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Supporting document 1

Risk and technical assessment – Application A1222

Steviol glycosides from *Yarrowia lipolytica*

Executive summary

This application from Avansya V.O.F (Avansya), a general partnership between Cargill Sweeteners Holding B.V. (Cargill) and DSM Food Specialties Stevia B.V. (DSM), seeks an amendment in the Australia New Zealand Food Standards Code (the Code) to permit the use of a purified mixture of steviol glycosides, called rebaudioside MD by the applicant, produced using a genetically modified (GM) strain of *Yarrowia lipolytica.* This GM yeast contains novel genes involved in steviol glycoside biosynthesis. Most of the novel genes were derived from *Stevia rebaudiana* Bertoni, with additional genes sourced from other plant species.

The food technology assessment concluded that rebaudioside MD manufactured from *Y. lipolytica* that has been modified to express steviol glycoside biosynthesis genes, meets the purity specifications listed in the Code. However, *Y. lipolytica* is not currently an approved organism for producing steviol glycosides via fermentation. The technological purpose of rebaudioside MD matches that of permitted steviol glycoside preparations and meets the proposed purpose as an intense sweetener food additive.

The host *Y. lipolytica* strain is neither pathogenic nor toxigenic and has a long history of safe use in foods. Analysis of the GM production strain confirmed the insertion and stability of steviol glycoside biosynthesis genes. The novel proteins produced by the *Y. lipolytica* strain are not known toxins or allergens, and the final rebaudioside MD did not contain detectable residual protein or DNA from the production organism after purification.

An acceptable daily intake (ADI) of 0-4 mg/kg bodyweight for steviol glycosides, expressed as steviol, was established by FSANZ in 2008. This ADI is appropriate for rebaudioside MD produced from the fermentation of *Y. lipolytica*, as it is chemically identical to the minor steviol glycosides extracted traditionally from the leaves of *Stevia rebaudiana* Bertoni and follows the same metabolic pathway in humans.

No public health and safety concerns have been identified with Avansya's rebaudioside MD produced from *Y. lipolytica* expressing the steviol glycoside biosynthesis pathway.

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1 Introduction

The application from Avansya V.O.F (Avansya), a general partnership between Cargill Sweeteners Holding B.V (Cargill) and DSM Food Specialties Stevia B.V (DSM), seeks an amendment to the Australia New Zealand Food Standards Code (the Code) for approval of a purified steviol glycoside mixture, rebaudioside MD, for use as an intense sweetener. The steviol glycoside mixture is produced by fermentation of simple sugars using *Yarrowia lipolytica* (*Y. lipolytica)* production strain VRM0014, expressing steviol glycoside biosynthesis pathway genes from *Stevia rebaudiana* Bertoni (*S. rebaudiana* Bertoni). This purified steviol glycoside mixture, referred to in this application as rebaudioside MD, is primarily comprised of rebaudioside M with a lesser amount of rebaudioside D, and may contain a mixture of other rebaudiosides in low amounts. Rebaudioside MD is produced for sale as a steviol glycosides preparation, containing a mixture of rebaudiosides[1].

Steviol glycosides preparations are required to conform to a relevant specification in Schedule 3 of the Code. Schedule 3 includes S3—35 which contains specifications for steviol glycosides extracted from the leaves of *S.rebaudiana* Bertoni using hot water extraction and enzymatic conversion. There is also another specification, being S3—39, for steviol glycosides produced by fermentation. Avansya's strain of *Y. lipolytica* is not listed in S3—39, therefore Avansya's rebaudioside MD is currently not a permitted steviol glycosides preparation.

Steviol glycosides, including rebaudioside MD, are already permitted for use as a food additive in the Code, with maximum permitted levels (MPL) in a variety of food categories and at Good Manufacturing Practices (GMP) levels in tabletop sweeteners in Schedule 15. Avansya states that rebaudioside MD provides an improved sensory profile and therefore a better sweetness quality when compared to major steviol glycosides derived from leaf, such as rebaudioside A and stevioside. Avansya also claims rebaudioside MD has similar stability to other steviol glycosides preparations, making it suitable for a wide variety of applications, functioning as a multi-purpose and low-calorie sweetener. Avansya's rebaudioside MD is stated to be chemically and substantially equivalent to rebaudioside MD produced from *Saccharomyces cerevisiae* strain CD15407 (previously submitted by Cargill under A1170).

