SUMMARY OF DATA FOR A GRAS CONCLUSION

Purified Steviol Glycosides (Rebaudioside M) produced by *Yarrowia lipolytica*

January 16, 2018

Prepared by: Katherine Vega
Senior Manager Regulatory Affairs
DSM Nutritional Products North America
45 Waterview Boulevard
Parsippany, New Jersey 07054

Jeanine van de Wiel
DSM Food Specialties
Alexander Fleminglaan 1
2613 AX Delft
Netherlands

DSM-ID4045-001

Contents

List of Tables and Figures

List of Annexes

Annex 1: Specifications for DSM Steviol Glycosides (Rebaudioside M) produced by *Yarrowia lipolytica*

Annex 2: Pariza & Johnson (2001) Decision Tree

Annex 3: Fermentation Media Components

Annex 4: Production Strain Control

Annex 5: Certificates of Analysis

Annex 6: rDNA detection in pilot batches of DSM Steviol Glycosides (Rebaudioside M) produced by *Yarrowia lipolytica*

Part 1: Signed Statements and Certification

1.1 Submission of GRAS Notice

DSM Food Specialties (DSM) is hereby submitting a Generally Recognized as Safe (GRAS) notice in accordance with the provisions of 21 CFR part 170, subpart E.

1.2 Name and Address of Notifier

DSM Nutritional Products North America
45 Waterview Blvd.
Parsippany, New Jersey, 07054, USA
Tel: 973-257-8500

1.3 Name of the Substance

The notified substance consists of purified steviol glycosides produced by *Yarrowia lipolytica* with rebaudioside M (Reb M) as the principal component. DSM proposes that the notified substance is appropriately described as rebaudioside M, Reb M, or steviol glycosides.

1.4 Intended Conditions of Use

DSM's purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica* will be marketed for use as a flavor and general-purpose sweetener in foods such as beverages, baked goods, confections, and dairy products intended for the general human population. It is not intended for use in infant formula or meat and poultry products. The intended use levels will vary by actual food category. The substance will be used at levels that do not exceed the amounts reasonably required to accomplish its intended effect in foods and in accordance with current Good Manufacturing Practices (cGMP).

1.5 Statutory Basis for the GRAS Conclusion

This GRAS conclusion is based upon scientific procedures in accordance with § 170.30(a) and (b).

1.6 Exclusion from Premarket Approval Requirements

The notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on the conclusion by DSM that the substance is GRAS under the conditions of its intended use.

1.7 Availability of Information

The complete data and information that are the basis of the GRAS conclusion will be made available to the Food and Drug Administration. Upon request, DSM will provide access to review and copy the data during customary business hours at its facility in Parsippany, New Jersey, or, upon request, will provide copies in electronic format or on paper.

1.8 Freedom of Information Act (FOIA) Exemptions

Parts 2 through 7 of this notification do not contain data or information that are exempt from disclosure under the Freedom of Information Act.

1.9 Certification

To the best of our knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to DSM and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

(b) (6)

Katherine Vega, PhD
Senior Manager, Regulatory Affairs

Part 2: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect of the Notified Substance

2.1 Identity

The notified substance consists of purified steviol glycosides with rebaudioside M (Reb M) as the primary component, produced by a strain of *Yarrowia lipolytica* genetically engineered to contain and express the steviol glycoside biosynthetic pathway of the stevia plant, *Stevia rebaudiana*.

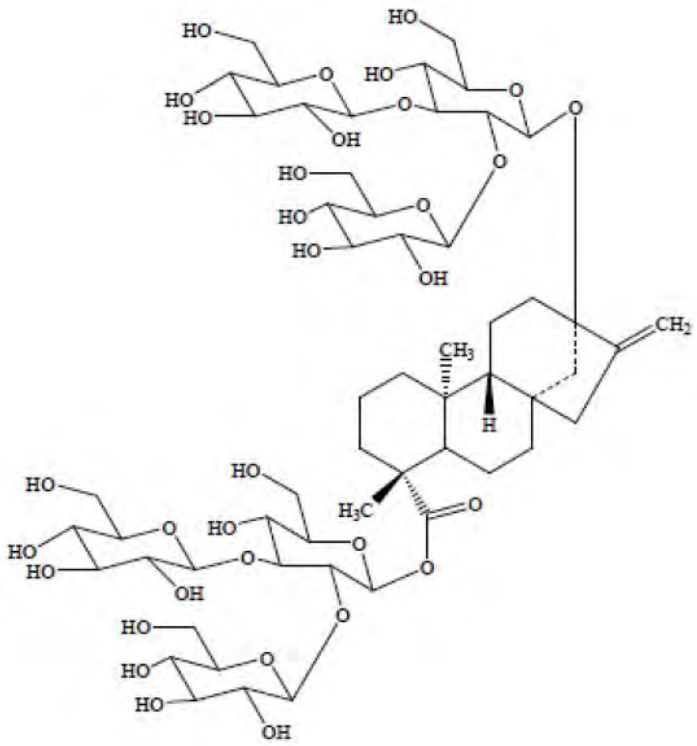
Chemical Name: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester

CAS Number: 1220616-44-3

Chemical Formula: $C_{56}H_{90}O_{33}$

Molar Mass: 1291.30 g/mol

Figure 2-1 Molecular structure of the steviol glycoside rebaudioside M



2.2 Specifications

The specifications for DSM purified steviol glycosides (Rebaudioside M) produced by *Yarrowia lipolytica* are shown below. [1] A specification sheet is also provided in Annex 1.

Physical properties

Appearance	Off-white to white powder
Odor	Odorless or slight characteristic
Moisture content by loss on drying	≤ 10%
Ash	≤ 1%
Solubility in purified water at room temperature (20°C)	Freely soluble to slightly soluble

Chemical Composition

Rebaudioside M (on dry basis)	≥ 95 %
Total steviol glycosides (on dry basis)	> 95 %
pH (1 gram dissolved in 1 l of water)	4.5 – 7.0
Heavy Metals	
Lead	< 1 ppm
Mercury	< 1 ppm
Cadmium	< 1 ppm
Arsenic	< 1 ppm

Microbiological Criteria

Total plate count	≤ 1000 CFU in 1 g
Yeast	≤ 100 CFU in 1 g
Mold	≤ 100 CFU in 1 g
Coliform	≤ 10 CFU in 1 g

Allergens: The finished goods are free of allergenic proteins, because there are no allergenic proteins in the fermentation media and the production organism is not known to produce allergenic proteins.

[1] Although there are no established regulatory specifications for food-grade rebaudioside M (Reb M), DSM has taken an approach similar to that of other GRAS notices with specifications based largely on those of JECFA and the Food Chemicals Codex (FCC, 2010) for steviol glycosides.

2.3 Description of the production organism

2.3.1 Classification of the organism: *Yarrowia lipolytica*

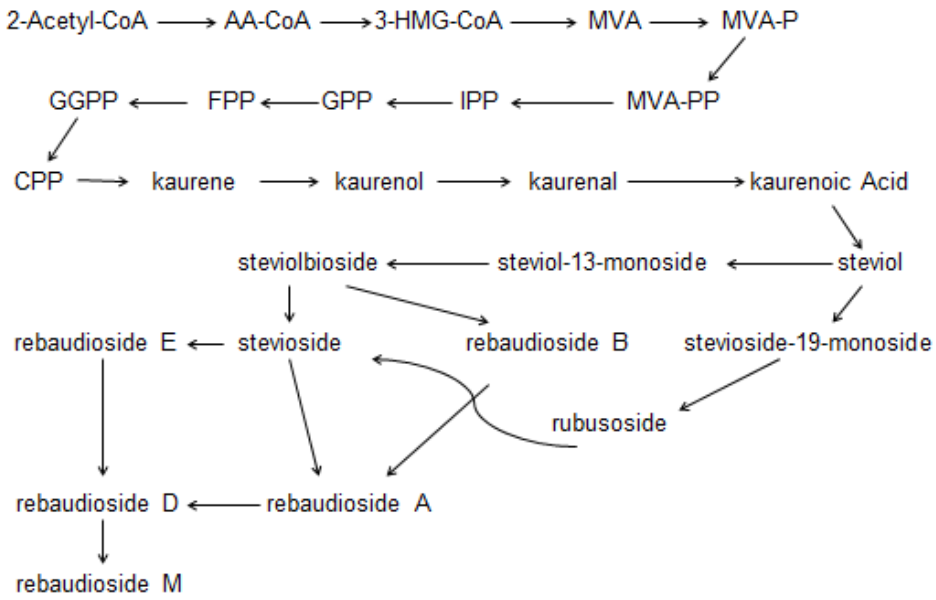
Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Saccharomycetes
Order:	Saccharomycetales
Family:	Dipodascaceae
Genus:	Yarrowia
Species:	lipolytica

2.3.2 Modifications to the production microorganism

The original strains used by DSM were obtained from the American Type Culture Collection (ATCC). The parent strains of *Yarrowia lipolytica* have been modified to overexpress the genes responsible for the production of steviol glycosides, especially rebaudioside M in this case. Most of the genes originate from the plant *Stevia rebaudiana* (but were produced synthetically and are adapted with respect to codon usage for optimal expression in the yeast). *Stevia rebaudiana* is the current botanical source of the steviol glycosides. The introduced DNA sequences are integrated in the genome of the host-organism, partly in pre-defined loci (targeted integration) but mostly randomly. As the yeast *Yarrowia lipolytica* is not known to harbor any genes encoding for toxins or otherwise harmful sequences, neither random nor targeted introduction of DNA sequences will lead to an increased risk due to unintended pleiotropic effects.

The pathway for the production of steviol glycosides is shown in Figure 2-2.

Figure 2-2 Overview of the steviol glycoside biosynthetic pathway



The mevalonate pathway serves as a supply of precursors for the production of steviol glycosides. The biosynthesis pathway is described in more detail in Brandle and Telmer, 2007.

2.3.3 Parental strains

Three parental strains of *Yarrowia lipolytica* were obtained directly from the ATCC and used to generate two starting strains. The intention was to begin the strain construction with two strains that had opposite mating types to allow for subsequent mating and natural polymorphic variation. Both strains were engineered with the steviol glycoside production pathways; these were mated, sporulated, and the spores were screened for high steviol glycoside production. The production strain was derived from one of these spores.

2.3.4 Genetic engineering of the production strain

The genetic engineering of the production organism is covered by several patents and patent applications listed in Table 2-1.

Table 2-1 List of patent and patent applications for the production organism

Subject matter	Priority	Filing	Published	Published as
Metabolic engineering of rebaudioside production *in S. cerevisiae* and *Y. lipolytica*	23 Jan 2012 16 Nov 2012	23 Jan 2013	01 Aug 2013	WO2013/110673
Extracellular production of rebaudiosides in metabolically engineered cells	31 May 2013	2 Jun 2014	04 Dec 2014	WO2014/191580
Rebaudioside production in deletion mutants	31 May 2013	2 Jun 2014	04 Dec 2014	WO2014/191581
Reb M production in metabolically engineered cells	15 Jul 2013	15 Jul 2014	22 Jan 2015	WO2015/007748
UGT enzymes	16 Mar 2015	16 Mar 2016	22 Sep 2016	WO2016/146711
UGT enzymes	23 Mar 2015	23 Mar 2016	29 Sep 2016	WO2016/151046

The production strain for DSM's ingredient is essentially the same as a strain of *Yarrowia lipolytica* used by DSM for production of rebaudioside A (Reb A), which was the subject of a prior GRAS notice filed by U.S. FDA as GRN No. 632 (March 18, 2016) with no objections.

DSM developed the new strain from the same parents as the Reb A production strain, using the same genetic engineering techniques with a few minor exceptions, to favor the production of Reb M (as described in several patent applications filed by DSM, Table 2-1).

As in the production of Reb A, the fermentation broth contains not only Reb M but also several other steviol glycosides that are largely removed during the purification and isolation steps.

2.3.5 Antibiotic resistance

The final production strain does not contain any antibiotic resistance genes. The strain is susceptible to antibiotics and to antifungals. When tested, the genetic changes introduced into the *Yarrowia lipolytica* do not affect antifungal susceptibility. Antibiotic markers were used in strain construction, and these were removed with Cre-Lox system. Cre-Lox was expressed from a plasmid, and loss of the plasmid was screened for (loss of antibiotic resistance). Loss of all markers was checked with a phenotypic test, and periodically confirmed with PCR or genomic sequencing.

2.3.6 History of safe use of *Yarrowia lipolytica*

Yarrowia lipolytica was previously classified as *Candida lipolytica* (van der Walt and von Arx, 1980). In addition to *C. lipolytica*, other names that have been used for this yeast include *Endomycopsis lipolytica, Saccharomycopsis lipolytica, Mycotorula lipolytica*, and *Yallowia lipolytica*.

Yarrowia lipolytica is generally regarded as a biosafety class 1 microorganism (Groenewald *et al.*, 2013). It has been used extensively at manufacturing scale without documented toxic, allergenic, or other harmful effects on the health of humans or other animals.

Y. lipolytica is an avirulent yeast species historically used for the production of citric acid and the flavor chemical, γ-decalactone. In accordance with U.S. food regulation 21 CFR 173.165, *Y. lipolytica* (described by its previous classification, *Candida lipolytica*) is permitted for use as a secondary direct food additive for fermentation production of citric acid.

In addition to approval as a secondary direct food additive in citric acid production, *Y. lipolytica* is routinely found associated with cheeses and meats (Prillinger *et al.*, 1999; Ferreira and Viljoen, 2003; Lanciotti *et al.*, 2005; Viljoen *et al.*, 1993; Gardini *et al.*, 2001). In March of 2011, FDA issued a *No Questions* letter regarding the production of an eicosapentaenoic acid (EPA)-rich triglyceride by *Yarrowia lipolytica.* (GRN No. 355) In November 2011, FDA did not object to a conclusion by Baolingbao Biology Co., Ltd. of Shangdong, China that erythritol produced from glucose *via* biotransformation by a strain of *Yarrowia lipolytica* is GRAS (GRN No. 382).

Y. lipolytica has an extensive history of genetic modification and safe use both in research laboratories and in a variety of industrial applications. This includes non-recombinant modifications, such as strain improvement through classical genetics and use of chemical or physical mutagens to enable competitive processes for the commodity chemical citric acid, the peach aroma γ-decalactone, and specific lipase enzymes.

