstrated the lack of this DNA. FSA and FSS reviewers were satisfied that the updated PCR analysis demonstrated a lack of residual DNA. The views of the AEJEG and Committee on Toxicity (COT) were considered in this safety assessment, which represents the opinion of the FSA and FSS on the modification of the production for steviol glycosides using enzymatic bioconversion. The FSA and FSS concluded that the Applicant had satisfactorily demonstrated that the manufacture of rebaudioside D via enzymatic conversion would not pose any toxicological concern under the proposed uses.

This is a joint FSA and FSS publication.

1. Introduction

The Food Standards Agency (FSA) and Food Standards Scotland (FSS) have undertaken a safety assessment for a change in the specifications of steviol glycosides in Great Britain, to include a new manufacturing method via enzymatic conversion for steviol glycosides including rebaudioside D, under the common authorisation procedure for food additives, food enzymes and food flavourings legislation, assimilated EU Regulation 1331/2008. To support the safety assessment by FSA and FSS, the Additives and Enzymes Joint Expert Group (AEJEG) provided risk assessment advice to the FSA and FSS outlined in this opinion.

Steviol glycosides are a class of compounds which function as low-calorie sweeteners that are currently authorised for use in a range of food groups, which includes but is not limited to edible ices; fruit and vegetables in vinegar, oil or brine; fruit and vegetable preparations excluding compote; and table-top sweeteners in powder form, with a varying range of restrictions.

The dossier was evaluated in line with Article 3 of assimilated Regulation 1331/2008 and has considered the aspects of the food additive and its modification of specification. This, and the guidance put in place by European Food Safety Authority (EFSA) for food additive applications, has formed the basis and structure for the assessment (EFSA ANS, 2012). The assessment has considered the aspects of the food additive and its production.

Information regarding the identity of the substance including existing and proposed specifications were provided. In addition, information on the manufacturing process, presence of impurities, stability of the substance, fate in food, existing authorisations and risk assessments, and biological and toxicological data were provided. This information was considered satisfactory.

Following the final review by the Joint Expert Group on Additives, Enzymes, and other Regulated Products (AEJEG) in July 2024, the AEJEG advised that the new method for the production of steviol glycosides from a genetically modified production strain of *Komagataella phaffi* was safe under the proposed conditions of use.

2. Assessment

2.1. Overview

As part of the Regulated Products authorisation process, a request was received for the Joint Expert Group on Additives, Enzymes, and other Regulated Products (AEJEG) to provide risk assessment advice on the application for a change in the steviol glycoside specification in the United Kingdom to include a new manufacturing method for steviol glycosides, including rebaudioside D.

The Applicant, SweeGen, Inc., is requesting approval for a modification of the specifications of the already authorised food additive steviol glycosides (E960) to include a new manufacturing process. This process consists of the production of rebaudioside D via enzymatic conversion using uridine diphosphate (UDP)-glucosyltransferase and sucrose synthase produced by a strain of *K. phaffi.*

Whilst it was a Member State of the European Union (EU), the United Kingdom (UK) accepted the assessments of the European Food Safety Authority (EFSA) in respect to authorisations for regulated food and feed products. Since the end of the transition period, the Food Standards Agency/Food Standards Scotland (FSA/FSS) has adopted equivalent technical guidance and quality assurance processes to be able to undertake Great Britain risk assessments for regulated product applications.

During the transition period of the UK leaving the EU, RP1194 'Rebaudioside M Produced Via the Enzyme Modification of Steviol Glycosides from Stevia' was given a favourable scientific opinion by EFSA (EFSA FAF, 2019). The FSA/FSS agreed that this opinion could be adopted for this application as FSA/FSS would have contributed to the opinion whilst the UK was still a Member State. It was therefore considered that the EFSA Opinion would be accepted as the UK Opinion.

Where EFSA, prior to the end of the transition period, evaluated an application for the product for which an application is now made to GB, FSA/FSS has decided to make use of the EFSA risk assessment, where appropriate, in forming its opinion. RP1245 was identified as a near identical application to RP1194, using the same production organism and manufacturing process. As the RP1194 opinion had been adopted by the

FSA/FSS, it had initially been agreed that the ESFA Opinion on the EU equivalent application to RP1245 could be used to form the basis of the UK Opinion due to the near identical nature of the applications.

In reviewing the output of the EFSA risk assessment, the reviewers verified that the standard approach as outlined in the relevant guidance had been followed and the arguments made were consistent with the data summarised in the opinion. Consideration was given to the processes undertaken to ensure the opinion is robust and whether there are any aspects that would require further review, such as specific issues for the countries of GB.

The EFSA Opinion on the modifications of specifications to include rebaudioside D produced via enzymatic conversion, was published in 2022 (EFSA FAF, 2022). The FSA/FSS reviewed the RP1245 equivalent EFSA Opinion and identified the same concerns surrounding the possible presence of DNA encoding for the kanamycin resistance gene within the final product that EFSA had identified. Upon reviewing the information provided by the Applicant, the FSA/FSS agreed with the conclusions of EFSA. Following the receipt of further information, the AEJEG was asked to assess only the information presented by the Applicant, in addition to the original application, and addressing these concerns.