If approved, Avansya's rebaudioside MD will be an alternative sweetener to other steviol glycosides preparations.

1.1 Objectives of the assessment

The objectives of this risk assessment were to:

- determine whether the proposed purpose is clearly stated and that Avansya's rebaudioside MD achieves its technological function in the quantity and form proposed to be used as a food additive;
- evaluate any potential public health and safety issues that may arise from the use of Avansya's rebaudioside MD, produced by fermentation of simple sugars using a *Y. lipolytica* production strain, expressing steviol glycoside biosynthesis pathway genes.

[1] Note that the steviol glycosides preparation that was the subject of A1170 is referred to as rebaudioside MD. It is a mixture of rebaudioside M and rebaudioside D. Under this application (A1222) the distribution of steviol glycosides is similar and the preparations are considered chemically equivalent. For consistency, the preparation under this application is also referred to as rebaudioside MD.

2 Food technology assessment

The food technology assessment of this application is similar to that of earlier applications, A1170 and A1207, which also relate to the production of steviol glycosides using a microbial fermentation production method for their steviol glycosides preparation.

2.1 Identity of the substance

Steviol glycosides are a group of compounds naturally occurring in the *S. rebaudiana* Bertoni (stevia) plant. The major glycosides present in the extract of the leaves from the stevia plant are stevioside and rebaudioside A. The minor glycosides include rebaudioside M and rebaudioside D and about 40 other steviol glycosides (JECFA 2019a).

All steviol glycosides share the same steviol backbone structure but have different sugar moieties attached, as conjugated glycosides. The sugar moieties include but are not limited to glucose, rhamnose, xylose, fructose, galactose and deoxyglucose, which can be attached in various combinations, quantity and orientation (FAO 2017, JECFA 2017).

Table B.6.4.1-1 of the application shows the compositional analyses for 5 commercial lots of rebaudioside MD. The distribution of steviol glycosides present in rebaudioside MD will vary, and is typically 85 – 96% rebaudioside M, and 3 – 11% rebaudioside D, with lesser amounts of other steviol glycosides.[2]

The chemical information for rebaudiosides M and D is provided in Table 1 below (FAO 2017, JECFA 2017).

Table 1 *Chemical information for rebaudiosides M and D*

	Rebaudioside M	**Rebaudioside D**
Chemical name	13-[(*O*-β- D-glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-*O*-β- D-glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl ester	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
Molecular formula	$C_{56}H_{90}O_{33}$	$C_{50}H_{80}O_{28}$
Molecular weight g mol^{-1}	1291	1129
CAS[3] number	1220616-44-3	63279-13-0

[2] Percentages expressed as % dry weight
[3] Chemical Abstracts Service

2.2 Physical and chemical properties

Avansya's rebaudioside MD is a white to light yellow powder with a characteristic sweet taste, consistent with the description of commercial steviol glycoside preparations in the most recent Chemical and Technical Assessment (CTA) published by the Food and Agriculture Organization of the United Nations (FAO)/JECFA for steviol glycosides (FAO 2017).

The applicant states that the rebaudioside MD preparation produced by the genetically modified *Y. lipolytica* strain VRM0014 is chemically and substantially equivalent to rebaudioside MD that is obtained from *S. cerevisiae* strain CD15407 (S3—39) (the subject of Application A1170).

Section B.2.1 of the application demonstrates that steviol glycosides produced by *Y. lipolytica* VRM0014 have the same HPLC retention times as steviol glycosides from *S.rebaudiana* Bertoni and establish that steviol glycosides from these two sources are chemically identical.

Rebaudioside MD is freely soluble in water. Further information on the physical and chemical properties is contained in section 2.5 below.

2.3 Method of production

Rebaudioside MD is a purified steviol glycoside mixture that is produced via fermentation of simple sugars (including dextrose and sucrose) using a *Y. lipolytica* production strain that has been engineered to produce steviol glycosides. Rebaudioside MD is manufactured in accordance with current Good Manufacturing Practices (cGMP[4]). Following fermentation, rebaudioside MD is purified in accordance with the methodologies outlined in the 2017 CTA (FAO 2017). A detailed description of the manufacturing process, including the raw materials, processing aids and equipment used in the production process can be found on pages 24-28 of the application.