Y. lipolytica is one of the more intensively studied yeast species and subject to in-depth reviews. Barth and Gaillardin (1997) published a history of *Y. lipolytica* research, including a review of the physiology, biochemistry and cell structure with detail on occurrence in nature, life cycle, and genetic and molecular data. Barth and Gaillardin (1997) also provide a comprehensive review on the available data on the physiology, cell biology, molecular biology and genetics of *Y. lipolytica*. The environmental and industrial applications of *Y. lipolytica* have been reviewed most recently by Bankar *et al.* (2009).

Furthermore, recombinant DNA technologies have been employed to facilitate the expression of many heterologous proteins in *Y. lipolytica* production systems (Madzak *et al.*, 2004). More recently, recombinant *Y. lipolytica* strains have been developed with the future goal of producing essential fatty acids for the human and animal nutrition sectors (see, for example, US Patent 8,323,935 B2 and US Patent 20130149754).

In a review of the safety of *Yarrowia lipolytica*, Groenewald *et al.* (2013) concluded that, in rare cases, the organism may lead to opportunistic infections in severely immunocompromised or otherwise seriously ill people. However, these infections can be effectively treated with standard antifungals or, in some cases, they resolve spontaneously.

In addition, the use of *Y. lipolytica* for cheese ripening has been reported to stimulate the production of biogenic amines, notably the production of tyramine, putrescine, cadaverine, and phenylethylamine (Groenewald *et al.*, 2013). However, the concentrations of biogenic amines associated with this use of *Y. lipolytica* (up to 120 mg/kg) were concluded not to give any reason for health concerns.

In a report by the European Food Safety Authority (EFSA) on risk-based control of biogenic amine formation in fermented foods (EFSA, 2011a), histamine and tyramine are considered as the most toxic biogenic amines. Although only limited published information is available, it has been reported that no adverse health effects were observed after exposure to the following biogenic amine levels in food (per person per meal): a) 50 mg histamine for healthy individuals, but below detectable limits for those with histamine intolerance; b) 600 mg tyramine for healthy individuals not taking monoamine oxidase inhibitor (MAOI) drugs, but 50 mg for those taking third-generation MAOI drugs, or 6 mg for those taking classical MAOI drugs. EFSA also concluded that this level of 6 mg of tyramine per person per meal would be easily exceeded by the consumption of fermented food (EFSA, 2011a). This level of 6 mg tyramine in one or two usual servings per person per day was described by McCabe-Sellers *et al.* (2006) as a clinically significant content in food, being sufficient to cause a mild adverse event. Although this level is relevant for sensitive persons only (individuals treated with classical MAOI drugs), it was used in our assessment as an acceptable threshold per day. For comparison, a 42-day oral toxicity study conducted with Wistar rats receiving tyramine orally at 0, 200, 2000 or 10,000 mg/kg feed resulted in a no-observable-adverse-effect level (NOAEL) of 2000 mg/kg feed (180 mg/kg bw/day) (Til *et al.*, 1997).

This acceptable threshold of 6 mg tyramine per person per day, derived from data available in literature for sensitive persons, is equivalent to a threshold of 0.1 mg tyramine/kg bw/day for a 60-kg bw person. Based on this threshold, a maximum level of tyramine (and therefore of biogenic amines in general) was derived in DSM's Reb M by using the ADI of 4 mg steviol equivalents/kg bw/day established by JECFA, equivalent to 16.2 mg DSM's Reb M/kg bw/day.[2] A maximum level of 6 mg biogenic amines per g DSM's Reb M (or 6000 ppm) is therefore considered acceptable.

For practical reasons, DSM uses the level of nitrogen in Reb M as an indication of the presence of biogenic amines. The maximum level of nitrogen for commercial production of Reb M is set at 100 ppm, and total nitrogen has been below 20 ppm in the Reb M batches produced until now (NBK-017589-005-1012, NBK-017589-005-1035, NBK-017589-005-113, NBK-0017589-008-001, NBK-017589-010-001). However, even if we were to assume that all nitrogen (100 ppm) in Reb M was from biogenic amines, which is highly improbable, the concentration of biogenic amines in Reb M would still be well below the acceptable level of 6000 ppm. Specifically, 100 ppm nitrogen, all coming from tyramine, would correspond to a tyramine level

[2] ADI adjusted by a factor of 0.25 based on the ratio of molecular weights of steviol (318.45 g/mol) and rebaudioside M (1291.3 g/mol).

of 979 ppm. Therefore, the possible presence of these compounds at low levels in Reb M is not a safety concern.

DSM conducted a comprehensive search of the scientific literature for safety and toxicity information on *Y. lipolytica*, from 2013, when the extensive review of Groenewald *et al.* was published, to the present time. The search terms were 'lipolytica' / 'lipolytica and *safe', 'lipolytica and *tox' and the data bases searched included PubMed, Toxnet, U.S. FDA GRAS notices, CDAT, NTP, GESTIS, IPCS INCHEM, TSCATS, US EPA, EFSA, EU Scientific Committees, Health Canada, and NICNAS. From the 6 hits identified, only one was considered relevant to the safety of the microorganism. Zinjarde (2014) reaffirmed the safety of the microorganism in a review of the different food-related applications of *Y. lipolytica*.

It can also be noted that EFSA added *Y. lipolytica* to the list of microorganisms for which a Qualified Presumption of Safety (QPS) assessment may be considered in the future (EFSA, 2013). In conclusion, *Yarrowia lipolytica* is deemed "safe-to-use".

Yarrowia lipolytica is a safe strain for production of food ingredients, as reported in the literature. The modifications DSM employed did not introduce antibiotic production or resistance genes into the organism. The modifications did not introduce any toxin-production genes into the organism. The modifications inserted the genes of the *Stevia rebaudiana* and *Arabidopsis thaliana* plants, both of which have a history of safe use, or equivalent genes from suitable edible plant sources, *e.g.*, tomato (*Solanum lycopersicum*) or lettuce (*Lactuca sativa*). There have been dozens of GRAS notices filed by U.S. FDA for highly purified *stevia* leaf extracts in the form of steviol glycosides, none of which generated questions from the agency, and *Arabidopsis* is an edible species of cress. The other gene added to the organism is from the fungus *Giberella fujikuroi*, also known as *Fusarium fujikuroi*, a well-known organism that has no history of causing disease in humans.

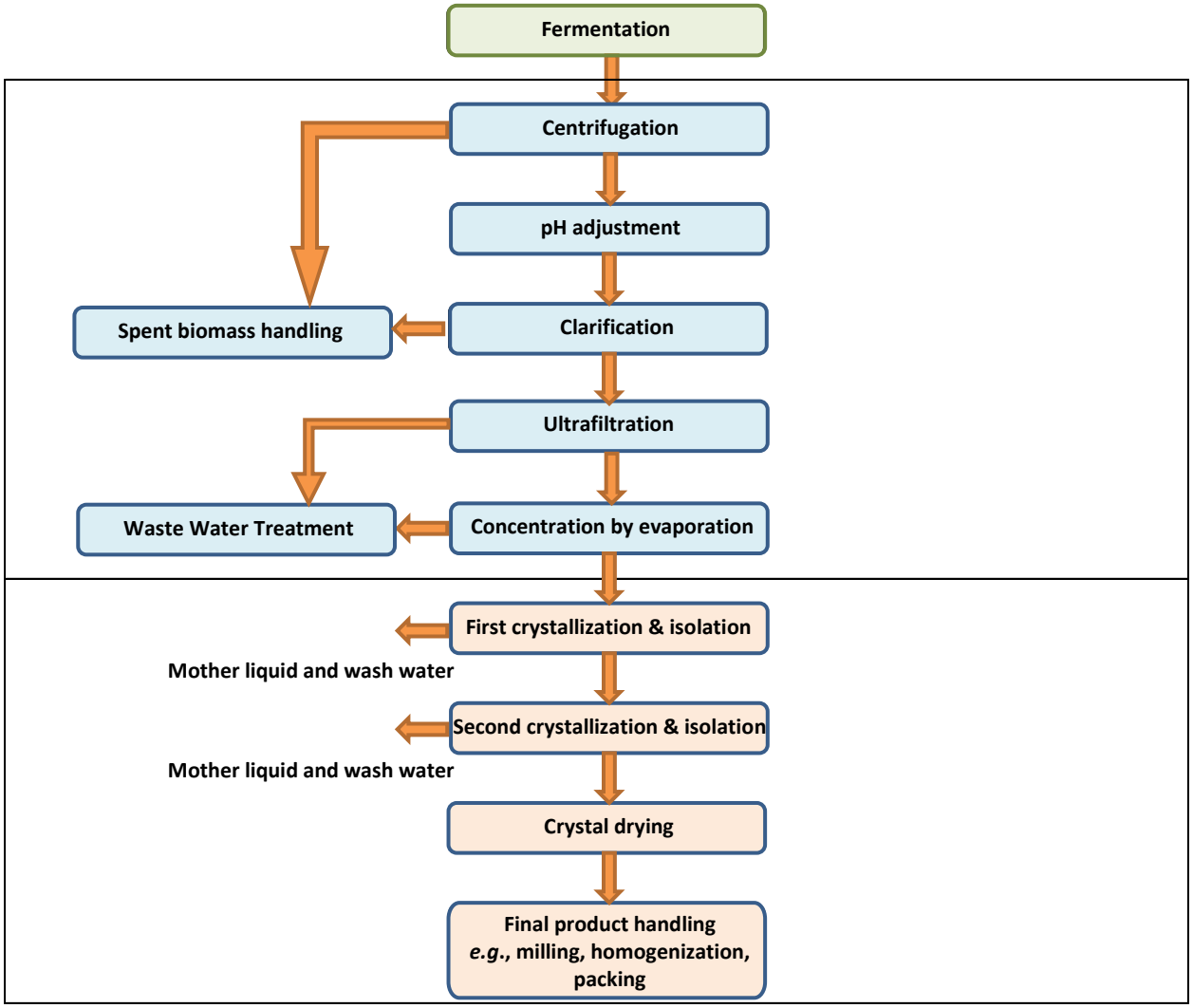
DSM also employed the Pariza and Johnson decision tree (see Annex 2) to determine if that well-accepted rubric revealed any questions about the use of the genetically engineered *Yarrowia lipolytica*. Since the decision tree did not reveal any concerns, and the aforementioned characteristics of the production organism indicate it is not unsafe, DSM concludes that the use of the genetically engineered *Yarrowia lipolytica* presents no known safety concerns.

2.4 Manufacturing process for DSM steviol glycosides (rebaudioside M)

2.4.1 Overview

The manufacturing process for DSM purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica* consists of the following steps: fermentation (Reb M formation); recovery, concentration, and crystallization; and quality control of the finished product. An overview of the process is provided in Figure 2-3. All equipment is made of stainless steel or other materials suitable for food contact.

Figure 2-3 Overview of the manufacturing process for DSM steviol glycosides (Reb M) produced by *Yarrowia lipolytica*



2.4.2 Raw Materials

The raw materials used for the fermentation and recovery of the product are suitable for the intended use, leading to the required safety status of the product. The raw materials used for the media are of food-grade quality and meet predefined quality standards that are strictly monitored and controlled by the Quality Assurance Department of DSM. The fermentation medium composition has been developed for optimum production of Reb M (see Annex 3).

2.4.3 Fermentation Process

DSM's steviol glycosides (Reb M) ingredient is manufactured by submerged fed-batch pure culture fermentation using the genetically modified strain of *Yarrowia lipolytica* described above. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During each step of fermentation, physical and chemical control measures are incorporated, and microbiological analyses are performed to ensure absence of foreign microorganisms and confirm strain identity.

The fermentation process consists of three steps: pre-culture fermentation, seed fermentation and main fermentation. The entire process is performed in accordance with current Good Manufacturing Practices (cGMP).

Biosynthesis and excretion of steviol glycosides occurs during the main fermentation. To produce the material of interest, a carefully controlled, submerged, aerobic fed-batch fermentation process is employed under aseptic conditions, using either a stirred tank or air-lift fermenter.

Growth of the production organism and increase of Reb M production are checked at the end of the main fermentation by analysis of aseptically-collected samples. Recovery takes place during and after fermentation is stopped.

2.4.4 Recovery Process

The major part of the production organism is removed by centrifugation, and the supernatant is heat-treated to kill-off any remaining microorganism. The supernatant is subsequently clarified by centrifugation or filtration, followed by ultrafiltration for the removal of large proteins, concentration, two crystallization steps where potable water is used to remove non-Reb M substances, and drying. The result is a dry powder consisting of ≥ 95 % Reb M.

2.4.5 Methods used to control the product specifications

Representative samples from each production batch are subjected to evaluation by the quality control department to ensure conformance to the established specification, following the method indicated for each material characteristic.

2.4.6 Method to ensure stability of the production organism

DSM maintains a master cell bank of several hundred vials of each production strain stored at -70 °C. A working cell bank is maintained at each production facility and is replenished from the master cell bank, as needed. Each shipment of cultures to a production site is checked for identity, viability and microbial purity, using different temperatures (25, 30 and 37 °C) and media, by enrichment and viewing morphology (colony shape and microscopy) before release. A general overview of the strain control process is provided in Annex 4.

2.4.7 Global capabilities

DSM has multiple fermentation facilities located in the major industrial markets, each of which is able to manufacture Reb M following the process above and under cGMP. The manufacturing may also be done on behalf of DSM by tolling companies, in accordance with the standards established by DSM for the product, cGMP, and any other requirements that might apply to food-production facilities.

2.5 Batch Analyses

DSM produced five batches at its Netherlands facility for analytical purposes. The results of analysis of these batches are summarized in Table 2-2 and compared to the tentative DSM, FCC and JECFA specifications. The analyses were done in-house. Certificates of analysis are provided in Annex 5.