EFSA's 2022 scientific opinion highlighted that whilst there was no toxicological concern with the product, there were still reservations that residual DNA encoding the kanamycin resistance gene could be present in the finished product. Therefore, the safety of the product could not be concluded upon.

Following EFSA's assessment, the Applicant supplied polymerase chain reaction (PCR) analysis results to the FSA/FSS, alongside the primer sequences targeting the DNA encoding for the kanamycin resistance gene, as this had been raised as an issue within the 2022 EFSA Opinion.

AEJEG Members concluded that the additional information provided was sufficient to allow for an evaluation of the proposal for a change in the steviol glycoside specification in the UK to include a new manufacturing method for steviol glycosides including rebaudioside D.

In conclusion, the AEJEG advised the FSA/FSS that sufficient information had been provided to allow for an evaluation of the proposal for rebaudioside D produced via enzymatic bioconversion, and that there were no concerns over safety of the proposed process.

2.2. Details of the EFSA Opinion

EFSA released a scientific opinion in 2022 detailing the safety evaluation that had been conducted on the equivalent application received by EFSA for RP1245. This opinion specified that whilst there was no toxicological concern, it had not been demonstrated that DNA encoding for the kanamycin resistance gene had been removed.

2.2.1. Background

The Applicant, SweeGen, Inc., applied to EFSA to allow for an amendment of the current E960 steviol glycoside specifications to include a new method of manufacturing rebaudioside D via enzymatic bioconversion of purified stevia leaf extract. It was mentioned that EFSA had previously produced a scientific opinion on rebaudioside M, which included the same amendments to the specification as those proposed by the Applicant of RP1245; however, the new Application requested the addition of rebaudioside D to the current specifications.

The method of enzymatic bioconversion proposed the use of two enzymes (sucrose synthase and uridine 5'-diphospho(UDP)-glucosyltransferase (UGT)) derived from strains of genetically modified *Komagataella phaffii* (*K. phaffi*, UGT-A). The strains are subjected to fermentation and the enzymes produced are isolated. The rebaudioside D is produced by mixing the enzymes with stevia leaf extract before purification and isolation are conducted to manufacture the rebaudioside D.

2.2.2. EFSA's 2022 Assessment

The Applicant described the food additive as having a white to off-white colour, that the additive is 202 times sweeter than sucrose and meets the existing solubility requirement for steviol glycosides of 'freely soluble to slightly soluble in water' in line with EU Regulation (EU) No 231/2012.

The Applicant had provided EFSA with data from 5 non-consecutive batches, conducted in triplicate, that had been analysed using a modified version of the high-performance liquid chromatography (HPLC) method outlined by The Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2010). This data showed a rebaudioside D content of >98%, with small amounts of other steviol glycosides detected in the remaining content (rebaudioside A < 2%, rebaudioside B < 0.5%). A further five batches were analysed using the 2017 JECFA HPLC methodology; this data showed a rebaudioside D content of >98%, rebaudioside M content of approximately 1.5%, and non-quantifiable levels of rebaudioside E. The Applicant explained that varying levels of other steviol glycosides would appear within the product due to the manufacturing and purification steps, as well as the composition of the stevia leaf extract material used within

the production process. The final rebaudioside D product contained >95% rebaudioside D content, with the presence of other steviol glycosides including rebaudiosides A, B, E, and M. It was discussed by the EFSA Panel that the 2017 JECFA HPLC methodology to analyse the samples was not followed fully, as the Applicant had quantified based on area percentage of the peaks within the HPLC chromatogram. Despite this divergence from the methodology, the Panel agreed that the presented data demonstrated the final concentration of rebaudioside D would be >95 % purity.

The Panel commented that *K. phaffi* UGT-A and the UGT-A fusion enzyme that had been used in the rebaudioside D manufacturing process had also been used to produce rebaudioside M; therefore, the Panel requested that the Applicant explain how the methodology favours the production of rebaudioside D over other steviol glycosides. The Applicant responded that the enzyme employed to convert the stevia leaf extract starting material, UGT-A, facilitates the conversion of rebaudioside A into rebaudioside D via the addition of a glucose unit to rebaudioside A. It was further explained that a stevia leaf extract containing a higher content of rebaudioside A would be more efficient in producing rebaudioside D. It was also clarified that the resulting mixture produced after the enzymatic bioconversion stage has a large percentage of rebaudioside D, with smaller amounts of other steviol glycosides possibly present. The Applicant explained that during the crystallisation process, it was possible to advantageously select the rebaudioside D using the different solvent solubility of the steviol glycosides.

2.2.3. Proposed Amendments

The Applicant requested amendments to the specifications already outlined in Regulation (EU) No 231/2012 (assimilated EU Regulation 231/2012 within the UK) for ‘E 960c(i) rebaudioside M produced via enzyme modification of steviol glycosides from stevia’.

The Applicant reasoned that as the same production strain and enzyme would be used during the manufacturing process, rebaudioside D should fall under the same specifications as rebaudioside M produced via enzymatic bioconversion. The current (Table 1) and newly proposed (Table 2) specifications have been outlined to highlight where the Applicant proposed that new specifications would diverge from those already captured within the regulation.