2.4 Product stability

General stability of steviol glycosides in products

JECFA concluded at its 68th meeting in 2007 that steviol glycosides are sufficiently thermally and hydrolytically stable for use in foods, including acidic beverages, under normal conditions of processing and storage (JECFA, 2007). Study results made available to JECFA for the 82nd meeting supported that the stability results can be extended to include steviol glycoside extract preparations containing higher levels of new glycosides added to the definition and appearing in commercial products, mainly rebaudioside D and rebaudioside M (FAO 2017). These publications therefore support the general stability of steviol glycosides, including a preparation such as the applicants rebaudioside MD.

Stability of Avansya's rebaudioside MD over shelf life

To assess the stability of the steviol glycosides preparation, Avansya provided results of a 3-month conventional and accelerated stability study conducted on 3 non-consecutive commercial lots of their rebaudioside MD preparation. Continued time point testing is underway.

For the conventional shelf life study (25°C and 60% relative humidity), the results are

[4]cGMP are practices established by the United States Food and Drug Administration (US FDA) within its Code of Federation Regulations to ensure the safe production of food.

provided in Table B.1.2.2-1 of the application, and support stability for up to three months.

Under accelerated testing conditions (40°C and 75% relative humidity), available results are provided in Table B.1.2.2.-2 of the application, and also support stability of the rebaudioside MD preparation under accelerated conditions for up to three months.

The parameters measured were moisture content, loss on drying, steviol glycoside content (total and individual), and microbial parameters.

2.5 Specifications for the substance

International specifications for purity of steviol glycosides are provided within primary sources of specification within section S3—2 of Schedule 3 (Identity and purity). These are S3—2(1)(b) [the FAO JECFA Monograph], S3-2(1)(c) [the Food and Chemicals Codex], or S3—2(1)(d) [European Commission Regulation No 231/2012 (EU 2012) laying down specifications for food additives]. All these international steviol glycosides specifications stipulate that the total percentage of steviol glycosides must be greater than or equal to 95% of the preparation, on a dry basis.

The current JECFA steviol glycosides monograph is monograph 20 from the 84th JECFA meeting in 2017 (JECFA 2017). It is important to note that the steviol glycosides specifications from the 87th JECFA meeting in 2019 (JECFA 2020) have not yet been discussed by the Codex Committee on Food Additives (CCFA) or ultimately ratified by the Codex Alimentarius Committee (CAC). Therefore these specifications are not yet part of the official JECFA Combined Compendium of Food Additive Specifications.

Schedule 3 also contains a specification relevant to this application, being S3—39 – Specification for steviol glycosides from fermentation. Avansya's strain of *Y. lipolytica* is not listed in S3—39, but the purity requirements are relevant. Under the Code, steviol glycosides must meet an assay value of not less than 95% total steviol glycoside content, on a dry weight basis, however the individual steviol glycosides can be in any combination and ratio.

Table B.6.4.1-1 of the application also shows the total steviol glycosides expressed as % dry weight. The total steviol glycoside content ranges from 97 – 100%. The applicant's purified rebaudioside MD preparation therefore meets or exceeds the ≥95% steviol glycoside purity definition for steviol glycosides from *S. rebaudiana* established by JECFA (JECFA 2017).

To confirm the success of the purification steps in the manufacturing process and to confirm the absence of residual protein in the rebaudioside MD preparation, samples from 5 non-consecutive lots of final product were assayed by the bicinchoninic acid (BCA) protein assay, carried out in triplicate. As stated in section B.6.4.2 of the application, no residual protein was detected in any of the test samples, above the limit of detection of 25 ppm for the assay.

To confirm the absence of residual novel DNA in the rebaudioside MD preparation, samples from the same 5 non-consecutive lots of final product were assayed by polymerase chain reaction (PCR). In all rebaudioside MD samples, no genomic DNA was detected above 10 ng/g detection parameters, confirming the absence of novel DNA in the rebaudioside MD preparation (final product).