Table 2-2 Batch analysis data for DSM purified steviol glycosides (rebaudioside M) produced by *Yarrowia lipolytica*

Parameter	Method	DSM Tentative Specs	JECFA Spec Steviol Glycosides	FCC10 Spec Steviol Glycosides	Batch Number				
					NBK-017589-005-1012	NBK-017589-005-1035	NBK-017589-005-113	NBK-0017589-008-001 (VVJ1602A)	NBK-017589-010-001
Product characteristics									
Appearance	Visual	Off-white to white powder	White to light yellow powder	White or light yellow powder	Off-white to white powder	Off-white to white powder	Off-white to white powder	Off-white to white powder	Off-white to white powder
Odor	Smell	Odourless or slight characteristic	Odourless or having a slight characteristic odour		Odourless	Odourless	Odourless	Odourless	Odourless
Moisture content	Karl Fisher	≤ 10%	NMT 6%	NMT 6%	2.6%	3.0%	4.4%	2.3%	6.5%
Ash	JECFA	≤ 1%	NMT 1%	NMT 1%	<1%	<1%	<1%	<1%	<0.3%
Solubility in purified water at room temperature (20°C)		Soluble in water at a level greater than 1000 ppm (>1 g/L)	Freely soluble in water	Freely to sparingly soluble in water	>1 g/L	>1 g/L	>1 g/L	1.1 g/L	1.0 g/L
Total steviol glycosides (on dry basis)	FCC (LC-UV) method copied from Reb A and qualified	> 95 %	NLT 95%	NLT 95%	98.6%	99.7%	100%	99.0%	100%
Rebaudioside M (on dry basis)	FCC (LC-UV) method copied from Reb A and qualified	≥ 95 %	NA	NA	97.7%	99.1%	99.8%	98%	98%
pH (1 gram dissolved in 1 l of water)	FCC	4.5 – 7.0	4.5 – 7.0	4.5 - 7.0	6.58	6.59	6.61	6.85	6.9

Parameter	Method	DSM Tentative Specs	JECFA Spec Steviol Glycosides	FCC10 Spec Steviol Glycosides	Batch Number				
					NBK-017589-005-1012	NBK-017589-005-1035	NBK-017589-005-113	NBK-0017589-008-001 (VVJ1602A)	NBK-017589-010-001
Lead	SLD A1603 NEN-EN-ISO 11885 (ICP-AES)	<1 ppm	<1 ppm	≤1 ppm	<0.3 ppm	<0.3 ppm	<0.3 ppm	<0.3 ppm	<0.3 ppm
Mercury	SLD A1603 NEN-EN-ISO 11885 (ICP-AES)	<1 ppm	NS	NS	<0.02 ppm	<0.02 ppm	<0.02 ppm	<0.02 ppm	<0.02 ppm
Cadmium	SLD A1603 NEN-EN-ISO 11885 (ICP-AES)	<1 ppm	NS	NS	<0.02 ppm	<0.02 ppm	<0.02 ppm	<0.02 ppm	<0.01 ppm
Arsenic	SLD A1603 NEN-EN-ISO 11885 (ICP-AES)	<1 ppm	<1 ppm	≤1 ppm	<0.02 ppm	<0.02 ppm	<0.02 ppm	<0.02 ppm	<0.02 ppm
Recombinant DNA (see Annex 6)	PCR	absent by test			absent	absent	absent	absent	absent
Microbiology									
Total Plate Count	European and US Pharmacopeias, membrane filtration	≤1000 cfu in 1g	NA	NA	10	200	20	<5	65
Yeast	European and US Pharmacopeias, membrane filtration	≤ 100 CFU in 1 g	NA	NA	<10	<10	<10	<10	<10
Mold	European and US Pharmacopeias, membrane filtration	≤ 100 CFU in 1 g	NA	NA	<10	20	<10	<10	<10
Coliforms	SLD M9849 ISO 21528-1 2004	≤ 10 CFU in 1 g	NA	NA	<0.3	<0.3	<0.3	<0.3	<3

2.6 Stability

By virtue of having similar chemical composition, the chemical stability DSM purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica* is expected to be comparable to that of other steviol glycosides. As such, the sections that follow briefly discuss information from prior GRAS notices about the stability of other steviol glycoside preparations. In addition, DSM provides the results of a stability study of purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica*, showing no significant reduction in the Reb M concentration after up to 12 months of storage at 25 and 40 °C.

In general, degradation products of steviol glycosides may be expected to be products resulting from cleavage of glucose units from the steviol backbone, isomerization, or oxidation. In scientific opinions about steviol glucosides, the European Food Safety Authority (EFSA) noted that several degradation products have been identified in steviol glycosides. Some of them share the same steviol aglycone backbone and differ only with respect to the number of glucose units, while the remaining compounds have slight structural differences in the aglycone backbone, such as an endocyclic double bond, additional hydroxyl group, or an isosteviol aglycone instead of steviol. These degradation products were shown to increase under different storage (pH, temperature, and time) and food production conditions.

Despite the lack of sufficient toxicity data, the EFSA panel concluded that the safety of related steviol glycosides and degradation products can be extrapolated from the presence of sufficient amounts of the compounds in the test materials used in existing studies, and that, under the conditions of intended use of steviol glycosides, exposure to these compounds at the levels typically present in high-purity steviol glycoside preparations is not expected to be associated with any adverse effects following oral intake by humans (EFSA Journal, 2010; 2015).

DSM monitors accordingly for the possible presence of any degradation products that might be of concern, establishing limits where it may be necessary, while recognizing the EFSA panel's conclusion that normal use of steviol glycosides as sweeteners is unlikely to results in significant consumer exposures to these degradation products.

2.6.1 Stability of Rebaudioside A

Chang and Cook (1983) investigated the stability of pure stevioside and rebaudioside A in carbonated phosphoric and citric acidified beverages. Some degradation of each sweetening component was detected after 2 months of storage at 37°C. However, no significant change was noted following 5 months of storage of stevioside and 3 months of storage of rebaudioside A at room temperature or below. Exposure to 1 week of sunlight did not affect stevioside, but a loss of approximately 20% of rebaudioside A was observed. Heating at 60°C for 6 days resulted in 0- 6% loss of rebaudioside A.

Merisant (GRN No. 252) conducted stability testing on rebaudioside A (1) as a powder, (2) as a pure sweetener in solution, and (3) in both cola-type and citrus carbonated beverages. No degradation was detected when the powder was stored at 105˚C for 96 hours. It was concluded that the powder was stable when stored for 26 weeks at 40±2˚C with relative humidity of 75±5%. Both published and unpublished testing results from Merisant revealed that rebaudioside A in carbonated citric acid beverages and phosphoric acid beverages did not significantly degrade during prolonged storage at refrigeration, normal ambient, or elevated ambient temperatures. Minimal loss of rebaudioside A was detected after storage at 60˚C, with considerable degradation noted after 13 hours at 100˚C for carbonated beverage solutions and

pure sweetener solutions.

Cargill (GRN No. 253) also conducted extensive stability testing on rebaudioside A as a powder under various storage conditions and under a range of pH and temperatures. Additionally, Cargill also investigated rebaudioside A stability in several representative food matrices at room temperature and elevated temperatures. Stability profiles were created for tabletop sweetener applications, mock beverages including cola, root beer and lemon-lime, thermally processed beverages, yogurt, and white cake. The results of stability testing revealed some degradation products that had not been detected in bulk rebaudioside A. These degradation products were structurally related to the steviol glycosides that are extracted from the leaves of *Stevia rebaudiana* Bertoni.

All degradation products were found to share the same steviol aglycone backbone structure as found in stevioside and rebaudioside A, but they differed in the glucose moieties present. The results of stability testing revealed that rebaudioside A is stable in various food matrices following several days or weeks of storage. The extent and rate of degradation is dependent on pH, temperature, and time. When placed in beverages, rebaudioside A is more stable in the pH range 4 to 6 and at temperatures from 5˚C to 25˚C.

In photostability studies of the dry powder and in mock beverages to ascertain rebaudioside A behavior under defined conditions of fluorescent and near UV light exposure, rebaudioside A was found to be photo stable under the defined conditions of analysis (Clos *et al.*, 2008).

In addition to the stability reports for purified rebaudioside A described above, in a GRAS notice by Sunwin and WILD Flavors (GRN No. 304) on purified steviol glycosides with rebaudioside A and stevioside as the principal components, stability was investigated using a 0.04% solution of Reb A 80% in acidic solutions between pH 2.81 and 4.18. In this study, the solutions were stored at 32°C for 4 weeks, and the Reb A content was determined at 1, 2 and 4 weeks. Reb A 80% was found to be very stable at pH 3.17 and above. At pH 2.81, after 4 weeks of storage under accelerated conditions only a 7% loss of Reb A was noted. Sunwin and WILD Flavors also studied the stability of Reb A 80% in simulated beverages using 0.1 % citric acid (pH 3.2). The solutions were pasteurized and stored for 8 weeks at 4° and 32°C, and little difference in sweetness perception was found under these conditions.

2.6.2 Stability of Rebaudioside M

PureCircle (GRN No. 473) conducted a stability test on a batch of rebaudioside X (later renamed rebaudioside M) at a concentration of 500 mg/L. Samples were stored in sealed amber glass vials for up to 26 weeks either at (1) 5°C and ambient relative humidity (RH) (50 to 55%) or (2) 40°C and 75% RH. Analyses for steviol glycosides were conducted in accordance with JECFA's assay method (JECFA, 2010) and were measured upon study initiation, and after 12, 24, and 26 weeks of storage. Minimal degradation (<4%) of rebaudioside X was observed when stored as a solution under either set of conditions. Additionally, minimal changes were observed in the other detected steviol glycosides over the study period.

GLG Life Tech (GRN No. 512) conducted a stability test on a sample of their high-purity rebaudioside M (>95%) at 25 ± 5°C and 60 ± 5% relative humidity for a period of 8 weeks. Minimal degradation (<1%) of rebaudioside M and total steviol glycosides was observed.

Blue California (GRN No. 667) conducted a 6-month accelerated stability study of 5 lots of their Reb-M 95%. The samples were stored at 40 ± 2°C and relative humidity of 75 ± 5%. Reb-M 95% was observed to be

stable over the course of the accelerated stability study.

Prakash *et al.* (2014) reported that rebaudioside M is stable for at least one year at ambient temperature and under controlled humidity conditions. Rebaudioside M shows similar stability as that of rebaudioside A in both low and high pH applications.

The stability data in the scientific literature for stevioside, the JECFA report, and the extensive stability testing for the structurally similar rebaudioside A (as presented by Merisant, Cargill, and Sunwin & WILD Flavors) and rebaudioside M (as presented by PureCircle Ltd. in GRN 473, along with GLG Life Tech's stability testing results in GRN 512, each filed by FDA with no objections) support the position that high-purity steviol glycoside preparations, including those where rebaudioside M is the primary component, are stable and well-suited for the intended food uses.

In addition to the existing information about the stability of various steviol glycosides, DSM began a stability study of its purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica* (food-grade sample number NBK-0017589-008-001, VVJ1602A RW; produced Oct. 2016, released Nov. 2016) in December of 2016. The results, as summarized in Table 2-3, show no statistically significant reduction in Reb M concentration after up to 12 months of storage at 25 and 40 °C, suggesting it can withstand short-term exposure to excessive storage conditions typically encountered during transportation and distribution.

Table 2-3 Stability data for DSM purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica*

Parameter (Method)	Limits Release/ end of shelf specs.	Condition	Temp/ RH	Time (months)						
				0	1	2	3	6	9	12
				%	%	%	%	%	%	%
		D-number		D34916	D34981	D35017	D35069	D35246	D35454	D35635
Reb M analyses (FCC LC-UV method)		1	25 C/ 60%	96.2 (98.8*)	95.1 (99.9*)	95.6 (99.2*)	94.4 (99.2*)	93.7 (97.4*)	95.0 (98.4*)	95.8 (98.5*)
		2	40 C/ 75%	96.2 (98.8*)	94.0 (99.2*)	94.4 (97.9*)	93.3 (97.5*)	92.9 (96.3*)	93.9 (97.3*)	93.7 (96.1*)
				%	%	%	%	%	%	%
		C-number		C36348	C36348	C36348	C36467	C36348	C36348	C36348
Moisture (TGA)		1	25 C	2.6	4.8	3.7	4.9	3.8	3.4	2.7
		2	40 C	2.6	5.2	3.6	4.4	3.5	3.5	2.5
X-rite A1882 v3	a/b/L	1	25 C	-0.5/ 0.5/ 90.8						
	a/b/L	2	40 C	-0.5/ 0.5/ 90.8						
AW A10054 v1	< 50%	1	25 C	2.3%						
		2	40 C	2.3%						

* On dry basis (corrected for moisture).

Part 3: Dietary Exposure

3.1 Intended Food Uses

DSM's ingredient, purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica*, is intended to be used as a general purpose non-nutritive sweetener[3] in various foods. It is not intended for use in infant formulas or meat and poultry products.

DSM anticipates that this ingredient will be used in a variety of foods such as beverages, dairy products, baked goods, and confections, in a manner similar to other non-nutritive sweeteners, including other stevia-derived substances described in prior GRAS notices that generated no questions from FDA (see section 6). DSM purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica* may also be used in other food categories, within the limits of cGMP.

3.2 Estimated Daily Intake

DSM's ingredient is equivalent in chemical and physical characteristics, and sensorial properties, to other commercially available high-purity steviol glycosides, including those derived from the stevia plant. Therefore, it can be used in various foods and beverages at the same levels, and the resulting consumer exposures from such uses would not be expected to differ significantly from what has been previously reported and reviewed by U.S. FDA as part of several prior GRAS notices.

The estimated daily intake of steviol glycosides has been reported in several publications, as well as in several GRAS notices to U.S. FDA. In 2006, JECFA determined a very conservative estimate of human exposure to steviol glycosides through food consumption in the U.S. and other countries. It was assumed that steviol glycosides would replace all sweeteners used in or as food, which is highly unlikely, applying the minimum reported relative sweetness comparison of steviol glycosides and sucrose of 200:1 (JECFA, 2006).

In 2010, an EFSA Panel calculated the anticipated human exposure to steviol glycosides by using the maximum use levels of steviol glycosides in the different food categories and individual food consumption data for European child and adult populations (EFSA, 2010). The EFSA values were based on the assumption that all the products consumed contained steviol glycosides. This is not probable because not every consumer is interested in consuming stevia glycoside-sweetened products and also, there are other sweetener alternatives to steviol glycosides. Ng *et al.*, 2012 calculated that only 6% of the products purchased from 2005 to 2009 in the USA contained non-caloric sweeteners.

In 2011, EFSA revised its dietary exposure assessment of steviol glycosides, taking into account the revised proposed uses. For European children (aged 1-14), the revised maximum average intake was lowered to 6.4 mg/kg bw/day (expressed as steviol equivalents) and the high intake estimate was lowered to 12.7 mg/kg bw/day; for adults, the range was from 2.3 as the maximum for the average consumer to 6.8 mg/kg bw/day steviol equivalents as the maximum for the high consumer (EFSA, 2011b). The lower estimates for children were still in excess of the current EFSA Acceptable Daily Intake (ADI) of 4.0 mg steviol equivalents/kg bw/day.

[3] As defined in 21 CFR 170.3(o)(19), non-nutritive sweeteners are substances having less than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.

EFSA also noted that the primary source of low/no calorie sweeteners in the diet was beverages and that excess exposure due to consumption of several of the food categories considered was not likely. Carbonated beverages, particularly soda, typically contain 12% sucrose or high fructose corn syrup to obtain the equivalent sweetness of sucrose.