Table 1. Existing specifications for “E 960 Steviol Glycosides” in the United Kingdom (taken directly from the dossier supplied to the FSA/FSS by the Applicant).

Parameter	Specification
Definition	The manufacturing process comprises 2 main phases: the first involving water extraction of the leaves of the *Stevia rebaudiana* Bertoni plant and preliminary purification of the extract by employing ion exchange chromatography to yield a

Parameter	Specification
	steviol glycoside primary extract, and the second involving recrystallisation of the steviol glycosides from methanol or aqueous ethanol resulting in a final product containing not less than 95 % of the below identified 11 related steviol glycosides, 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 *Stevia rebaudiana* plant, have been identified in small amounts (0.10 to 0.37 % w/w).
Chemical name	Steviolbioside: 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid Rubusoside: 13-β-D-glucopyranosyloxykaur-16-en-18-oic acid, β-D-glucopyranosyl ester Dulcoside A: 13-[(2-O-α–L-rhamnopyranosyl-β–D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester Stevioside: 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester Rebaudioside A: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D- glucopyranosyl ester Rebaudioside B: 13-[(2-O-β–D-glucopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid Rebaudioside C: 13-[(2-O-α–L-rhamnopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester Rebaudioside D: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester Rebaudioside E: 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester Rebaudioside F: 13[(2-O-β-D-xylofurananosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester Rebaudioside M: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester
Trivial name, molecular formula and conversion factors	Steviol, $C_{20}H_{30}O_3$, conversion factor 1.00. Steviolbioside, $C_{32}H_{50}O_{13}$, conversion factor 0.50. Rubusoside, $C_{32}H_{50}O_{13}$, conversion factor 0.50. Dulcoside A, $C_{38}H_{60}O_{17}$, conversion factor 0.40. Stevioside, $C_{38}H_{60}O_{18}$, conversion factor 0.40. Rebaudioside A, $C_{44}H_{70}O_{23}$, conversion factor 0.33. Rebaudioside B, $C_{38}H_{60}O_{18}$, conversion factor 0.40. Rebaudioside C, $C_{44}H_{70}O_{20}$, conversion factor 0.34. Rebaudioside D, $C_{50}H_{80}O_{28}$, conversion factor 0.29. Rebaudioside E, $C_{44}H_{70}O_{23}$, conversion factor 0.33. Rebaudioside F, $C_{43}H_{68}O_{22}$, conversion factor 0.34. Rebaudioside M, $C_{56}H_{90}O_{33}$, conversion factor 0.25.
Trivial name, CAS number and molecular weight (g/mol)	Steviol, (no CAS listed), 318.46. Steviolbioside, 41093-60-1, 642.73. Rubusoside, 64849-39-4, 642.73. Dulcoside A, 64432-06-0, 788.87. Stevioside, 57817-89-7, 804.88. Rebaudioside A, 58543-16-1, 967.01. Rebaudioside B, 58543-17-2, 804.88. Rebaudioside C, 63550-99-2, 951.02. Rebaudioside D, 63279-13-0, 1,129.15. Rebaudioside E, 63279-14-1, 967.01. Rebaudioside F, 438045-89-7, 936.99. Rebaudioside M, 1220616-44-3, 1,291.30.
Assay	Not less than 95 % steviolbioside, rubusoside, dulcoside A, stevioside, rebaudiosides A, B, C, D, E, F, and M, on the dried basis, in any combination and ratio.
Description	White to light yellow powder, approximately between 200 and 350 times sweeter than sucrose (at 5 % sucrose equivalency).
Identification	Freely soluble to slightly soluble in water.

Parameter	Specification
(Solubility)	
Identification (pH)	Between 4.5 and 7.0 (1 in 100 solution).
Purity (Total ash)	Not more than 1 %.
Purity (Loss on drying)	Not more than 6 % (105 °C, 2 h).
Purity (Residual solvent)	Not more than 200 mg/kg methanol.
Purity (Residual solvent)	Not more than 5,000 mg/kg ethanol.
Purity (Arsenic)	Not more than 1 mg/kg.
Purity (Lead)	Not more than 1 mg/kg.

Table 2. Proposed specifications for "E 960c(i) Rebaudioside M and D produced via enzyme modification of Steviol Glycosides from Stevia" in the United Kingdom (taken directly from the dossier supplied to the FSA/FSS by the Applicant).