Table 2 *Comparison of Avansya's rebaudioside MD analyses compared to requirements of JECFA[a], FCC[b], EU[c] and relevant Code (S3—39) purity specifications*

Parameter	Avansya	JECFA	EU	FCC	Code (S3—39)
Appearance/description	White to light yellow powder	White to light yellow powder	White to light yellow powder	White to light yellow powder	White to light yellow powder
Purity (%) SG (dried basis)	97.2, 100.2, 99.7, 97.5, 99.4	≥ 95	≥ 95	≥ 95	≥ 95
Solubility	Freely soluble in water	Freely soluble in 50:50 ethanol:water	Freely soluble to slightly soluble in water	Freely soluble in 50:50 ethanol:water	Freely soluble in water
pH (1% solution)	5.4, 4.8, 5.2, 4.9, 5.0	4.5-7.0	4.5-7.0	4.5-7.0	4.5-7.0
Total ash (%)	<1.0, <0.06, <0.04, <0.04, <0.06	≤1	≤1	≤1	≤1
Loss on drying (105°C, 2 hr)	0.5, 0.85, 3.0, 2.7, 1.3	≤6	≤6	≤6	≤6
Residual ethanol (mg/kg)	0.45, 0.08, 0.24, 0.39, .033	≤5000	≤5000	≤5000	≤5000
Residual methanol (mg/kg	All <200	≤200	≤200	≤200	≤200
Lead (mg/kg	All <1.0	≤1.0	≤1.0	≤1.0	≤1.0
Arsenic (mg/kg)	All <1.0	≤1.0	≤1.0	≤1.0	≤1.0
Cadmium (mg/kg)	All <1.0	-	-	-	≤1.0
Mercury (mg/kg)	All <1.0	-	-	-	≤1.0
Total (aerobic) plate count (CFU/g)	All <10	≤1000	-	-	-
Yeast and moulds (CFU/g)	All <10	≤200	-	-	-
E. coli	All negative	Negative/1 g	-	-	-
Salmonella	All negative	Negative/25 g	-	-	-
Protein (ng/ml)*	All ND below 25 ppm		-	-	-
DNA (pg/ul)*	All ND below 10 ng/g		-	-	-

Table notes

a Joint FAO/WHO Expert Committee on Food Additives (JECFA 2017)

b Food Chemicals Codex

c European Union, European Commission Regulations No 231/2012 and 2016/1814

* Values for five non-consecutive lots of Avansya's rebaudioside MD

ND Not detected

2.6 Analytical methods for detection

There are well established internationally recognised analytical methods of detection for steviol glycosides. The applicant's analytical method of analysis of its rebaudioside MD is by a modified high-performance liquid chromatography (HPLC) method. The method is confidential commercial information, with a summary provided in section B.8 of the application. There are two analytical methods of analysis using HPLC provided within the 2017 JECFA Steviol glycosides specification (pages 52 – 56) (JECFA 2017). The applicant's method is modified from a JECFA HPLC method, to optimise the analysis to account for steviol glycosides with a higher degree of glycosylation i.e. rebaudiosides M and D.

2.7 Technological purpose

Steviol glycosides extracted or derived from the leaves of *S. rebaudiana* Bertoni, including rebaudioside MD, are already permitted for use as food additives in the Code, with the International Numbering System (INS) assignation 960. The technological purpose of steviol glycosides as a food additive is that of an intense sweetener, which replaces the sweetness normally provided by sugars in food, without contributing significantly to their available energy. Rebaudioside MD, similar to other already permitted steviol glycosides preparations for use in food and beverages in Australia and New Zealand, will be used as an intense sweetener for the replacement of sucrose in reduced-calorie or no-sugar-added products. Steviol glycosides are permitted at various maximum permitted levels in a variety of food classes and at GMP level for tabletop sweeteners in Schedule 15. The technological purpose of this particular rebaudioside MD from the applicant does not differ from currently permitted steviol glycosides, rather it is the production method that differs.

2.8 Technological justification

The primary reason for developing alternative methods to the traditional extraction methods for steviol glycosides is that not all glycosides are produced to the same degree in the leaves of *S. rebaudiana* Bertoni. Those steviol glycosides present at higher levels are called major glycosides (such as rebaudioside A and stevioside). As noted in section 2.1 above, rebaudiosides M and D are minor glycosides, as they are present at much lower levels in the leaf. The applicant states that its rebaudioside MD preparation provides improved sensory characteristics over major steviol glycosides such as stevioside and rebaudioside A, and also has similar stability, making it suitable for a wide variety of applications, functioning as a multi-purpose and low-calorie sweetener. The fermentation method used by Avansya is technologically justified in that it yields a higher amount of rebaudioside M and D, compared to the low levels in the stevia leaf.