In 2011, the Center for Disease Control reported that the 95th percentile consumer of carbonated sugar-sweetened beverages drank four 12 oz. cans per day. JECFA noted that individuals who consumed no-calorie beverages consumed as much as the sugar-sweetened beverage consumer. Four 12-oz. cans weigh approximately 1 kg and, at 12% sugar, the amount of sugar in 1 kg beverage would be 120 grams. At 200 times the sweetness of sucrose, the quantity of Reb M consumed at the 95th percentile level would be 600 mg. In a 60-kg adult, the exposure from carbonated beverages would be 10 mg Reb M/kg bw/day, or 2.5 mg steviol equivalent/kg bw/day.[4] This is below the ADI of 0-4 mg steviol equivalents/kg bw/day established in 2008 by JECFA for steviol glycosides.

In 2014, EFSA completed a revision of the dietary exposure assessment for steviol glycosides based on the authorized uses and the proposed extension at that time, and by using the latest EFSA food consumption database (EFSA, 2014). The revised estimate was considerably reduced, since it resulted in a maximum average intake of 2.4 mg/kg bw/day (expressed as steviol equivalents) for toddlers and 1.0 mg/kg bw/day for adults, and in 95th percentile estimates from 0.3 to 4.3 mg/kg bw/day for the elderly and toddlers, respectively (EFSA, 2014). Except for the upper range of exposure for toddlers, these revised exposure estimates remain below the ADI for all age groups.

Even in a worst-case scenario, where DSM's purified steviol glycosides (Reb M) would replace all steviol glycosides currently used on the market, which is highly unlikely, the intake of DSM's ingredient will still not exceed the ADI established by JECFA, 4.0 mg steviol/kg bw/day, equivalent to 16.2 mg Reb M/kg bw/day.[4]

3.3 Estimated exposure based on caloric sweetener consumption

The approach used by Renwick (2008) to predict rebaudioside A exposure based on sucrose intake data has been successfully used in other GRAS notices to estimate exposure to various sweeteners, including other forms of rebaudioside M (see GRN No. 512 and 667). DSM has employed a similar strategy for calculating exposure to its purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica*.

Using daily intakes of intense sweeteners, Renwick (2008) predicted dietary exposures to rebaudioside A in average and high consumers, respectively, to be: 1.3 and 3.4 mg/kg bw/day for the general population; 2.1 and 5.0 mg/kg bw/day for children; and 1.4 and 4.5 mg/kg bw/day for individuals (adults and children) with diabetes (see Table 3-1). These values were derived assuming a relative sweetness for rebaudioside A that is 200 times that of sucrose.

The relative sweetness intensity of rebaudioside M generally ranges from 200-350 times that of sucrose, suggesting the estimates of Renwick (2008) for Reb A are representative of possible exposures to DSM's purified steviol glycosides (Reb M) when used as a substitute. As Table 3-1 shows, these values represent intakes up to 1.1 mg steviol equivalents/kg bw/day (4.5 mg Reb M/kg bw/day) in adults and 1.2 mg

[4] Converted using a factor of 0.25 based on the ratio of molecular weights of steviol (318.45 g/mol) and rebaudioside M (1291.3 g/mol).

steviol/kg bw/day (5 mg Reb M/kg bw/day) for children. All predicted intake values are well below the JECFA ADI of 4.0 mg steviol/kg bw/day (equivalent to 16.2 mg Reb M/kg bw/day).[5]

Table 3-1 Estimated daily intakes of DSM purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica* based on sucrose intake data

Population Group	Intakes of intense sweeteners (mg sucrose/kg bw/day)[a]		Predicted intakes[b] (mg/kg bw/day)			
			as Reb M[c]		as Steviol[d]	
	Average consumer	High consumer	Average consumer	High consumer	Average consumer	High consumer
Non-diabetic adults	255	675	1.3	3.4	0.32	0.85
Diabetic adults	280	897	1.4	4.5	0.35	1.1
Non-diabetic children	425	990	2.1	5.0	0.52	1.2
Diabetic Children	672	908	3.4	4.5	0.85	1.1

[a] From Renwick (2008).

[b] Based on the approach used by Renwick (2008) to predict rebaudioside A intakes (by substitution) from sucrose data.

[c] Calculated by dividing the sucrose intake by the average relative sweetness value of 200 for DSM's steviol glycosides (Reb M).

[d] Reb M intakes converted using the ratio (0.25) of molecular weights of steviol (318.45 g/mol) and rebaudioside M (1291.3 g/mol) to account for the proportion of rebaudioside M that represents steviol.

The USDA reported in a publication entitled *USDA, ERS, Sugar and Sweeteners Outlook yearbook* (last updated 2014) that the per capita availability of caloric sweeteners in 2014 was 131 lbs/person/year (USDA Table 50). They also noted that approximately 27% of the sweeteners are lost due to waste at the production and consumer level (USDA Table 51). This means actual consumption is approximately 95.7 lbs sweetener/person/yr or 43.4 kg sweetener/person/yr. This is equivalent to 0.119 kg sweetener/person/day, or 119 g sweetener/person/day. For a person with a body weight of 60 kg, consumption would be approximately 1.98 g sweetener/kg bw/day. Based on an assumed relative sweetness of 200 times that of sucrose, the estimated consumption of DSM's purified steviol glycosides (Reb M) as the sweetener in all foods would be approximately 9.9 mg/kg bw/day, below the JECFA ADI (4 mg steviol/kg bw/day = 16.2 mg Reb M/kg bw/day). It is important to note that these exposure values are greatly exaggerated, because DSM's Reb M is not expected to replace all the sweeteners used in food and beverages due to both technical and sensorial barriers.

[5] Converted using a factor of 0.25 based on the ratio of molecular weights of steviol (318.45 g/mol) and rebaudioside M (1291.3 g/mol).

Part 4: Self-limiting levels of use

It is expected that, as with other steviol glycosides, the levels of use of DSM's purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica* are self-limiting due to organoleptic factors and consumer taste considerations.

Part 5: Common use in food prior to 1958

The elements of this section do not apply.

Part 6: Narrative of the basis for the GRAS conclusion

6.1 Overview

To make a conclusion that the use of its ingredient, purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica*, as a general purpose non-caloric sweetener in foods is GRAS, DSM relied largely on information discussed in several prior GRAS notices for other highly-purified steviol glycoside preparations that were filed and accepted by U.S. FDA with no objections. These notices are summarized in Table 6-1.

Table 6-1 GRAS notices submitted to U.S. FDA for use of purified steviol glycosides

GRN No.	Substance	Date of Closure
252	Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni	Dec 17, 2008
253	Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni	Dec 17, 2008
275	Purified steviol glycosides with rebaudioside A as the principal component	Jun 11, 2009
278	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	Jul 20, 2009
282	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	Aug 11, 2009
287	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	Aug 28, 2009
303	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	Mar 22, 2010
304	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	Mar 22, 2010
318	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	May 15, 2010
323	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	Jul 9, 2010
329	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	Sep 10, 2010
337	Enzyme-modified steviol glycosides preparation (EMSGP)	Jun 17, 2011
348	Stevioside purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (stevioside)	Jul 14, 2011
349	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	Jul 14, 2011

GRN No.	Substance	Date of Closure
354	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	Jul 15, 2011
365	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	Aug 18, 2011
367	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	Jul 8, 2011
369	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	Oct 11, 2011
375	Enzyme-modified steviol glycosides	Sep 2, 2011
380	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	Nov 28, 2011
388	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	Jan 9, 2012
389	Steviol glycosides with stevioside as the principal component	Jan 18, 2012
393	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	Jan 23, 2012
395	Steviol glycosides with rebaudioside A and stevioside as the principal components	Jan 24, 2012
418	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	Jun 7, 2012
448	Enzyme-modified steviol glycosides	May 3, 2013
452	Enzyme-modified steviol glycosides	Jul 1, 2013
456	Rebaudioside D purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside D)	Jul 1, 2013
461	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	Aug 14, 2013
467	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	Nov 25, 2013
473	Purified steviol glycosides with rebaudioside X as the principal component	Dec 17, 2013
493	High purity steviol glycosides (minimum purity 95%)	May 30, 2014
512	High purity Rebaudioside M	Oct 24, 2014
516	Steviol glycosides with rebaudioside A and stevioside as the principal components	Oct 31, 2014
536	High purity rebaudioside C	Feb 12, 2015
548	High purity rebaudioside D	Apr 22, 2015
555	High purity steviol glycosides (minimum purity 95%) consisting primarily of rebaudioside A.	Apr 21, 2015
607	Glucosylated steviol glycosides (minimum purity 80%)	Oct 14, 2016
619	Purified steviol glycosides	May 27, 2016
626	Steviol glycosides produced in *Saccharomyces cerevisiae*	May 27, 2016
632	Rebaudioside A from *Yarrowia lipolytica*	Jun 24, 2016
638	High purity steviol glycosides (minimum purity 97%) consisting primarily of rebaudioside A	Jul 10, 2016
656	Enzyme-modified steviol glycosides	Sep 28, 2016
662	Glucosylated steviol glycosides (minimum purity 95%)	Sep 29, 2016
667	Rebaudioside M	Feb 17, 2017

GRN No.	Substance	Date of Closure
702	Purified steviol glycosides	Sep 28, 2017
715	Rebaudioside D	Oct 24, 2017
733	Purified steviol glycosides	Pending
744	Steviol glycosides consisting primarily of rebaudioside M	Pending
745	Steviol glycosides consisting primarily of rebaudioside M	Pending

Four GRAS notices, GRN Nos. 512, 626, 632, and 667, were considered of particular relevance.

GRAS GRN No. 632 was submitted by DSM in 2015 (filed in 2016), and established the use of a strain of *Yarrowia lipolytica* genetically modified to biosynthesize steviol glycosides consisting primarily of rebaudioside A (Reb A), similar to the production of the current notified substance. This notice also discussed the safety of steviol glycosides in general, along with the findings of safety studies of DSM's Reb A specifically.

GRAS notice GRN No. 626 was submitted in 2016 by Cargill for purified steviol glycosides (rebaudiosides A, B, C, D, E, F, M, stevioside, steviolbioside, rubusoside, and dulcoside A) produced through fermentation using a genetically modified strain of *Saccharomyces cerevisiae*, an approach similar to that of DSM.

GRAS notices GRN Nos. 512 and 667 were for high-purity (≥ 95%) Reb M ingredients similar in composition, specifications, and proposed food uses to DSM's steviol glycosides (Reb M), as Table 6-2 illustrates.

Table 6-2 DSM's purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica vs.* rebaudioside M in other GRAS notices

	Current GRAS notice	GRN No. 667	GRN No. 512
Company	DSM Food Specialties	Blue California	GLG Life Tech Corporation
Year submitted	2017	2016 (2017 FDA response letter)	2014
Substance	Rebaudioside M (≥ 95%)	Rebaudioside M (≥ 95%)	Rebaudioside M (≥ 95%)
Source	Produced by fermentation using a strain of *Yarrowia lipolytica* genetically engineered to contain and express the *Stevia rebaudiana* biosynthetic pathway for steviol glycosides	Synthesized from *Stevia rebaudiana* Bertoni extract by a genetically-modified *Pichia pastoris* strain	Obtained from the leaves of *Stevia rebaudiana* Bertoni through extraction and multiple purification steps
Specifications			
Appearance and color	Off-white to white powder	Powder, white	Powder, white to off-white
Solubility	Freely soluble to slightly soluble	Soluble in water	Sparingly soluble
Purity	≥ 95% (Reb M)	≥ 95% (Reb M)	≥ 95% (Reb M)
Residual Ethanol	NS	< 1000 ppm	≤ 5000 ppm
Residual Methanol	NS	< 200 ppm	≤ 200 ppm
Loss on Drying	≤ 10%	≤ 6%	≤ 4%
pH, 1% solution	4.5-7.0	5-7	4.5-7.0

	Current GRAS notice	GRN No. 667	GRN No. 512
Total ash	≤ 1%	≤ 1%	< 1%
Arsenic	< 1 ppm	< 0.5 ppm	< 1 ppm
Lead	< 1 ppm	< 0.5 ppm	< 1 ppm
Mercury	< 1 ppm	< 0.5 ppm	< 1 ppm
Cadmium	< 1 ppm	< 0.5 ppm	< 1 ppm
Total Plate Count (CFU/g)	≤ 1000	< 3000	< 1000
Total Coliform (CFU/g)	≤ 10	< 100	NS
Yeast & Mold	≤ 100 (each)	< 100	< 100
Salmonella spp	NS	Negative	Negative 25 g
Staphylococcus aureus	NS	NS	Negative
E. coli (mgn/g)	NS	Negative	Negative
Proposed food uses	General-purpose sweetener, excluding meat, poultry products and infant formulas	General-purpose sweetener, excluding meat, poultry products and infant formulas	General-purpose sweetener, excluding meat, poultry products and infant formulas
Proposed use levels	In accordance with GMP	In accordance with GMP	In accordance with GMP
Maximum dietary exposure (expressed as steviol equivalents)	Adults: 1.1 mg/kg bw/day Children: 1.2 mg/kg bw/day Based on Renwick (2008) estimates (for Reb A) and presumed relative sweetness of 200 times that of sucrose for Reb M.	Adults: 1.7 mg/kg bw/day Children: 1.88 mg/kg bw/day Based on Renwick (2008) estimates (for Reb A) and presumed relative sweetness of 200 times that of sucrose for Reb M (Note: actual number used appears to have been 130 times).	Adults: 0.55 mg/kg bw/day Children: 0.61 mg/kg bw/day Based on Renwick (2008) estimates (for Reb A) and presumed relative sweetness of 380 times that of sucrose for Reb M.

NS = no specification established.

In addition to the safety of the production organism (discussed in section 2.3), other elements considered pivotal to a safety assessment of DSM's purified steviol glycosides (Reb M) are discussed below.

In making its GRAS conclusion, DSM also sought the opinion of a panel of scientific experts. The Expert Panel concluded that: (1) there is reasonable certainty that no harm will result from the use of DSM's ingredient as a non-nutritive sweetener in foods for the general U.S. population (excluding infant formulas and meat and poultry products) at levels resulting in consumer exposures within the ADI of 0-4 mg steviol/kg bw established by JECFA for steviol glycosides; and (2) such uses would be considered generally recognized as safe (GRAS) based on scientific procedures, and that other qualified experts would agree. The Panel's opinion statement is provided in section 7.