Parameter	Specification
Definition	Rebaudioside M is a steviol glycoside composed predominantly of rebaudioside M with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside B, rebaudioside D, rebaudioside I, and stevioside. Rebaudioside D is a steviol glycoside composed predominantly of rebaudioside D with minor amounts of other steviol glycosides such as rebaudioside A, rebaudioside B, and rebaudioside M. Rebaudioside M or D is obtained via enzymatic bioconversion of purified steviol glycoside leaf extracts (95% steviol glycosides) using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeasts *K. phaffi* (formerly known as *Pichia pastoris*) UGT-a and/or *K. phaffi* UGT-b that facilitate the transfer of glucose from sucrose and UDP-glucose to steviol glycosides via glycosidic bonds. Rebaudioside M is produced using enzymes from both yeasts, whereas rebaudioside D is produced using enzymes only from *K. phaffi* UGT-a. After removal of the enzymes by solid-liquid separation and heat treatment, the purification involves concentration of the rebaudioside M or D by resin adsorption, followed by recrystallisation of rebaudioside M or D resulting in a final product containing not less than 95 % of rebaudioside M, or not less than 95 % rebaudioside D. Viable cells of the yeasts *K. phaffii* UGT-a and/or *K. phaffii* UGT-b or their DNA shall not be detected in the food additive.
Chemical name	Rebaudioside D: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester Rebaudioside M: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester; 13-[(O-b-D-Glucopyranosyl-(1-2)-O-[b-D-glucosylpyranosyl-(1-3)]-b-Dglucosylpyranosyl)oxy]kaur-16-en-18-oic acid (4-)-O-b-D-glucosylpyranosyl-(1-2)-O-[b-Dglucosylpyranosyl-(1-3)]-b-Dglycosylpyranosyl ester
Trivial name, molecular formula and conversion factors	Rebaudioside D, $C_{50}H_{80}O_{28}$, conversion factor 0.29. Rebaudioside M, $C_{56}H_{90}O_{33}$, conversion factor 0.25.
Trivial name, CAS number and molecular	Rebaudioside D, 63279-13-0, 1 129.15. Rebaudioside M, 1220616-44-3, 1 291.30.

Parameter	Specification
weight (g/ mol)	
Assay	Not less than 95 % rebaudioside M, on the dried basis; or Not less than 95% rebaudioside D, 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.
Identification (pH)	Between 4.5 and 7.0 (1 in 100 solution).
Purity (Total ash)	Not more than 1 %.
Purity (Loss on drying)	Not more than 6 % (105 °C, 2h).
Purity (Residual solvent)	Not more than 5 000 mg/kg ethanol.
Purity (Arsenic)	Not more than 0.5 mg/kg.
Purity (Lead)	Not more than 0.25 mg/kg.
Purity (Cadmium)	Not more than 0.25 mg/kg.
Purity (Mercury)	Not more than 0.1 mg/kg.
Residual protein	Not more than 5 mg/kg.
Particle size	Not less than 74 µm [using a mesh #200 sieve with a particle size limit of 74 µm].

The EFSA 2022 Panel assessed and agreed that data supplied showed that rebaudioside D would be produced in line with the proposed specifications that had been suggested by the Applicant.

The EFSA 2022 Panel considered that although the Applicant requested to expand the specifications of rebaudioside M to include rebaudioside D produced via enzymatic conversion, rebaudioside D should be included in the legislation under a separate entry. This is because the final product would be a different steviol glycoside with regards to the chemical composition and production process, as the manufacture of rebaudioside M used two enzymes, UGT-A and UGT-B, whereas the process to manufacture rebaudioside D only used UGT-A.

The Applicant stated that the final concentration of rebaudioside D would be >95%. The EFSA 2022 Panel had been supplied with data supporting this and agreed that a statement of 'not less than 95% of rebaudioside D' was an appropriate reflection of the product.

The EFSA 2022 Panel discussed the Applicant's request to modify the specifications of the toxic elements within the final product. Data provided by the Applicant allowed the Panel to agree with the newly proposed

maximum limits for arsenic, lead, cadmium, and mercury, although it was suggested that for arsenic a lower maximum level should be set within the specifications.

The Applicant had proposed a maximum level of residual ethanol at 5,000 mg/kg. It was noted by the EFSA 2022 Panel that data showed the level of residual ethanol within the product did not exceed 20 mg/kg.

Analytical data provided by the Applicant showed no microbiological contamination in any batch tested.

The existing specifications for rebaudioside M listed the residual protein maximum limit at 5 mg/kg. The Applicant had conducted a bicinchoninic acid (BCA) assay with a limit of detection of 5 mg/kg. No residual proteins were detected above this limit.

The Applicant provided EFSA with analytical data to demonstrate the absence of viable cells or residual DNA within the final product. The Panel agreed that the absence of viable cells had been appropriately demonstrated. However, the experimental data showing absence of residual DNA were inconclusive and the Panel noted that the *K. phaffi* UGT-A DNA encoding the kanamycin resistance had still been detected in some samples. Therefore, the presence of DNA in the final product could not be ruled out by the Panel at this stage. It was noted that the concern of residual DNA being present would be the possibility of the gene propagating in microbiota.

The Applicant provided data showing that no kaurenoic acid would be present within the final product; this was accepted by the EFSA 2022 Panel.

No pesticide residues were detected in the batches sampled by the Applicant.

2.2.4. Manufacturing Process

The Applicant stated that rebaudioside D would be manufactured by enzymatic bioconversion using purified stevia leaf extract converted using the enzymes UDP-glucosyltransferase and sucrose synthase both derived from genetically modified *K. phaffi.*

The Applicant specified that all raw materials, process aids and purification equipment used during the production process follow international standards.

2.2.5. Enzyme Production

Genetically modified strains of *K. phaffii* are used to produce the enzymes UDP-glucosyltransferase and sucrose synthase as these aid in the conversion of the purified steviol leaf extract.