2.9 Food technology conclusion

The food technology assessment concludes that the applicant's rebaudioside MD preparation produced by fermentation of simple sugars using the applicant's genetically modified *Y. lipolytica* strain VRM0014, expressing steviol glycoside biosynthesis pathway genes, meets the purity parameters of specifications currently listed in the Code – but not the specific method of production. These purity parameters are also consistent with international purity specifications for steviol glycosides. The rebaudioside MD preparation is also thermally and hydrolytically stable for food use. Its technological purpose matches that of permitted steviol glycosides preparations produced by the currently permitted methods and meets the proposed purpose as an intense sweetener food additive.

3 Risk assessment

3.1 Safety assessment of the genetically modified production strain

Some information relevant to this section is Confidential Commercial Information (CCI), so full details cannot be provided in this public report.

3.1.1 History of use

3.1.1.1 Host organism

Y. lipolytica is an ascomycete yeast that is widespread in nature. Although the primary habitat of *Y. lipolytica* is unknown, it is often found in marine environments, including hypersaline water (Groenewald et al, 2014; Kurtzman, 2011; Butinar et al, 2005). *Y. lipolytica* is often naturally found in foods, primarily those with high proportions of fat and/or protein, such as fermented dairy and meat products (Groenewald et al, 2014).

Y. lipolytica is generally classified as a biosafety level 1 organism, based on the United States Public Health Service Guidelines[5], and is not known to cause disease in healthy adult humans. However, opportunistic infections by *Y. lipolytica* occur occasionally in immunocompromised individuals, generally related to the use of intravenous catheters (Groenewald et al, 2014). There are no reports of toxin production by *Y. lipolytica*, apart from their role in biogenic amine formation in cheese and meat (Groenewald et al, 2014).

Y. lipolytica has been granted Qualified Presumption of Safety[6] (QPS) status by the European Food Safety Authority (EFSA), with the qualification that the strain is for production purposes only, which implies the absence of viable cells of the production organism in the final product. During the manufacturing process the fermentation broth (containing *Y. lipolytica*) undergoes heat treatment to kill the yeast cells, followed by purification of the rebaudioside MD product. The host organism was not detected in the final product, so would not enter the food chain. This was confirmed by analysis of five non-consecutive lots of final rebaudioside MD product, with residual DNA and protein levels below the level of detection[7]. Therefore there is no safety concern.

The production strain *Y. lipolytica* VRM0014 was derived from three parental *Y. lipolytica* strains (ATCC 76861, 76982 and 201249), all of which are classed as biosafety level 1 organisms. The taxonomy of the production strain was confirmed as *Y. lipolytica*, based on 100% identity of the 18s rRNA sequence.

3.1.1.2 Gene donor organism(s)

The applicant has provided information about the identity and source of donor genetic material used in the construction of the production organism. Donor genes introduced into the production strain were sourced from biosafety level 1 organisms or derived through chemical synthesis, where they were codon optimised for expression in *Y. lipolytica*. All

[5] For more information see the following CDC webpage: https://www.cdc.gov/biosafety/publications/bmbl5/index.htm

[6] For more information see the following EFSA webpage: https://www.efsa.europa.eu/en/topics/topic/qualified-presumption-safety-qps

[7] The genomic DNA limit of detection was 10ng/g, with genomic DNA extracted from the production strain used as a positive control. The residual protein limit of detection was 25 ppm, with samples compared against an analytical standard of high purity rebaudioside M. Refer to Sections B.6.4.2-B.6.4.3 of the application.

donor genes were introduced using standard transformation techniques such that there is no potential for carryover of any pathogenic, toxigenic or allergenic factors from any of the donor organisms.

Genes involved in steviol glycoside biosynthesis were obtained from *S. rebaudiana* Bertoni, a member of the daisy family (Asteraceae), which includes lettuce and artichoke. The leaves of *S. rebaudiana* Bertoni have been used in South America for centuries, including in drinks such as maté, a green herbal tea (Samuel et al., 2018). The leaves of *S. rebaudiana* contain steviol glycosides, which provide sweetness to the tea. Thus, this plant has a long history of safe use. Other genes for steviol glycoside production were obtained from other plants.