6.2 Absorption, distribution, metabolism, excretion (ADME)

Studies about the absorption, distribution, metabolism, and excretion (ADME) of steviol and steviol glycosides have been discussed extensively in other GRAS notices. Briefly, the available data suggest that steviol glycosides are not absorbed intact, but as the aglycone steviol. The successive removal of glucose

units by microflora in the colon is required for absorption, and it is generally accepted that all steviol glycosides share the same metabolic fate.

The more recent findings of Purkayastha *et al.* (2015; 2016) are consistent with the assumptions above regarding the metabolism of steviol glycosides. *In vitro* metabolism experiments with rebaudiosides A, B, C, D, E, F and M and pooled human fecal homogenates showed that glycosidic side chains containing glucose, rhamnose, xylose, fructose and deoxyglucose, including combinations of α(1-2), β-1, β(1-2), β(1-3), and β(1-6) linkages, were degraded to steviol, mostly within 24 hours. At a lower concentration (0.2 mg/mL), rebaudioside M showed complete hydrolysis after 24 hours, whereas limited hydrolysis was seen at the higher concentration (2 mg/mL) due to lower solubility, as has been previously reported.

The authors proposed based on these findings that the rate of hydrolysis is essentially similar for those steviol glycosides containing differing numbers of glucose units at either the R1 and R2 position of the steviol backbone, and the number and location of the attached glucose units appear to have no significant impact on the rate of hydrolysis in the human gastrointestinal tract.

In vitro and *ex vivo* studies in various animal species have shown that steviol is rapidly absorbed from the gastrointestinal tract (Wingard *et al.*, 1980; Geuns *et al.*, 2003a, 2003b; Koyama *et al.*, 2003a). Absorbed steviol is taken up by the portal vein and transported to the liver for further metabolism (Koyama *et al.*, 2003b; Nakayama *et al.*, 1986). In the liver, steviol has been shown to undergo conjugation with glucuronic acid, leading to the formation of steviol glucuronide (Geuns *et al.*, 2003b). Early studies performed *in vitro* with rat and human liver microsomes reported the formation of oxidative metabolites of steviol (steviol-16,17α-epoxide, 15α-hydroxysteviol) (Compadre *et al.*, 1988; Koyama *et al.*, 2003a). *In vivo*, these steviol metabolites have been identified in hamsters (Hutapea *et al.*, 1999), but not in rats (Roberts and Renwick, 2008) or humans (Geuns *et al.*, 2007).

Following oral administration of either steviol glycosides or steviol to rats, steviol was primarily excreted in the feces *via* the bile, while a small proportion is also observed in the urine (Wingard *et al.*, 1980; Nakayama *et al.*, 1986; Roberts and Renwick, 2008).

The fate of radiolabeled ^{3}H-stevioside administered orally to Wistar rats was studied by Nakayama *et al.* (1986). A slow increase in radioactivity of the blood was observed, reaching its peak at 8 hours. After 1 hour, the highest concentration was observed in the small intestine, followed by the stomach and then the cecum. After 4 hours, the level in the cecum was higher than in other tissues. At 72 hours, radioactivity excreted into the bile was 40.9% of the original dose. At 120 hours, the percentages of radioactivity excreted into the feces, expired air and urine were 68.4%, 23.9% and 2.3%, respectively. It was concluded from these observations that enterohepatic circulation occurs in rats. Stevioside is metabolized by cecal flora to steviol and sugars, which are thereafter absorbed from the cecum, distributed throughout the body, and excreted mainly into feces and expired air.

Nikiforov *et al.* (2013) reported the detection of very low plasma levels of parent compound (≤1.5 µg/mL) following administration of rebaudioside A or D to rats at 2000 mg/kg body weight/day in the diet for 1 day and 21 days. Free steviol (≤12 µg/mL) and glucuronide-conjugated steviol (≤40 µg/mL) were the primary metabolites detected in the plasma. The detection of low levels of parent compound is not considered to be associated with any safety concerns, since studies conducted with rebaudioside A and D have not shown any adverse toxicological findings.

No free steviol was detected in the blood of human volunteers following ingestion of stevioside or rebaudioside A, but steviol glucuronide and, in some cases, low concentrations of the unchanged steviol

glycoside were detected in the plasma (Geuns and Pietta, 2004; Geuns *et al.*, 2007). None of the dihydroxy or monohydroxy metabolites of steviol identified in rats or hamsters, particularly those potentially mutagenic, were detected in human plasma. Similar to what has been observed in rats, the presence in plasma of consecutive peaks of steviol glucuronide indicates enterohepatic circulation of steviol in humans (Kraemer and Maurer, 1994).

Steviol glucuronide was also reported to be the main metabolite found in the urine of volunteers exposed to stevioside or rebaudioside A (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Wheeler *et al.*, 2008). Additionally, very small amounts of the unchanged glycoside or steviol were also recovered in urine. Steviol was reported to be the main metabolite found in the feces of humans following stevioside or rebaudioside A intake (Geuns and Pietta, 2004; Geuns *et al.*, 2007; Wheeler *et al.*, 2008). It should also be noted that no parent steviol glycoside has been detected in human plasma or urine from any of these studies.

The shared metabolic fate of different steviol glycosides, and the apparent interspecies similarities in metabolism, suggest the extensive data base of safety information from humans and experimental animal studies employing a variety of steviol glycosides is directly relevant to the safety of DSM's purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica*.

6.3 Safety data of steviol glycosides

6.3.1 Overview

Steviol glycosides extracted from the *Stevia rebaudiana* Bertoni plant have been commercialized and used safely as sweeteners since the 1970s (see Carakostas *et al.* 2008).

The safety of steviol glycosides has been discussed extensively as part of reviews by various authorities such as JECFA on multiple occasions, the European Food Safety Authority (EFSA, 2010), Food Standards Australia New Zealand (FSANZ, 2008), and Health Canada (Health Canada, 2012). In addition to evaluations by these authoritative bodies, dozens of GRAS notices have been submitted to U.S. and have been filed with no objections (Table 6-1).

Early studies of steviol glycosides employed crude and/or poorly-characterized extracts, and raised several safety concerns (reviewed in other GRAS notices). However, subsequent studies with purified and/or standardized steviol glycosides have since resolved these issues, and enabled the Joint FAO/WHO Expert Committee on Food Additives (JECFA) to establish an acceptable daily intake 0-4 mg/kg bw for steviol glycosides, expressed as steviol.

Steviol glycoside safety studies discussed in prior GRAS notices are summarized in Tables 6-3 to 6-8. A subset of studies employing high-purity steviol glycosides is discussed in more detail below. Other studies of less pure substances are included in the summary tables for completeness, but are not discussed further, because they are considered less relevant to a discussion about purified steviol glycosides.

DSM also conducted literature searches in December 2017 using the terms "stevia," "steviol" or "rebaudioside," with "safety" or "toxic*" or "adverse" for any new information not described in prior GRAS notices. No new information relevant to the safety of purified steviol glycosides was identified.

6.3.2 Acute toxicity

Table 6-3 summarizes acute toxicity studies of steviol glycosides.

Toskulkao *et al.* (1997) reported a median lethal dose (LD_{50}) of >15 g/kg bw following oral administration of stevioside (purity 96%) to mice, rats and hamsters. Other acute toxicity studies were conducted with steviol glycosides not complying with JECFA specifications. For example, rebaudioside A, rebaudioside B, stevioside, and steviolbioside (purity not specified) were reported to produce no toxic effects when administered to male Swiss-Webster mice as a single gavage dose of 2 g/kg bw (Medon *et al.*, 1982).

6.3.3 Short-term and Subchronic Toxicity

Several short-term and subchronic toxicity studies of high-purity steviol glycosides have been conducted in experimental animals (Curry and Roberts, 2008; Nikiforov and Eapen, 2008) or stevioside of high purity (Aze *et al.*, 1991; Geuns *et al.*, 2003b). These studies are summarized in Table 6-4.

Rebaudioside A (> 97% purity) was administered to Wistar rats at concentrations up to 10% (100,000 ppm) of the diet in a 4-week dose-range finding study, and at concentrations up to 5% (50,000 ppm) of diet in a 13-week toxicity study (Curry and Roberts, 2008). No deaths, adverse clinical signs, changes in clinical chemistry and hematology parameters, and no treatment-related pathological findings were reported in these studies. The only observations that could be linked to treatment were effects on body weight, food intake and food conversion efficiency. Indeed, dietary concentrations greater than 2.5% (25,000 ppm) were associated with statistically significantly lower body weight gains in both sexes, particularly during the first days of the studies. Despite this decrease in body weight gain, no clear differences in food consumption could be seen in the 13-week study, and limited effects on food conversion efficiency were observed (Curry and Roberts, 2008).

Similar effects have been observed in other studies with intense sweeteners administered at a high level, with decreases in body weight gain ranging from 3.7 to more than 20% for neotame, sucralose or saccharin in comparison to control (Flamm *et al.,* 2003). In its evaluations, JECFA did not consider these changes in body weight gain to be of toxicological significance (JECFA, 2009). Similarly, JECFA considered that the decrease in body weight gain observed in rats receiving rebaudioside A for 13 weeks can be attributed to lower palatability and decreased caloric density of the diet. In addition, several changes in clinical chemistry and hematological parameters were observed (Curry and Roberts, 2008).

Mean plasma urea and creatinine concentrations were slightly higher in some of the treated groups; significantly lower concentrations of bile acids were observed. The increases in mean plasma urea and creatinine were not considered a sign of renal toxicity, because they were small, fell within the historical control range, and were not associated with any changes in macroscopic and microscopic observations of the kidneys. Bile acid levels were lower but within the normal range of historical controls, except for the high-dose male group. This effect was attributed to the metabolism and excretion of a large amount of rebaudioside A, and to the fact that biliary elimination is the main pathway of excretion in rats; therefore, it was not considered an adverse effect. Overall, the NOAEL was determined to be 5% (50,000 ppm) rebaudioside A in the diet, corresponding to 4161 and 4645 mg/kg bw/day, respectively, for males and females. This would be equivalent to 1370 mg and 1530 mg steviol/kg bw/day in males and females (Curry and Roberts, 2008; JECFA, 2009).

In another study, rebaudioside A (99.5% purity) was administered orally to Sprague-Dawley rats for 13 weeks up to doses of 2000 mg/kg bw/day (Nikiforov and Eapen, 2008). No adverse effects on body weight gains, terminal body weights, clinical and functional observations, hematology, serum chemistry or urinalysis were reported. No organ weight changes, macroscopic or microscopic tissue changes were observed that could be attributed to the treatment (Nikiforov and Eapen, 2008). A slight decrease in food

conversion efficiency observed in high-dose males was associated with decreased body weights and body weight gains. These observations corroborate the effects observed at higher doses by Curry and Roberts (2008). These effects were attributed to the lower nutritive value of the rebaudioside A-containing diets. Other observations included a tendency toward reduced serum bile acids, decreased urine volumes, and slight changes in serum electrolytes in treated groups, consistent with the results of Curry and Roberts (2008). In the absence of toxic effects, a NOAEL of 2000 mg/kg bw/day, corresponding to 660 mg steviol/kg bw/day, was proposed (Nikiforov and Eapen, 2008).

6.3.4 Genotoxicity

The genotoxic potential of steviol glycosides has been extensively studied *in vitro* and *in vivo*. Table 6-5 provides an overview of these studies. A critical review of the genetic toxicity of steviol glycosides and steviol was also published by Brusick (2008).

Overall, steviol glycosides do not show evidence of genotoxicity. Among the numerous studies performed, a single Comet assay was reported to show effects indicative of DNA damage (Nunes *et al.*, 2007). Groups of 5 male Wistar rats received stevioside (88.6% purity) in the drinking water at concentrations of 0 or 4 mg/mL for 45 days. This resulted in increased numbers of cells, including blood, liver, brain, and spleen cells, with "tails" and statistically significantly higher total "tail" scores (measure of tail length and overall size) compared to untreated rats (Nunes *et al.*, 2007). However, the validity of this study has been questioned by others, due to methodological concerns (Geuns, 2007; Williams, 2007). The JECFA (2009) and the EFSA Panel (2010) each considered that this study does not provide substantive evidence of a genotoxic potential for stevioside, also due to the fact that similar findings were not seen in earlier studies in mice using steviosides of higher or lower purities.

6.3.4.1 In vitro genotoxicity of steviol and steviol metabolites

Several *in vitro* studies have reported on the genotoxicity of steviol and some of its oxidative derivatives, notably in the presence of a metabolic activation system (Pezzuto *et al.*, 1985; 1986; Terai *et al.*, 2002). It is yet to be noted that the primary evidence for steviol genotoxicity comes from very specific bacteria tests or purified plasmid DNA that lack DNA repair capabilities. As reviewed by Brusick (2008), the genetic toxicity of steviol and some of its derivatives, exhibited in strain TM677, was not reproduced in the same bacteria having normal DNA repair processes. Studies of DNA damage and micronucleus formation performed in rats, mice and hamsters have also demonstrated the absence of genotoxicity of steviol *in vivo* up to doses of 8000 mg/kg bw (Temcharoen *et al.*, 2000).

More importantly, the available toxicokinetic data indicate the absence of free steviol from the systemic circulation of humans. Therefore, any possible concerns raised by the few genotoxic results of steviol observed *in vitro* are fully addressed by the fact that the genotoxic potential of steviol is not expressed *in vivo*, by the negative genotoxicity findings for steviol glycosides *in vitro* and *in vivo*, and by the absence of steviol in the human systemic circulation.

6.3.5 Chronic toxicity

Chronic toxicity studies of steviol glycosides are summarized in Table 6-6. As noted previously, the results of these studies are relevant for the safety evaluation of DSM's Reb M, since all steviol glycosides are converted to steviol in the gut *via* the same metabolic pathway.

Neither of two studies in rats exposed for 2 years to dietary concentrations of stevioside showed any evidence of adverse effects or carcinogenicity. The first study led to a NOAEL of 1.2% of the diet, equivalent to 600 mg stevioside/kg bw/day (Xili *et al.*, 1992). As subsequent study, which was more robust, led to a NOAEL of 2.5% of the diet, equivalent to 970 mg stevioside/kg bw/day in males, or 388 mg steviol equivalents/kg bw/day (Toyoda *et al.*, 1997). The NOAEL from the latter study was used by JECFA to establish an Acceptable Daily Intake (ADI) for steviol glycosides of 4 mg steviol equivalents/kg bw/day, by applying a 100-fold uncertainty factor (JECFA, 2008; 2009).