2.2.6. Rebaudioside D Production

A buffer agent is mixed with UGT-A fusion enzyme and agitated. The purified stevia leaf extract is added to the mixture. The resultant solution has a high proportion of rebaudioside D, which is then collected and subjected to a heat treatment to denature the enzymes prior to filtration, which removes the enzymes from the product.

2.2.7. Rebaudioside D Purification

The filtered mixture is passed through a column containing a macroporous resin; buffers are passed through before the rebaudioside D is eluted with ethanol. The subsequent solution is then condensed. The condensate product is chilled and the rebaudioside D crystalises before being collected and purified. The purification involves rinsing and dissolving the crystals in ethanol which is then treated with activated charcoal – this removes any impurities. The rebaudioside D is then recrystalised before being dried and processed.

2.2.8. Raw Materials and Processing Aids

Sucrose and UDP-glucose were used as a glucose source in the process.

It was noted that a macroporous resin was used during manufacture.

2.2.9. Characterisation of the Enzymes' Production Organism

Genetically modified *K. phaffi* is used to produce the enzymes UDP-glucosyltransferase and sucrose synthase used within the production process of rebaudioside D. UGT-A enzyme is produced from the *K. phaffi* strain A.

2.2.10. Characteristics of the Recipient Strain

The parental strain was listed as being *K. phaffi* ATCC20864.

2.2.11. Characteristics of the Donor Sequence

Within the 2022 EFSA opinion, the Applicant stated that: 'The UGT-A fusion enzyme consists of a sequence encoding UDP glucosyltransferase from barley (*Hordeum vulgare*) in frame with the SUS gene from bean (*Vigna radiata*) catalysing the glycosylation of UDP. The expression cassette UGT-A contains the AOXI (alcohol oxidase) promoter, the α-factor signal for protein secretion from *Saccharomyces cerevisiae*, in frame with the GCW61 gene encoding a cell wall protein from *K. phaffii* and the AOX1 terminator from *K. phaffii*. The plasmid vector pHKA-UGTA derives from the expression vector pPICαA and contains five copies of the expression cassette of UGT-

A, the pUC origin of replication, the HIS4 gene (involved in histidine biosynthesis) from the expression vector pPIC9K and a kanamycin resistance gene used as a selectable marker for transformation in E. coli.'

2.2.12. Description of the Genetic Modification Process

The pHKA-UGTA plasmid was cloned into the recipient strain and integrated into the HIS4 locus. This resulted in the *K. phaffi* A strain.

2.2.13. Safety Aspects of the Production Strain

K. phaffi ATCC20864 (parental strain) qualifies for Qualified Presumption of Safety (QPS) status. The production strain (*K. phaffi* A) contains the kanamycin resistance gene. The potential presence of this gene in the final food additive was noted by the EFSA FAF Panel in 2022 as a possible safety concern.

2.2.14. Absence of Viable Cells of the Production Strain in the End Product

The Applicant provided the EFSA 2022 Panel with analytical data showing the absence of any viable cells in the final product, which the Panel accepted.

2.2.15. Absence of DNA of the Production Strain in the End Product

PCR was used to demonstrate the lack of DNA encoding for the kanamycin resistance gene within the final product. The EFSA 2022 Panel deemed the data invalid as the DNA was detected even in samples that were listed as only containing water. A second and third set of PCR experiments were conducted and presented to the EFSA Panel; however, the same issues persisted, and the results were considered to be inconclusive. Due to the lack of definitive data showing the lack of DNA, the EFSA Panel concluded that they could not accept that the recombinant DNA would not be present in the final product.

2.2.16. Method(s) of Analysis in Food

The Applicant did not provide the EFSA 2022 Panel with information regarding method(s) of analysis within food. It is stated that the Panel assumed that the available analysis of other steviol glycosides could be applied to rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract.

2.2.17. Stability, Reaction and Fate in Food of the Proposed Food Additive

The Applicant had provided an accelerated 6-month stability study whereby 5 batches of the product were stored at 40 ± 2°C and a relative humidity of 75 ± 5%. It was reported that the product remained stable under the conditions.

2.2.18. Proposed Uses and Use Levels

The Applicant stated that rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract is proposed for use as high-intensity sweetener in food and beverages under the same conditions as those already approved for steviol glycosides within the EU.

2.2.19. Exposure Data

Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf was proposed to be used in the same way and at the same levels as already authorised steviol glycosides (E960). As such, further exposure data was not presented by the Applicant.

The EFSA 2022 Panel discussed that if rebaudioside D produced via enzymatic bioconversion of purified stevia leaf were consumed in place of other steviol glycosides, the previous estimate of steviol glycoside (E960) exposure would not be exceeded. The previous estimate of exposure to steviol glycosides (E960) were below the derived acceptable daily intake (ADI) of 4 mg/kg bw/day for all population groups except in one country whereby the toddler ADI exposure estimate was 4.3 mg/kg bw/day.

2.2.20. Anticipated Exposure to Toxic Elements from Proposed Specifications

Data was supplied to the EFSA 2022 Panel concerning content levels of numerous toxic elements. Arsenic (As, <0.02–0.09 mg/kg), lead (Pb, 0.10–0.16 mg/kg), cadmium (Cd, < 0.01 mg/kg), and mercury (Hg, <0.01 mg/kg) were all measured. The proposed maximum limits for these elements were included within the proposed amendments to the specifications.