3.1.2 Description of DNA to be introduced and method of transformation

Steviol glycoside production genes were introduced into the *Y. lipolytica* host using multiple expression cassettes. An expression cassette includes an open reading frame and any associated regulatory elements, e.g. a gene in the rebaudioside M pathway, a promoter and a terminator. The introduced genes encode proteins required for steviol glycoside biosynthesis and transport, and to improve the overall production efficacy of the *Y. lipolytica* production strain, i.e. to direct the metabolism of *Y. lipolytica* towards steviol glycoside production.

Expression cassettes were inserted into the host's genome using standard transformation techniques (e.g. lithium acetate/polyethylene glycol) and involved multiple steps. First, expression cassettes were introduced into two *Y. lipolytica* strains of opposing mating types. Next, these strains were mated to produce diploids, which were then sporulated to produce haploid progeny. A single haploid stain was genetically modified further. The final production strain VRM0014 has DNA integrated either randomly or site-specifically (e.g. at a pre-defined locus based on homologous recombination). Classical mutagenesis was used to improve the strain.

Antibiotic resistance markers were also used in the construction of the production strain. Resistance to antibiotics was used to select transformants with DNA integrations. The presence of antibiotic resistance markers in the final production organism was examined (see Section 3.1.3).

3.1.3 Characterisation of inserted DNA

Whole genome sequencing was performed on the final production organism. Analysis of this data showed that the genes required for steviol glycoside biosynthesis were integrated into the host genome. The applicant provided data that confirmed the final production strain was antibiotic resistance marker free.

3.1.4 Genetic stability of the inserted genes

Data on the stability of the production strain was provided by the applicant as an indirect means to show genetic stability of the inserted genes. That is, if the inserted genes were not stably maintained or expressed there would be a loss of steviol glycoside production.

Over the course of a number of fermentations the performance of the production strain in maintaining steviol glycoside production was shown. It can be concluded that the inserted genes have been stably integrated into the production strain's genome.

3.1.5 Safety of novel proteins

The potential toxicity of each protein added to the *Y. lipolytica* production strain was

evaluated using a bioinformatic approach, where the novel protein sequence was queried against known venom, toxins or virulence factors annotated in the UniProt[8] database using a BLAST search.

The bioinformatics search did not identify any known venoms or toxins under the search parameters. Potential virulence factors were matched to multiple novel proteins introduced into the genetically modified *Y. lipolytica* VRM0014 production strain, most notably cytochrome P450 monooxygenases. The specific identity of the novel protein and the identity of the potential virulence factors has been classified as commercial confidential information and is not described in this supporting document. Importantly, the *Y. lipolytica* VRM0014 production strain and any residual DNA (with a limit of detection of 10ng/g) is removed from the final commercial product during the manufacturing process.

FSANZ has reviewed the bioinformatic results supplied by the applicant as part of the safety assessment. Considering the identity of the donor genes, the nature of the potential virulence factors identified in bioinformatic analysis and that the production strain and any residual DNA and protein are removed from the final commercial product; the novel proteins produced by *Y. lipolytica* VRM0014 do not pose a safety risk to consumers.

3.2 Toxicological assessment

FSANZ established an ADI for steviol glycosides of 0-4 mg/kg bw/day steviol in 2008 as a part of application A540 (FSANZ 2008). The ADI was derived by applying a 100-fold safety factor to the NOEL of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol) based on a two-year rat study.

FSANZ updated the hazard assessment for steviol glycosides as a part of the following applications:

- A1207: Rebaudioside M as a Steviol Glycoside from *Saccharomyces cerevisiae* (2021)
- A1183: Enzymatic production of Rebaudioside E (2020)
- A1176: Enzymatic production of Steviol Glycosides (2019)
- A1172: Enzymatic production of Rebaudioside D (2019)
- A1170: Rebaudioside MD as a Steviol Glycoside from *Saccharomyces cerevisiae* (2019)
- A1157: Enzymatic production of Rebaudioside M (2018)
- A1149: Addition of Steviol Glycosides in Fruit Drinks (2018)
- A1137: Broaden Definition of Steviol Glycosides (2017)
- A1108: Rebaudioside M as a Steviol Glycoside Intense Sweetener (2015)

These assessments did not identify a need to change the ADI initially established by FSANZ.