6.3.6 Developmental and Reproductive Toxicity

Studies in rats and hamsters have shown no effects of purified steviol glycoside preparations on fertility and offspring development (Mori *et al.*, 1981; Yodyingyuad and Bunyawong, 1991; Usami *et al.*, 1995; Curry *et al.*, 2008). These studies are summarized in Table 6-7. Most recently, Curry *et al.* (2008) observed no adverse reproductive or developmental effects in a 2-generation study in rats receiving rebaudioside A at up to 2.5% (25,000 ppm) of the diet, corresponding to approximately 2048 and 2273 mg/kg bw/day, respectively, for males and females.

6.3.7 Human Studies

In addition to studies evaluating the metabolism and pharmacokinetics of steviol glycosides in humans (section 6.2), other human studies have examined the effects of purified steviol glycosides and Stevia extracts on various endpoints. These studies are summarized in Table 6-8.

Maki *et al.* (2008a) investigated the effects of daily consumption of 1000 mg rebaudioside A (97% purity) on the blood pressure (resting, seated systolic/diastolilc, mean arterial) and heart rate of healthy volunteers; serum chemistry, hematology, urinalysis, and adverse events were monitored. Study participants received four 250-mg capsules or placebo with each of two meals for 4 weeks. No clinically-significant differences were observed in any of the parameters measured.

Likewise, administration of (1000 mg/day) rebaudioside A (97% purity) to individuals with type 2 diabetes mellitus for 16 weeks had no clinically-significant effects on glucose homeostasis parameters (glycosylated hemoglobin, fasting blood glucose, insulin, C-peptide), blood pressure, body weights, serum chemistry, hematology, urinalysis, or adverse events reported (Maki *et al.*, 2008b).

Table 6-3 Summary of acute oral toxicity studies

Reference	Species	Sex	Test material	LD_{50}
Medon *et al.* (1982)	Mouse	Male	Rebaudiosides A and B, stevioside, steviolbioside (purity unspecified)	>2 g/kg bw
Toskulkao *et al.* (1997)	Mouse	Male and Female	Stevioside (96% purity) Steviol (90% purity)	>15 g/kg bw
Toskulkao *et al.* (1997)	Rat	Male and Female	Stevioside (96% purity) Steviol (90% purity)	>15 g/kg bw
Toskulkao *et al.* (1997)	Hamster	Male and Female	Stevioside (96% purity)	>15 g/kg bw
Toskulkao *et al.* (1997)	Hamster	Male and Female	Steviol (90% purity)	Males: 5.2 g/kg bw Females: 6 g/kg bw

Adapted from GRAS notice GRN No. 667.

Table 6-4 Summary of short-term and subchronic toxicity studies

Reference	Animal model (no./sex/group)	Test Material	Dosage/Duration	NOAEL (mg/kg bw/day)	Results and Remarks
Akashi and Yokoyama (1975)[a]	Rat (strain unspecified)	Stevioside (purity unspecified)	Oral doses up to 2500 mg/kg bw/ 3 months	2500	No effects noted at doses tested. Experimental details such as body weight, organ weight, blood analysis, urine chemistry, gross necropsy and histopathology not discussed.
Mitsuhashi (1976)[a]	Rat (strain unspecified)	Stevioside (purity unspecified)	Dietary concentrations up to 7%/ 3 months	Not reported	No effects noted at doses tested. Experimental details such as body weight, organ weight, blood analysis, urine chemistry, gross necropsy and histopathology not discussed.
Aze *et al.* (1991)[b]	F344 rat (10/sex/group)	Stevioside (95.6% purity)	0, 0.31, 0.62, 1.25, 2.5, 5% in diet/ 13 weeks	2500 mg/kg bw/day	No effects observed on mortality, body weight or food consumption. Clinical chemistry investigation revealed increased LDH levels & histopathological investigation indicated increased incidence of single-cell liver necrosis in all male treated groups, but not in clear dose-response relationship. Investigators did not consider these changes to be treatment related due to small magnitude & low severity of changes, the lack of clear dose relationship & limitation to males only. Organ weights, urine chemistry & gross necropsy not discussed. Authors concluded that 5% stevioside in diet is tolerable dose for 2-year study.
Yodyingyuad and Bunyawong (1991)[c]	Hamster (10/sex/group)	Stevioside (90% purity)	0, 0.5, 1.0, 2.5 g/kg bw/day/ duration unclear/ 3 months	2500	F_0, F_1 & F_2 generations in reproductive study dosed for 90 days. Histological examination showed no effect at any dose. Weights of organs, blood analysis, urine chemistry & gross necropsy not discussed. The F1 & F2 hamsters continued to receive stevioside (via drinking water for one month, then at same dose as parents).
Awney *et al.* (2011)	Sprague-Dawley rat	Stevioside (97% purity)	15 or 1500 mg/kg bw/day in drinking water for 12 weeks	15	Treatment with high-dose stevioside caused significant changes in several investigated toxicological parameters. Among hematological parameters, significant changes noted in all except WBCs, RBCs, and PCV%, and in all clinical chemistry parameters except proteins, total lipids, ATL and AST.

Table 6-4 Summary of short-term and subchronic toxicity studies

Reference	Animal model (no./sex/group)	Test Material	Dosage/Duration	NOAEL (mg/kg bw/day)	Results and Remarks
Curry and Roberts (2008)	Wistar rat (10/sex/group)	Rebaudioside A (97% purity)	0, 2.5, 5, 7.5, and 10% of diet/ 4 weeks	Males: 9938 Females: 11,728 (10% level)	Reductions in body weight gain associated with reduced food consumption early in the study. Slight, but statistically significant, differences in several investigated toxicological parameters noted at ≥ 7.5% of diet.
Curry and Roberts (2008)	Wistar rat (20/sex/group)	Rebaudioside A (97% purity)	0, 1.25, 2.5, and 5% of diet/ 13 weeks	Males: 4161 Females: 4645 (5% level)	Reductions in body weight gain attributable to initial taste aversion and lower caloric density of the diet were observed in high-dose male and female groups. Inconsistent reductions in serum bile acids and cholesterol were attributed to physiological changes in bile acid metabolism due to excretion of high levels of rebaudioside A via the liver. All other hepatic function test results and liver histopathology were within normal limits. Significant changes in other clinical pathology results, organ weights and functional observational battery test results were not observed. Macroscopic and microscopic examinations of all organs, including testes and kidneys, were unremarkable with respect to treatment-related findings.
Nikiforov and Eapen (2008)	Sprague-Dawley rat (20/sex/group)	Rebaudioside A (99.5% purity)	Diet providing 0, 500, 1000, or 2000 mg/kg bw/day	≥ 2000 mg/kg bw/day	Lower mean body weight gains in males receiving 2000 mg/kg bw/day. No effects on other measured parameters.
Eapen (2008)	Beagle dog (4/sex/group)	Rebaudioside A (97.5% purity)	Diet providing 0, 500, 1000, or 2000 mg/kg bw/day for 6 months	≥ 2000 mg/kg bw/day	No effects observed in any measured parameter, which included mortality, clinical observations, home cage, open field and functional observations and measurements, hematology and serum chemistry findings, urinalysis findings, final body weights, gross necropsy observations, organ weights, or histological changes.

Table 6-4 Summary of short-term and subchronic toxicity studies

Reference	Animal model (no./sex/group)	Test Material	Dosage/Duration	NOAEL (mg/kg bw/day)	Results and Remarks
Rumelhard *et al.* (2016)	Sprague-Dawley rat	Rebaudioside A produced fermentatively by *Yarrowia lipolytica* (>95% purity)	Diet providing 0, 500, 1000, or 2000 mg/kg bw/day for 90 days	≥ 2000 mg/kg bw/day	No effects observed in any measured parameter, which included mortality and moribundity, clinical examinations, body weights, food consumption, functional observation battery and motor activity data, ophthalmic examination, clinical pathology (hematology, coagulation, serum chemistry, urinalysis), gross pathology, organ weights, and histopathology.

Adapted primarily from GRAS notice GRN No. 667; some studies as described in GRN No. 282.
[a] As reported by Geuns (2003); [b] As reported by Carakostas *et al.* (2008); [c] Abstract only.

Table 6-5 Summary of genotoxicity studies

Reference	Endpoint	Test System	Material	Concentration/Dose	Result
Medon *et al.* (1982)	Forward mutation	*S. typhimurium* TM677	Stevioside (purity unspecified)	Not specified	Negative[a]
Kerr *et al.* (1983)	Mutation	*D. melanogaster* Muller 5 strain	Stevioside (purity unspecified)	2% in feed	Negative
Ishidate *et al.* (1984)	Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside (85% purity)	12 mg/mL	Negative[b]
Pezzuto *et al.* (1985)	Forward mutation	*S .typhimurium* TM677	Stevioside (purity unspecified)	10 mg/plate	Negative[a]
Pezzuto *et al.* (1985)	Forward mutation	*S .typhimurium* TM677	Steviol (purity unspecified)	Up to 10 mg/plate	Mixed results[f]
Suttajit *et al.* (1993)	Reverse mutation	*S. typhimurium* TA98, TA100	Stevioside (99% purity)	50 mg/plate	Negative[a]
Suttajit *et al.* (1993)	Chromosomal aberration	Human lymphocytes	Stevioside (purity unspecified)	10 mg/mL	Negative
Suttajit *et al.* (1993)	Reverse mutation	S. typhimurium TA98 and TA100	Steviol (purity unspecified)	20 mg/plate	Negative[a]

Table 6-5 Summary of genotoxicity studies

Reference	Endpoint	Test System	Material	Concentration/Dose	Result
Matsui *et al.* (1996)	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, TA1537	Stevioside (83% purity)	5 mg/plate[b] 1 mg/plate[c]	Negative
Matsui *et al.* (1996)	Forward mutation	*S. typhimurium* TM677	Stevioside (83% purity)	10 mg/plate	Negative[a]
Matsui *et al.* (1996)	Gene mutation (umu)	*S. typhimurium TA1535/pSK1002*	Stevioside (83% purity)	5 mg/plate	Negative[a]
Matsui *et al.* (1996)	Gene mutation	*B. subtilis* H17 rec+, M45 rec-	Stevioside (83% purity)	10 mg/disk	Negative[a]
Matsui *et al.* (1996)	Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside (83% purity)	8 mg/mL 12 mg/mL	Negative
Matsui *et al.* (1996)	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, and TA1537	Steviol (99% purity)	5 mg/plate	Negative[a]
Matsui *et al.* (1996)	Forward mutation	*S. typhimurium* TM677	Steviol (purity unspecified)	Up to 10 mg/plate	Mixed results[f]
Matsui *et al.* (1996)	Gene mutation (umu)	*S. typhimurium* TA1535/pSK1002	Steviol (99% purity)	Up to 2500 µg/plate	Positive[a]
Matsui *et al.* (1996)	Gene mutation	*B. subtilis* H17 rec+, M45 rec-	Steviol (99% purity)	10 mg/disk	Negative[a]
Matsui *et al.* (1996)	Gene mutation	Chinese hamster lung fibroblasts	Steviol (99% purity)	400 µg/mL	Positive[g]
Matsui *et al.* (1996)	Micronucleus formation	MS/Ae mice	Steviol (99% purity)	100 mg/kg bw	Negative
Klongpanichpak *et al.* (1997)	Reverse mutation	*S. typhimurium* TA98 and TA100	Stevioside (purity unspecified)	50 mg/plate	Negative
Klongpanichpak *et al.* (1997)	Reverse mutation	*S. typhimurium* TA98 and TA100	Steviol (96% purity)	2 mg/plate	Negative
Oh *et al.* (1999)	Gene mutation	Mouse lymphoma L5178Y cells, TK+/- locus	Stevioside (purity unspecified)	5 mg/mL	Negative[a,d]
Oh *et al.* (1999)	Micronucleus formation	ddY mouse bone marrow and regenerating liver	Stevioside (purity unspecified)	62.5-250 mg/kg bw	Negative

Table 6-5 Summary of genotoxicity studies

Reference	Endpoint	Test System	Material	Concentration/Dose	Result
Oh *et al.* (1999)	Gene mutation	Mouse lymphoma L5178Y cells, TK+/- locus	Steviol (purity unspecified)	340 µg/mL	Negative[a,b]
Oh *et al.* (1999)	Micronucleus formation	ddY Mouse regenerating liver	Steviol (purity unspecified)	50-200 mg/kg	Negative[d]
Nakajima (2000a)	Chromosomal aberration	CHL/IU Chinese hamster lung fibroblasts	Reb A (purity unspecified)	1.2 - 55 mg/mL	Negative[a]
Nakajima (2000b)	Micronucleus formation	BDF1 mouse bone marrow	Reb A (purity unspecified)	500-2000 mg/kg bw per day for 2 days	Negative[e]
Temcharoen *et al.* (2000)	Micronucleus formation	Swiss mouse bone marrow	Steviol (~90% purity)	8000 mg/kg	Negative[h]
Temcharoen *et al.* (2000)	Micronucleus formation	Wistar rat bone marrow	Steviol (~90% purity)	8000 mg/kg	Negative[h]
Temcharoen *et al.* (2000)	Micronucleus formation	Syrian golden hamster bone marrow	Steviol (~90% purity)	4000 mg/kg	Negative[h]
Sekihashi *et al.* (2002)	DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver	Stevia extract (Stevioside 52%; Reb A 22%)	250-2000 mg/kg bw	Negative[e]
Sekihashi *et al.* (2002)	DNA damage (comet assay)	TK6 and WTK1 cells	Steviol (purity unspecified)	62.5-500 µg/mL	Negative[a]
Sekihashi *et al.* (2002)	DNA damage (comet assay)	Male DBF1 mouse stomach, colon liver; male CRJ:CD1 mouse liver kidney, color and testes	Steviol (>99% purity)	250-2000 mg/kg	Negative
Sasaki *et al.* (2002)	DNA damage (comet assay)	Male ddY mouse stomach, colon, liver, kidney, bladder, lung, brain, bone marrow	Stevia (purity unspecified)	2000 mg/kg bw	Negative[e]
Terai *et al.* (2002)	Forward mutation	*S. typhimurium* TM677	Steviol (purity unspecified)	Not specified	Positive[g]
Wagner and Van Dyke (2006)	Bacterial Mutagenicity	5 *Salmonella* strains with and without exogenous metabolic activation system	Reb A (99.5% purity)	1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate	No mutagenic response