It had been previously discussed that if rebaudioside D were to replace other steviol glycosides, the exposure would not exceed previously calculated limits of steviol glycosides.

It was explained within the opinion that steviol has a molecular weight of 318.45 Da and rebaudioside D a molecular weight of 1,129 Da, and when a conversion factor is applied the steviol equivalency of rebaudioside D is

0.29 (Table 2). It was calculated that 'an exposure of 4.3 mg/kg bw per day expressed as steviol equivalents equates to approximately 15 mg/kg bw per day expressed as rebaudioside D'.

The toxic element levels within rebaudioside D, when combined with the estimated intakes of other steviol glycosides, could result in an exposure which can be compared with reference points and health-based guidance values that have been outlined within Table 3.

Table 3. Reference points/health-based guidance values (HBGV) for toxic elements present in (E960c). This table was taken directly from the 2022 EFSA opinion and had not been supplied to the FSA/FSS by the Applicant.

Impurity/ constituentHBGV/ RP (µg/kg bw)	Basis/Reference Point
Lead (Pb)/0.5 (Benchmark Dose Lower ($BMDL_{01}$)	The reference point is based on a study demonstrating perturbation of intellectual development in children with the critical response size of 1 point reduction in IQ. The EFSA CONTAM Panel mentioned that a 1-point reduction in IQ is related to a 4.5% increase in the risk of failure to graduate from high school and that a 1-point reduction in IQ in children can be associated with a decrease of later productivity of about 2%. A risk cannot be excluded if the exposure exceeds the $BMDL_{01}$ (margin of exposure (MOE) lower than 1). EFSA CONTAM panel (2010)
Mercury (Hg)/4 (Tolerable Weekly Intake (TWI))	The HBGV was set using kidney weight changes in male rats as the pivotal effect. Based on the $BMDL_{10}$ of 0.06 mg/kg bw per day, expressed as mercury, and an uncertainty factor of 100 to account for inter- and intra-species differences, with conversion to a weekly basis and rounding to one significant figure, a TWI for inorganic mercury of 4 µg/kg bw per week, expressed as mercury was established. EFSA CONTAM Panel (2012)
Cadmium (Cd)/2.5 (TWI)	The derivation of the reference point is based on a meta-analysis to evaluate the dose–response relationship between selected urinary cadmium and urinary beta-2-microglobulin as the biomarker of tubular damage recognised as the most useful biomarker in relation to tubular effects. A group-based BMDL5 of 4 µg Cd/g creatinine for humans was derived. A chemical-specific adjustment factor of 3.9 was applied to account for human variability in urinary cadmium within each dose-subgroup in the analysis resulting in a reference point of 1.0 µg Cd per g creatinine. In order to remain below 1 µg Cd/g creatinine in urine in 95% of the population by age 50, the average daily dietary cadmium intake should not exceed 0.36 µg Cd/kg bw, corresponding to a weekly dietary intake of 2.5 µg Cd/kg bw. EFSA CONTAM Panel (2009b)
Arsenic (As)/0.3-8 (BMDL01)	The reference point is based on a range of benchmark dose lower confidence limit (BMDL01) values between 0.3 and 8 µg/kg bw per day identified for cancers of the lung, skin and bladder, as well as skin lesions. In general, the MOE should be at least 10,000 if the reference point is based on carcinogenicity in animal studies. However, as the BMDL for As is derived from human studies, an interspecies extrapolation factor (i.e. 10) is not needed. EFSA CONTAM Panel (2009a)

It was explained by EFSA (2022) that a risk assessment (Tables 4 and 5) of the toxic impurities was taken into account to assess any possible health risks if they were present at the specified limits. The EFSA 2022 Opinion further outlined that the assessment could be performed by calculating

the margin of exposure by dividing the reference point by the exposure estimate or estimate the contribution of E960c to the health-based guidance value.

It was clarified by the FAF Panel that the numbers used within the calculations were used solely to support the assessment, and the maximum limits would be set by risk management.

Table 4. Risk assessment for toxic elements based on the highest reported analytical data. Adapted from the 2022 EFSA Opinion, not supplied to the FSA/FSS by the Applicant.

Exposure to proposed additive (mg/kg bw per day)	Margin Of Exposure for Arsenic at 0.09 mg/kg	Margin Of Exposure for Lead at 0.16 mg/kg	% of the total weekly intake for Cadmium at 0.01 mg/kg	% of the total weekly intake for Mercury at 0.01 mg/kg
4.3 [(a)] /15[(b)]	222–5,926	208	0.04	0.03

(a): Estimated exposure expressed as steviol equivalents, using MPLs and the proposed extension of use (toddlers, 95th percentile). Data from EFSA ANS Panel scientific opinion on the safety of the extension of use of steviol glycosides (E960) as a food additive (EFSA ANS Panel, 2015).
(b): Corresponding estimated exposure to the rebaudioside D preparation after application of the steviol equivalency factor of 0.29 as proposed in the amendment of the specifications

Table 5. Risk assessment for toxic elements based on the maximum limits as proposed by the Applicant. Adapted from the 2022 EFSA Opinion, not supplied to the FSA/FSS by the Applicant.