3.2.1 Toxicity of rebaudioside MD

All known steviol glycosides share a common metabolic pathway. Degradation of steviol glycosides occurs in the colon where gut microflora hydrolyse and utilise the attached sugar moieties as an energy source. The remaining steviol core is absorbed into the body where it is conjugated to steviol glucuronide in the liver and excreted, predominantly in the urine. The number of sugar moieties attached to the steviol glycoside and the types of sugar present in those moieties, do not have any marked effect on the rate of microbial hydrolysis.

The applicant supplied a microbial degradation study to demonstrate that the rebaudioside MD mixture produced by genetically modified *Y. lipolytica* follows the same metabolic fate as

[8] For more information or to access the UniProt database see the following webpage: https://www.uniprot.org/

other steviol glycosides and is degraded by gut microflora to free steviol.

FSANZ also conducted a literature search to capture scientific studies relevant to safety that have been published since A1207, using the PubMed and EBSCO online databases and the search terms 'steviol' or 'rebaudioside' and 'toxic', 'safety', 'hazard' or 'adverse'. Two relevant studies were located that address the safety and metabolic fate of steviol glycosides in humans.

In vitro anaerobic metabolism study of rebaudiosides MD in human fecal homogenates (2020). Regulatory status: Non-GLP

This study was submitted by the applicant and the test article, rebaudioside MD produced from *Y. lipolytica,* was representative of the commercial product under review.

The test system was prepared from stool samples from six healthy adults of each sex. Two pooled homogenates from male subjects and two from female subjects, were prepared by diluting, centrifugation and collection of the supernatant. The test article was mixed with each faecal homogenate sample in triplicate and incubated at 37°C, under anaerobic conditions, for 0, 4, 8, 12, 16, 24 or 48h. Metabolic deglycosylation of rebaudioside MD was determined by measuring the increasing concentration of free steviol in each test sample, using liquid chromatography mass spectrometry.

Results showed that rebaudioside MD underwent rapid deglycosylation to produce free steviol. Deglycosylation reached completion within 24 hours, independent of the faecal donor gender. Rebaudioside A extracted from *S. rebaudiana* Bertoni, used as the positive control, showed a comparable rate of deglycosylation to the test item.

Systematic review of steviol glycosides and key characteristics of carcinogens (Chappel 2021).

A systematic review was undertaken to analyse the available data investigating a potential link between steviol glycosides and carcinogenicity. The review considered *in vitro* and *in vivo* data, in mammalian and non-mammalian studies, that were identified in the PubMed and Embase scientific literature databases, and the ToxCast/Tox21 high throughput screening databases. Using predetermined criteria, study results were weighted on quality and relevance, and combined together to consider the total evidence available for each key characteristic of carcinogenicity.

The authors concluded that exposure to steviol glycosides does not pose a carcinogenic hazard to humans.

Metabolic fate of steviol glycosides in adults and children (Purkayastha 2020). Regulatory status: not GLP

This study compared the metabolic fate in faecal homogenate of steviol glycosides preparations produced by either stevia leaf extraction, enzymatic bioconversion or enzymatic glycosylation. Each test sample began with a commercial mixture of steviol glycosides extracted from stevia leaves that contained greater than 95% stevia glycosides. Faecal samples were collected from adult female, adult male or child (2-3 years old) donors. Steviol glycoside fate over time was measured using liquid chromatography mass spectrometry.

Stevia leaf extract was fully degraded to free steviol by faecal homogenate from each donor group, irrespective of donor age or gender.

This study is of limited value for regulatory purposes due to limitations in reporting (e.g. test

item description) and statistical analysis.

3.2.2 Allergenicity of rebaudioside MD

A bioinformatic approach was used to evaluate the allergenicity of the novel proteins introduced into the *Y. lipolitica* production strain. The protein sequence of each novel protein was queried against the AllergenOnline[9] database (queried in February 2020) using the full length protein sequences (>35% homology) or an 80 mer sliding window (>35% sequence homology). The bioinformatics search did not identify any known allergens using these search parameters. Furthermore, analytical results of five non-consecutive batches of the rebaudioside MD produced by *Y. lipolitica* VRM0014, which is the subject of this application, did not contain residual protein (with a limit of detection of 25 ppm) when tested using a bicinchoninic acid protein assay.