Table 6-5 Summary of genotoxicity studies

Reference	Endpoint	Test System	Material	Concentration/Dose	Result
Clarke (2006)	Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence and presence of exogenous metabolic activation system	Reb A (99.5% purity)	Cloning conc. of 500, 1000, 2000, 3000, 4000 and 5000 µg/mL	No mutagenic or clastogenic response
Krsmanovic and Huston (2006)	Mouse micronucleus	Micronucleus study consisted of 7 groups, each containing 5 male and 5 female ICR mice	Reb A (99.5% purity)	500, 1000, and 2000 mg/kg bw	No increase in micronuclei formation
Nunes *et al.* (2007)	DNA damage (comet assay)	Wistar rats; liver, brain and spleen	Stevioside (88.62% purity)	4 mg/mL (estimated to be 80-500 mg/kg bw/day) in drinking water for 45 days	Positive in all tissues examined, most notably in liver
Williams and Burdock (2009)	Bacterial Mutagenicity	4 *Salmonella* strains and 1 *E. coli* strain with and without exogenous metabolic activation system	Reb A (95.6% purity)	Up to 5000 µg/plate	No mutagenic response
Williams and Burdock (2009)	Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence and presence of exogenous metabolic activation system	Reb A (95.6% purity)	Up to 5000 µg/mL	No mutagenic or clastogenic response
Williams and Burdock (2009)	Chromosome Aberration	Chinese Hamster V79 cells	Reb A (95.6% purity)	Up to 5000 µg/mL	No mutagenic or clastogenic response
Williams and Burdock (2009)	Mouse micronucleus	Micronucleus study in groups of 5 male and 5 female NMRI mice	Reb A (95.6% purity)	Up to 750 mg/kg bw	No increase in micronuclei formation
Williams and Burdock (2009)	Unscheduled DNA synthesis	*In vivo* Wistar rat	Reb A (95.6% purity)	Up to 2000 mg/kg bw	No increase in unscheduled DNA synthesis

Table 6-5 Summary of genotoxicity studies

Reference	Endpoint	Test System	Material	Concentration/Dose	Result
Rumelhard *et al.* (2016)	Bacterial mutagenicity	4 Salmonella strains and 1 E. coli strain with and without exogenous metabolic activation	Rebaudioside A produced fermentatively by *Yarrowia lipolytica* (>95% purity)	Up to 5000 µg/plate	No mutagenic response
Rumelhard *et al.* (2016)	Micronucleus formation in cultured human lymphocytes	Cultured human lymphocytes in absence and presence of exogenous metabolic activation	Rebaudioside A produced fermentatively by *Yarrowia lipolytica* (>95% purity)	Up to 5000 µg/plate	Not clastogenic or aneugenic

Adapted primarily from GRAS notice GRN No. 667; some studies as described in GRN No. 282.

[a] With or without metabolic activation; [b] Without metabolic activation; [c] As calculated by Williams (2007); [d] Inadequate detail available; [e] Sacrificed at 30 hours after 2nd administration; [f] Negative without metabolic activation, and positive with metabolic activation; [g] With metabolic activation; [h] Killed at 24, 30, 48 and 72 h. Ratio of polychromatic to normochromatic erythrocytes was decreased at later time point(s) in females.

Table 6-6 Summary of chronic toxicity studies

Yamada *et al.* (1985)	F344 rat (70/sex/group; 30/sex/group in low-dose)	95.2% Steviol glycosides (75% stevioside; 16% Reb A)	0.1, 0.3, 1% of diet/ 22 months for males; 24 months for females	550 (high dose)	At 6 and 12 months, 10 males and 10 females sacrificed for analysis. General behavior, growth and mortality were the same among groups throughout experiment. At 6 months, protein urea significantly increased in females, and blood glucose increased in both sexes, although urinary glucose not detected. Weights of liver, kidney, heart, prostate & testes increased in males at 6 months, and weight of ovaries decreased in females in dose-dependent manner. Histopathological examination showed differences in various organs at 6 months that were unrelated to stevioside dose. These differences not seen at 12 months. Authors concluded there were no significant changes after 2 years.
Xili *et al.* (1992)[a]	Wistar rat (45/sex/group)	Stevioside (85%)	0, 0.2, 0.6, 1.2% of diet/ 24 months	794 (high dose)	After 6, 12, and 24 months, 5 rats from each group sacrificed for analysis. No effects on growth, food utilization, general appearance, mortality, or lifespan. No changes in hematological, urinary, or clinical biochemistry values. Histopathological analysis showed that the neoplastic and non-neoplastic lesion were unrelated to the level of stevioside in diet.
Toyoda *et al.* (1997)	F344 rat (50/sex/group)	Stevioside (95.6% purity)	*Ad libitum* 0, 2.5, 5% of diet/ ~24 months (104 weeks) Mid-dose calculates to 970 mg/kg bw/day in males	Not reported	Significant decrease in survival rates in males receiving 5%. General condition, body weight, food intake, mortality, hematological histopathological and organ weights evaluated. Body weight gains decreased in a dose-dependent manner in both sexes. Kidney weights significantly increased in 5% females. Tumors and non-neoplastic lesions found in all groups and not correlated to treatment. Conclusion: stevioside is not carcinogenic under these experimental conditions

Adapted primarily from GRAS notice GRN No. 667; some studies as described in GRN No. 282.

[a] Abstract only.

Table 6-7 Summary of reproductive and developmental toxicity studies

Reference	Animal model (no./sex/group)	Test Material	Dosage/Duration	NOAEL (mg/kg bw/day)	Results and Remarks
Planas and Kuc (1968)	Rat (14/group)	Crude stevia extract (otherwise unspecified)	0 or 5 % crude stevia extract/ 18 days	Not reported	Extract given orally to adult female rats for 12 days before mating with untreated males for 6 days. Fertility reduced to 21% of control, remaining lower during 50-60-day recovery. Histological examination, weights of organs, blood analysis, urine chemistry, and necropsy not discussed.
Mori *et al.* (1981)	Rat (11/sex/group)	Stevioside (96% purity)	0, 0.15, 0.75 or 3 % of diet/ 60 days	2000	Males given stevioside dose in diet for 60 days before and during mating with females that received same diet (as mated male) 14 days before mating and 7 days during gestation. No treatment-related effects on fertility or mating performance, or fetal development. Rats of each sex had slightly decreased body weight gain at highest dose with non-significant increase in number of dead and resorbed fetuses at highest dose.
Oliveira-Filho *et al.* (1989)[a]	Rat	Dried Stevia Leaves (otherwise unspecified)	0 or 0.67 g dried leaves/mL, 2 mL twice per day/ 60 days	Not reported	Prepubertal rats (25-30 days old) tested for glycemia; serum concentrations of thyroxine; tri-iodothyroxine; available binding sites in thyroid hormone-binding proteins; binding of ^{3}H-methyltrienolone (a specific ligand of androgen receptors) to prostate cytosol; zinc content of prostate, testis, submandibular salivary gland, and pancreas; water content of testes and prostate; body-weight gain; and final weights of testes, prostate, seminal vesicle, submandibular salivary gland and adrenal. The only difference due to treatment was seminal vesicle weight, which fell to 60% compared to control.
Yodyingyuad and Bunyawong (1991)	Hamster (10/sex/group)	Stevioside (90% purity)	0, 500, 1000, 2500 mg/kg bw/day/ duration unclear/ 3 months	2500	Males from each group mated to females from respective dose group. Each female allowed to bear 3 litters during course of experiment. Stevioside had no effect on pregnancies of females at any dose. F_1 and F_2 animals continued to receive stevioside (*via* drinking water for one month, at same dose as parents); showed normal growth and fertility. Histological examination showed no effect on reproductive organs at any dose.

Table 6-7 Summary of reproductive and developmental toxicity studies

Reference	Animal model (no./sex/group)	Test Material	Dosage/Duration	NOAEL (mg/kg bw/day)	Results and Remarks
Usami *et al.* (1995)[a]	Wistar rat (25-26 pregnant)	Stevioside (95.6% purity)[b]	0, 250, 500, 1000 mg/kg bw by gavage on days 6-15 of gestation	1000	Pregnant rats sacrificed on day 20 of gestation and examined for maternal and fetal body weights, number of live fetuses, sex distribution, resorptions or dead fetuses, and fetal malformations. No treatment-related effects observed. Authors concluded that orally administered stevioside was not teratogenic in rats.
Wasuntarawat *et al.* (1998)	Golden Syrian hamster (12-20 females/group)	Steviol (90% purity)	0, 250, 500, 750, 1000 mg/kg kg bw/day by gavage during days 6-10 of gestation	250	Signs of maternal toxicity noted within 3-4 days after treatment with ≥500 mg/kg bw/day. Dose-related fetal mortality was observed, but no dose-dependent teratogenic effects.
Melis (1999)	Wistar rat (10 males/group)	Crude stevia extract (otherwise unspecified)	0 or 0.67 g dried leaves/mL, 2 mL/ 60 days		Animals receiving stevia extract had lower plasma testosterone, lower sperm concentration, and lower relative weights of testis, epididymis, and seminal vesicles. No histopathological changes in testis, seminal vesicles, prostate, or epididymis.
Kumar and Oommen (2008)	Swiss albino mice (5 females/group)	Stevioside and stevia extract (purity unspecified)	500 or 800 mg/kg bw/ 15 days	800	No effect on reproductive parameters when administered before or during pregnancy. No changes seen in number of implantations or uterine resorptions. No gross anatomical or histopathologic effects seen in 16-day embryos.
Curry *et al.* (2008)	Wistar rat (F_0: 30/sex/group; F_1: 24-25/sex/group)	Rebaudioside A (97% purity)	0, 0.75, 1.25, or 2.5% of diet for two generations	2048-2273 (2.5% level)	Treatment was not associated with any signs of clinical toxicity or adverse effects on body weight, body weight gain, or food consumption. No treatment-related effects in F_0 or F_1 on mating performance, fertility, gestation lengths, oestrous cycles, or sperm motility, concentration, or morphology. The survival and general condition of F_1 and F_2 offspring, preweaning reflex development, overall body weight gains, and the timing of sexual maturation, were not adversely affected.

Adapted primarily from GRAS notice GRN No. 667; some studies as described in GRN No. 282.

[a] Abstract only; [b] As reported by European Commission (1999).

Table 6-8 Summary of human studies

Reference	Study Type	Test Material	Measured Parameters	Results
Temme *et al.* (2004)	4 male and 4 female healthy volunteers	Stevioside (97% purity), 250-mg capsule, 3 times per day for 3 days 288 mg steviol/day	24-hr urine samples taken before dosing on day 1 and after dosing on day 3. Fasting blood samples taken before dosing on day 1, and six samples taken at different time points on day 3 after dosing. Fasting blood pressure measurements were taken before the first capsule and at six different time intervals after the first dose. Urine was analyzed for creatinine, sodium, potassium, calcium, and urea. Blood was analyzed for plasma glucose, plasma insulin, alkaline phosphatase, alanine transaminase (ALT), glutamic-pyruvate transaminase (GPT), creatine kinase, and lactate dehydrogenase.	Clinical analyses of blood, blood pressure, and urine showed no differences between samples taken before or after dosing.
Wheeler *et al.* (2008)	Randomized, double-blind, crossover 8 healthy adult males	• 5 mg/kg rebaudioside A (98.7% purity) • 4.2 mg/kg stevioside (96.6% purity) (-1.6 mg/kg steviol equivalents) A single oral dose of each as an aqueous solution with at least 14 days in between treatments	ECG, serum chemistry, hematology, adverse events Pharmacokinetic endpoints: steviol and steviol glucuronide in plasma, urine, and feces (pre-dose to 72 hours post-dose)	Sporadic out-of-range clinical pathology results in several subjects. None considered treatment-related or clinically significant. Minor fluctuations in heart rate, with no apparent treatment-related trends. Primary route of elimination was in urine as steviol glucuronide (59% rebaudioside A and 62% stevioside); feces accounted for about 5% of the dose. Only a trace was recovered as steviol in urine.
Maki *et al.* (2008a)	Randomized, double-blind, placebo-Controlled 50 healthy adults with normal blood pressure	Placebo (microcrystalline cellulose) Rebaudioside A (97% purity), 1000 mg orally (four 250-mg capsules, 2 with morning meal and 2 with evening meal) per day for 4 weeks	Blood pressure (resting, seated systolic/diastolic, mean arterial) and heart rate monitoring, serum chemistry, hematology, urinalysis, adverse events	No significant differences of clinical significance in heart rate, blood pressure or clinical pathology.

Table 6-8 Summary of human studies

Reference	Study Type	Test Material	Measured Parameters	Results
Maki *et al.* (2008b)	Randomized, double-blind, placebo-controlled 60 individuals with type 2 diabetes	Placebo (microcrystalline cellulose) Rebaudioside A (97% purity), 1000 mg orally (four 250-mg capsules, 2 with morning meal and 2 with evening meal) per day for 16 weeks	Body weights, glycosylated hemoglobin, fasting glucose, insulin, and C-peptide, total cholesterol and triglycerides, blood pressure, serum chemistry, hematology, and urinalysis, adverse events	No significant differences in glucose homeostasis, body weights, fasting lipids, or blood pressure. Mean ALT, GGT, and % basophils increased significantly, from baseline (week -2) in Reb A group, but mean levels remained within normal range.

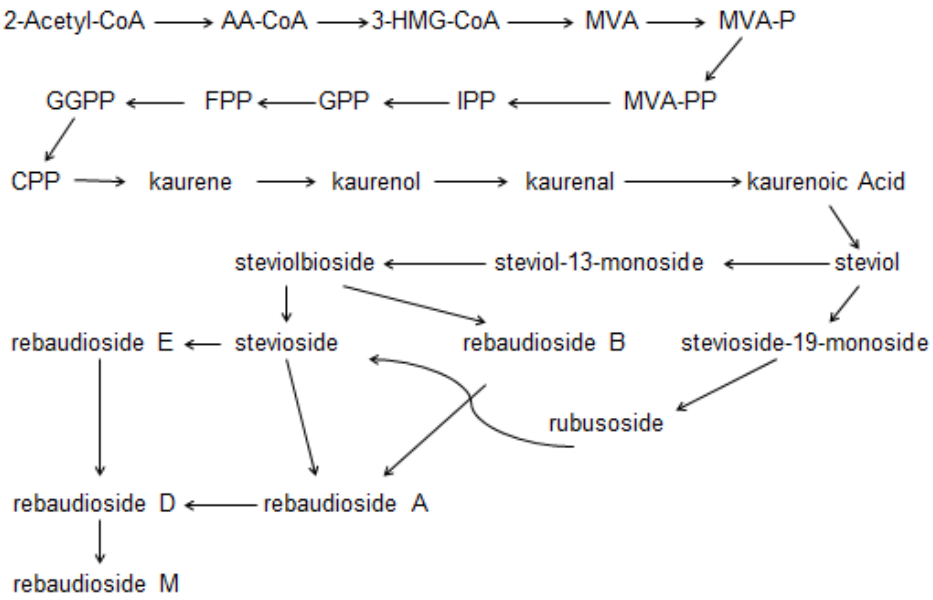
Adapted primarily from GRAS notice GRN No. 667; some studies as described in GRN No. 282.