Exposure to proposed additive (mg/kg bw per day)	Margin Of Exposure for Arsenic at 0.5 mg/kg	Margin Of Exposure for Lead at 0.5 mg/kg	% of the total weekly intake for Cadmium at 0.25 mg/kg	% of the total weekly intake for Mercury at 0.1 mg/kg
4.3 [(a)] /15[(b)]	40 - 1067	67	1.1	0.26

(a): Estimated exposure expressed as steviol equivalents, using MPLs and the proposed extension of use (toddlers, 95th percentile). Data from EFSA ANS Panel scientific opinion on the safety of the extension of use of steviol glycosides (E 960) as a food additive (EFSA ANS Panel, 2015).
(b): Corresponding estimated exposure to the rebaudioside D preparation after application of the steviol equivalency factor of 0.29 as proposed in the amendment of the specifications

The FAF panel discussed that the MOE values for arsenic could fall below the target level of 1,000. Therefore, the maximum limit of arsenic set within the specifications should be lower than the current limit proposed by the applicant.

2.2.21. Biological and Toxicological Data

The Applicant had submitted publications concerning the safety of steviol glycosides which were considered by the EFSA 2022 Panel.

2.2.22. *In vitro* Study Submitted by the Applicant

The Applicant provided the EFSA 2022 Panel with an *in vitro* metabolic study in human faecal homogenate samples, performed with bioconversion rebaudioside D and bioconversion rebaudioside M (Unpublished data supplied by the Applicant). The rebaudioside D and

rebaudioside M samples were incubated with 6 adult male and 6 adult female pooled faecal homogenate samples at a concentration of 0.2 mg/mL under anaerobic conditions (37°C, 4-72 hours). Rebaudioside A was used as a positive control. Liquid chromatography-mass spectrometry (LC-MS) was used to provide metabolic mass balance on the molar equivalent formation of the steviol metabolite over the time course. It was documented that there had been no difference between the male and female donors in the deglycosylation observed in the pooled faecal homogenates, and that the metabolism indicated rapid deglycosylation over the first 8 hours of metabolic incubation.

The results of this study were discussed by the EFSA 2022 Panel, who agreed that the results obtained reflected those of other opinions of rebaudioside D and steviol glycosides obtained from stevia leaf extract.

2.2.23. Toxicological Data

The Applicant did not conduct any toxicity studies to support the Application. The EFSA 2022 Panel considered that a read-across approach could be used due to the similarity in metabolic fates across steviol glycosides and the range of toxicity studies already available on other steviol glycosides that had been evaluated.

A read across from rebaudioside A to rebaudioside D was supported with a 28-day toxicity study (Nikiforov et al., 2013). The EFSA 2022 Panel agreed this study could be considered in support of demonstrating toxicological safety of this product; it showed a lack of adverse effects up to 2,000 mg/kg bw/day.

The Applicant provided the EFSA 2022 Panel with published articles in support of the Application, although the Panel did not deem these articles relevant.

The EFSA 2022 Panel concluded that whilst rebaudioside D produced via enzymatic bioconversion was of no toxicological concern, the presence of recombinant DNA encoding for the kanamycin resistance gene could not be ruled out and therefore the safety of the product had not been sufficiently demonstrated.

As rebaudioside D had already been discussed by EFSA (2022) with a subsequent scientific opinion published, the FSA/FSS deemed it appropriate to conduct a safety assessment that focussed on the new information provided by the Applicant to the FSA/FSS.

2.3. Information Considered by the AEJEG

The EFSA scientific opinion published in 2022 stated that whilst rebaudioside D manufactured via enzymatic conversion did not pose any toxicological concern, it could not be demonstrated that residual kanamycin DNA would not be present within the final product, and so a safety conclusion could not be reached at this time.

The FSA/FSS agreed with the 2022 EFSA opinion for RP1245; however, the Applicant presented the FSA/FSS with further information to be considered that covered EFSA's concern over the kanamycin resistance gene.

Due to the provision of new information, the Application was required to undergo assessment through the AEJEG.

The 2022 EFSA opinion and additional information regarding concern over the kanamycin resistance gene was used as a basis for the FSA/FSS assessment.

Upon reviewing the information provided by the Applicant, the FSA/FSS agreed with the conclusions of EFSA and used the 2022 Opinion as the basis for the assessment.

The Applicant provided PCR experiments demonstrating the lack of kanamycin resistance gene in the rebaudioside D product, testing 3 batches. The Applicant 'spiked' samples of rebaudioside D with the resistance gene DNA in different concentrations alongside regular rebaudioside D samples, then used the KanR primer to detect the presence of the DNA within the spiked samples to demonstrate that the primers worked.

After being presented with this information, the AEJEG requested the primer sequence, and asked if the primers used were a single pair of primers or nested primers, with a further request asking that if a single pair had been used, how the possible presence of gene fragments had been discounted.

The Applicant provided the primer sequence and confirmed that a single pair of primers had been used.