On that basis rebaudioside MD is highly unlikely to pose an allergen risk to consumers.

3.3 Assessment by other international bodies and food agencies

3.3.1 European Food Safety Authority

EFSA evaluated the safety of steviol glycosides as a food additive in 2010 and established an ADI of 4 mg/kg bw/day expressed as steviol equivalents, which was consistent with the ADI established by JECFA and FSANZ (EFSA 2010; FSANZ 2008; JECFA 2007). An updated assessment in 2015 (EFSA 2015) included rebaudioside D and M in the specifications for steviol glycosides. Currently, eleven steviol glycosides are authorised as a food additive in the European Union, with specifications requiring that the composition of purified food additive must contain more than 95% authorised steviol glycosides.

In 2020, the EFSA panel on Food Additives and Flavourings published an opinion on an applicant-submitted proposal to amend the EU specifications for steviol glycosides. The applicant proposed expanding the definition of steviol glycoside to include all 60 steviol glycosides present in *S. rebaudiana* Bertoni and to include microbial safety limits (EFSA 2020).

The EFSA panel supported the proposed introduction of microbial safety limits and by considering the common metabolic fate of all steviol glycosides in the colon, agreed that a read-across approach to toxicological safety assessment was appropriate to support the safety all 60 steviol glycosides. However, the EFSA panel noted that if the current steviol glycoside specifications were expanded to include all 60 steviol glycosides, this would change how product purity was calculated and thus products of lower quality than is currently accepted could be permitted into the European market (EFSA 2020).

3.3.2 Joint FAO/WHO Expert Committee on Food Additives

The FSANZ ADI is consistent with the ADI established by JECFA at the 69th meeting held in 2008 (JECFA 2009). JECFA re-assessed steviol glycosides at the 82nd meeting in 2016 and confirmed the existing ADI. The assessments confirmed that steviol glycosides share a metabolic pathway to steviol. The ADI, expressed as steviol, is therefore appropriate for all steviol glycosides.

The 87th JECFA meeting in 2019 developed a framework for steviol glycosides, building on JECFA's earlier assessments and superseding the JECFA specification developed at the 84th

[9] For more information or to access the AllergenOnline database see the following webpage: http://www.allergenonline.org

JECFA meeting in 2017. The 87th meeting also prepared four steviol glycosides specification annexes, for the different production methods (JECFA 2020). These are:

- Hot water extraction from the leaves of *S. rebaudiana* Bertoni (stevia) plant (Annex 1)
- Fermentation using GM microorganisms (Annex 2)
- Enzymatic modification (bioconversion) of the stevia plant extract using enzymes (Annex 3)
- Glycosylation of stevia plant extracts using enzymes to add glucose units to steviol glycosides (Annex 4, tentative pending further information to finalise).

The meeting confirmed that steviol glycosides prepared using these production methods, which comply with the specifications for different production methods and purity requirements, were considered equivalent in terms of safety (JECFA 2019a, JECFA 2019b).

These specifications are yet to be discussed by the CCFA or ratified by the Codex Alimentarius Committee (CAC). Therefore, the new specifications are not part of the official JECFA Combined Compendium of Food Additive Specifications.

3.4 Risk assessment discussion and conclusion

The safety assessment did not identify any concerns associated with the host organism *Y. lipolytica,* or the novel proteins expressed by the introduced genes for the biosynthesis of rebaudioside MD. The host *Y. lipolytica* production strain is neither pathogenic nor toxigenic and has a long history of safe use in foods. Characterisation of the genetically modified production strain confirmed both the insertion and stable inheritance of steviol glycoside biosynthesis genes. Neither the host organism, nor residual DNA or residual protein were detectable in the final rebaudioside MD preparation.

No new evidence of adverse effects of steviol glycosides has been identified that would justify a change in the ADI established by FSANZ in 2008. The applicant provided an *in vitro* anaerobic metabolism study demonstrating that the metabolic fate of rebaudioside MD is equivalent to other steviol glycosides previously assessed by FSANZ. Therefore the ADI of 0-4 mg/kg bw expressed as steviol, is appropriate for the rebaudioside MD preparation produced using genetically modified *Y. lipolytica* VRM0014 that is the subject of this application.

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