Members of the AEJEG agreed that the presence of DNA fragments could still not be discounted and requested that the PCR experiments be repeated using nested primers. In addition to this, the AEJEG requested that the original PCR experiments be re-run to include 5 batches to the original experimental design and specified that the new PCR experiments should include 5 batches also.

After receiving the updated PCR experiments, the AEJEG agreed that the safety of rebaudioside D produced by enzymatic bioconversion had been sufficiently demonstrated. This was justified by the evident validity of PCR experiments conducted by the Applicant that highlighted the lack of the kanamycin resistance gene that had been a concern of the AEJEG.

2.4. Discussion

In 2022, EFSA published a scientific opinion paper on the 'Safety of the proposed amendment of the specifications for enzymatically produced steviol glycosides (E960c): Rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract', whereby the Applicant had requested modification and to add rebaudioside D to the current specification of rebaudioside M produced via enzymatic bioconversion.

Rebaudioside D would be produced by the enzymatic bioconversion of purified stevia leaf extract using the enzymes UDP-glucosyltransferase and sucrose synthase to obtain a final product purity of >95% rebaudioside D, with other minor steviol glycosides present in much smaller amounts.

The 2022 EFSA opinion concluded that there was no toxicological concern for rebaudioside D, but the presence of recombinant DNA encoding for the kanamycin resistance gene could not be discounted and therefore a safety conclusion was not reached. After this decision, the Applicant provided the FSA/FSS with data that demonstrated the lack of the DNA within the final product. As the FSA/FSS had analysed the information provided by the Applicant and agreed with the 2022 EFSA Opinion, it was decided that a safety assessment only on the updated PCR experiments demonstrating the lack of DNA encoding for the kanamycin resistance gene was required to be conducted by the AEJEG.

In conclusion, the AEJEG advised the FSA/FSS that sufficient information had been provided to allow for an evaluation of the proposal for rebaudioside D produced via enzymatic bioconversion; there were no concerns over safety of the proposed process.

2.5. Conclusions of the AEJEG

The original 2022 EFSA scientific opinion concluded that whilst rebaudioside D produced via enzymatic bioconversion did not yield any toxicological concern, it could not be ruled out that recombinant DNA encoding for the kanamycin resistance gene would not be present. As a result of this published opinion, the Applicant provided the FSA/FSS with experimental data (PCR) demonstrating the absence of the DNA within the final product.

The AEJEG were satisfied that this additional experimental data adequately demonstrated that kanamycin resistance gene DNA fragments would not be present in the final food additive.

The AEJEG concluded that the manufacture of rebaudioside D produced via enzymatic bioconversion as described within this Application did not pose a toxicological risk to consumer's health.

The safety of rebaudioside D produced via this enzymatic bioconversion had been sufficiently demonstrated to show that it did not pose a concern to consumer's health.

3. Conclusions

To support the FSA and FSS in evaluating the application, the AEJEG was asked to review information submitted by the Applicant and any subsequent additional information requested and advise the FSA and FSS.

The COT also reviewed the AEJEG advice and agreed with the conclusions of the AEJEG.

The FSA and FSS agreed on the conclusions of the AEJEG, in that the proposed change in the steviol glycoside specification to include a new manufacturing process for rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract (≥95% steviol glycosides) using the enzymes uridine diphosphate (UDP)-glucosyltransferase (UGT) and sucrose synthase is safe under the proposed conditions of use and at the anticipated levels of intake.

The AEJEG advised the FSA and FSS that sufficient information had been provided to allow for an evaluation of the proposal for modification of the specifications of steviol glycosides produced from a genetically modified production strain of *K. phaffi*. There were no concerns over the safety of the proposed process.

Regarding the original inconclusive PCR data to demonstrate the lack of the kanamycin resistance gene provided to EFSA, the AEJEG advised the FSA and FSS that the updated PCR analysis provided by the Applicant was sufficient in demonstrating the lack of residual kanamycin resistance DNA in the final product. The FSA and FSS agreed with this conclusion.

The FSA and FSS agreed with the recommendation of the EFSA FAF panel that the current level of arsenic within the specification should be lowered to increase the MOE.

The FSA and FSS concluded in this assessment that the modification of the specifications to include a new manufacturing process for rebaudioside D produced via enzymatic bioconversion of purified stevia leaf extract as described within this application would not pose a risk to health. Therefore, there were no concerns over safety of the proposed process.

Abbreviations

Abbreviation	Definition
AEJEG	Joint Expert Group on Additives, Enzymes, and other Regulated Products
AOXI	Alcohol oxidase
BCA	Bicinchoninic acid
BMDL	Benchmark dose limit
COT	Committee on Toxicity
EFSA	European Food Safety Authority
EU	European Union
FSA	Food Standards Agency
FSS	Food Standards Scotland
GB	Great Britain
HBGV	Health Based Guidance Value
HPLC	High performance liquid chromatography
JECFA	The Joint Food and Agriculture Organisation/World Health Organisation Expert Committee on Food Additives
LC-MS	Liquid chromatography-mass spectrometry
MOE	Margin of exposure
PCR	Polymerase chain reaction
TWI	Tolerable weekly intake
UDP	Uridine diphosphate
UGT	Uridine 5'-diphospho(UDP)-glucosyltransferase

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