6.4 Other safety considerations

6.4.1 Intermediates and byproducts of steviol glycoside biosynthesis

DSM's ingredient, purified steviol glycosides (rebaudioside M), is produced through fermentation by a strain of *Yarrowia lipolytica* genetically modified to express the steviol glycoside biosynthetic pathway of the stevia plant (see Figure 6-1). The production strain and fermentation conditions were developed to favor biosynthesis of rebaudioside M (Reb M), but the fermentation broth also contains several other steviol glycosides, most of which are removed during the purification and isolation steps.

Figure 6-1 Overview of the steviol glycoside biosynthetic pathway



Intermediates in the biosynthetic pathway of steviol glycosides that may be present in small amounts in DSM's purified steviol glycosides (Reb M) produced by *Yarrowia lipolytica* include steviol and kaurenoic acid. Steviol is synthesized from kaurene *via* the mevalonate pathway, while kaurene is oxidized in a three-step reaction to kaurenoic acid by kaurene oxidase; steviol is also formed with the hydroxylation of kaurenoic acid by kaurenoic acid 13-hydroxylase.

DSM had previously discussed the possible presence of these intermediates as part of GRAS notice GRN No. 632 for steviol glycosides produced by a similar strain of *Yarrowia lipolytica* favoring rebaudioside A (Reb A) biosynthesis. DSM monitors for the presence of these intermediates and has established adequate limits. DSM had also noted previously that analysis of commercially available stevia extracts revealed the presence of steviol and kaurenoic acid.

The chemical structures of steviol and kaurenoic acid were classified following the Cramer Class rule (Cramer *et al.*, 1978) by means of the widely used Toxtree-v2.6.0 software. Both compounds can be classified as Cramer Class III compounds, to which the threshold of toxicological concern (TTC) value of 1.5 µg/kg bw/day may be applied (Kroes *et al.*, 2004; EFSA/WHO, 2015). The TTC approach establishes a level of exposure below which there would be no appreciable risk to human health. It is an approach commonly

used by FDA to assess the risk of food-contact substances migrating into food, and by JECFA to assess the safety of flavors (Munro, 1996); it is also recommended by WHO and EFSA to assess substances of unknown toxicity present at low levels in the diet (EFSA/WHO, 2015).

DSM determined that, to ensure exposure remains below 1.5 µg/kg bw/day, the maximum acceptable level of either steviol or kaurenoic acid in DSM purified steviol glycosides (Reb M) would be 93 mg/kg (93 ppm), based on the JECFA ADI of 4 mg steviol equivalents/kg bw/day, equivalent to 16.2 mg DSM Reb M/kg bw/day.

6.4.2 Biogenic amines

As noted previously in the review of the production organism (see section 2.3.6), there have been reports of the formation of biogenic amines by *Yarrowia lypolytica*. Although the production of these toxic compounds is unlikely under the controlled fermentation conditions used to produce DSM's purified steviol glycosides (Reb M), DSM monitors the finished material for nitrogen content and uses this as an indicator for the presence of these metabolites.

Part 7: Supporting Data and Information

7.1 Expert Panel Statement

Expert Panel Opinion Regarding the Generally Recognized as Safe (GRAS) Status of DSM Purified Steviol Glycosides from *Yarrowia lipolytica* with Rebaudioside M (Reb M) as the Principal Component

Background

DSM Food Specialties (DSM) commissioned an independent panel of experts (GRAS Expert Panel), qualified by their scientific training and national and international experience, to determine whether: (1) there is sufficient information available to support the safety of DSM's high-purity (≥95%) steviol glycosides with rebaudioside M (Reb M) as the principal component when used as a general purpose non-nutritive sweetener[1] in various foods, excluding infant formulas and meat and poultry products; and (2) there is a basis to conclude that this technical evidence of safety is generally known and accepted by qualified experts.

To assist the Panel in its review, DSM provided a comprehensive summary (GRAS dossier) with detailed information about the intended uses and use levels, manufacturing, specifications, and analytical data, along with a summary of data supporting the safety of DSM's Reb M.

DSM's Reb M consists of purified steviol glycosides with rebaudioside M as the principal component, produced through fermentation by a strain of *Yarrowia lipolytica* genetically modified to express the steviol glycoside biosynthetic pathway of the stevia plant, *Stevia rebaudiana*. DSM's Reb M is produced in accordance with Good Manufacturing Practices (GMP). The purified ingredient meets JECFA and FCC published specifications for steviol glycosides,[2] and is equivalent to other commercially available high-purity steviol glycoside preparations, including those derived from the stevia plant. As such, it may be appropriately described as rebaudioside M, Reb M, or steviol glycosides.

The maximum dietary exposure to Reb M (expressed as steviol equivalents) was estimated to be 1.1 mg/kg bw/day for adults and 1.2 mg/kg bw/day for children, based on an approach used by Renwick (2008)[3] for substituting the high-intensity sweetener sucrose, and a presumed relative sweetness of 200 times that of sucrose for Reb M.

The GRAS Expert Panel noted that DSM had previously submitted a GRAS notice for a similar ingredient, purified steviol glycosides with rebaudioside A (Reb A) as the principal component, derived through fermentation using *Yarrowia lipolytica*. The notice was filed by U.S. FDA as GRN No. 632 (March 18, 2016) with no questions regarding DSM's conclusion that use of the ingredient as a non-nutritive sweetener in various foods is GRAS.

---

[1] As defined in 21 CFR 170.3(o)(19), non-nutritive sweeteners are substances having less than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.

[2] Although there are no established regulatory specifications for food-grade rebaudioside M, DSM has taken an approach similar to that of other GRAS notices with specifications based on those of JECFA and the Food Chemicals Codex (FCC, 2010) for steviol glycosides.

[3] Renwick AG (2008). The use of a sweetener substitution method to predict dietary exposures for the intense sweetener rebaudioside A. Food and Chemical Toxicology 46: S61-69.

DSM informed the Panel that the Reb M production strain is essentially the same as that used to produce Reb A. The new strain was derived from the same parents as the Reb A production strain, using the same genetic engineering techniques, with a few minor exceptions. Upstream of the steviol glycoside biosynthetic pathway, DSM employed additional codon optimization to increase the efficiency of the gene products, enzymes in the pathway that push production in favor of Reb M rather than Reb A. The presence of excess glucose in the fermentation media also aids in pushing the output towards Reb M, which has two additional glucose molecules. As in the production of Reb A, the fermentation broth contains not only Reb M but several other steviol glycosides that are removed during the purification and isolation steps.

The Panel noted that evaluation of the Reb M production strain using the Pariza and Johnson (2001)[4] decision tree did not reveal any safety concerns.

In its GRAS dossier, DSM noted that there is an extensive body of literature on the safety of steviol glycosides and that this information would be relevant to the safety of DSM's purified steviol glycosides with Reb M as the principal component, because: (1) it is generally accepted that all steviol glycosides share the same metabolic fate, *i.e.*, not absorbed to any extent until converted to the aglycone steviol by colonic microflora; and (2) metabolism of steviol glycosides is similar between humans and experimental animals. This approach has been used in several other GRAS notices for steviol glycosides, most recently in GRN Nos. 512 and 667 for high-purity (≥95%) Reb M ingredients similar to DSM's Reb M in composition, specifications, and proposed food uses.

The GRAS Expert Panel concurred with DSM's determination that existing toxicity data for steviol glycosides in general (and the metabolite steviol) can be used to support the safety of Reb M, noting the following elements discussed in DSM's GRAS dossier (and other filed GRAS notices) as evidence of the general safety of steviol glycosides:

- Steviol glycosides extracted from the stevia plant have been commercialized and used safely as sweeteners since the 1970s (Carakostas *et al.* 2008)[5], and DSM's Reb M is a high-purity ingredient similar to other steviol glycoside ingredients commercially available.

- Aside from DSM's GRAS notice GRN No. 632 for Reb A from *Yarrowia lipolytica*, dozens of other GRAS notices for highly-purified steviol glycosides have been filed and accepted by U.S. FDA with no questions, many relying on the same body of data.

- Early studies of steviol glycosides employed crude and/or poorly-characterized stevia extracts, and raised several safety concerns. However, subsequent studies with purified and/or standardized steviol glycosides have since resolved these issues, and enabled the Joint FAO/WHO Expert Committee on Food Additives (JECFA) to establish an acceptable daily intake (ADI) of 0-4 mg/kg bw for steviol glycosides, expressed as steviol.

- The safety of steviol glycosides has been discussed extensively as part of reviews by various authorities such as JECFA on multiple occasions, the European Food Safety Authority (EFSA, 2010)[6], Food Standards Australia New Zealand (FSANZ, 2008)[7], and Health Canada (Health Canada, 2012).[8]

---

[4] Pariza MW and Johnson EA (2001). Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Regulatory Toxicology and Pharmacology 33:173-186.

[5] Carakostas MC, Curry LL, Boileau AC, Brusick DJ (2008). Overview: the history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. Food Chem Toxicol 46(7):S1-S10.

Expert Panel Opinion Statement

We, the members of the GRAS Expert Panel, have independently and collectively, critically evaluated all the relevant information, summarized in DSM's GRAS dossier or otherwise publicly available. It is our opinion as qualified experts that there is reasonable certainty that no harm will result from the use of DSM's high-purity (≥95%) steviol glycosides from *Yarrowia lipolytica*, with rebaudioside M (Reb M) as the principal component, as a non-nutritive sweetener in foods for the general U.S. population (excluding infant formulas and meat and poultry products) at levels resulting in consumer exposures within the ADI of 0-4 mg steviol/kg bw established by JECFA for steviol glycosides.

We further conclude that the such uses would be considered generally recognized as safe (GRAS) based on scientific procedures, and that other qualified experts would agree.

(b) (6)

13 July 2017

Stanley M. Tarka, Jr., Ph.D., F.A.T.S.
The Pennsylvania State University College of Medicine
Tarka Group, Inc.
Carlisle, PA

Date

(b) (6)

July 11, 2017

John A. Thomas, Ph.D., F.A.C.T., D.A.T.S.
Indiana University
Indianapolis, IN

Date

(b) (6)

July 10th, 2017

José L. Avalos, Ph.D.
Princeton University
Princeton, NJ

Date

[6] EFSA Journal (2010). Scientific Opinion on the safety of steviol glycosides for the proposed uses as a food additive. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 38(4):1537.

[7] FSANZ (2008). Final Assessment Report, Application A540, Steviol glycosides as intense sweeteners. Food Standards Australia New Zealand.

[8] Health Canada (2012). Information and Consultation Document on Health Canada's Proposal to allow the Use of the Food Additive Steviol Glycosides as a Table-Top Sweetener and as a sweetener in Certain Food Categories. Available at http://www.hc-sc.gc.ca/fn-an/consult/steviol/document-consultation-eng.php#a3.

7.2 References

Akashi H and Yokoyama Y (1975). Dried-leaf extracts of stevia, toxicological test. Shokuhin Kogyo 18(20):34-43. Cited In: GRAS notice GRN. No. 667.

Awney H A, Massoud MI, El-Maghrabi S (2011. 'Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing male rats. Journal of Applied Toxicology 31(5):431-438. Cited In: GRAS notice GRN. No. 667.

Aze Y, Toyoda K, Imaida K, Hayashi S, Imazawa T, Hayashi Y, Takahashi M (1991). Subchronic oral toxicity study of stevioside in F344 rats. Bulletin of the National Institute of Agricultural Sciences 48-54 (in Japanese). Cited In: GRAS notice GRN. No. 667.

Bankar AV, Kumar AR and Zinjarde SS (2009). Environmental and industrial applications of *Yarrowia lipolytica.* Appl Microbiol Biotechnol (2009) 84:847–865

Barth G and Gaillardin C (1997). Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. FEMA Microbiology Reviews 19:219-237.

Brandle, JE and Telmer, PG (2007). Steviol Glycoside Biosynthesis. Phytochemistry 68:1855-1863.

Brusick DJ (2008). A critical review of the genetic toxicity of steviol and steviol glycosides. Food Chem Toxicol 46:S83-S91.

Carakostas MC, Curry LL, Boileau AC, Brusick DJ (2008). Overview: the history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. Food Chem Toxicol 46(7):S1-S10.

Chang, SS and Cook, JM (1983). Stability Studies of Stevioside and Rebaudioside A in Carbonated Beverages. J. Agric. Food Chem 3(1):409-412.

Clarke J (2006). Mammalian cell gene mutation test (L5178YTTKtI—Mouse Lymphoma Assay)[with Rebaudioside A]. BioReliance, Rockville, MD. Unpublished Report (Study Number AB21TG. 704. BTL*)*. Cited In: GRAS notice GRN. No. 667.

Clos JF, DuBois GE and Prakash I, (2008). Photostability of Rebaudioside A and Stevioside in Beverages. J. Agric. Food Chem. 56, 8507–8513.

Compadre C, Hussain R, Nanayakkara N, Pezzuto J, Kinghorn A (1988). Mass spectral analysis of some derivatives and *in vitro* metabolites of steviol, the aglycone of the natural sweeteners, stevioside, rebaudioside A, and rubusoside. Biomedical & Environmental Mass Spectrometry 15(4):211-222.

Cramer GM, Ford RA and Hall RL (1978). Estimation of toxic hazard - a decision tree approach. Food and Cosmetic Toxicology, 16: 255-276

Curry L and Roberts A (2008). Subchronic toxicity of rebaudioside A. Food Chem Toxicol 46 (7):S11-S20.

Curry L, Roberts A, Brown N (2008). Rebaudioside A: Two-generation reproductive toxicity study in rats. Food Chem Toxicol 46 (7):S21-S30.

Eapen A (2008). A 6-Month Oral (Dietary) Toxicity Study of Chrysanta® 99-P in Beagle Dogs', WIL Research Laboratories, LLC. Unpublished Report (Study Number WIL-568011). Reb A Safety Evaluation Dossier–References VIII-4. Cited In: GRAS notice GRN No. 667.

EFSA Journal (2010). Scientific Opinion on the safety of steviol glycosides for the proposed uses as a food additive. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 38(4):1537.

EFSA Journal (2011a). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. EFSA Panel on Biological Hazards (BIOHAZ), 9(10): 2393

EFSA Journal (2011b). Revised exposure for steviol glycosides for the proposed uses as a food additive, 9(1): 1972

EFSA Journal (2013). Anticipation of future notifications to EFSA of microbial taxonomic units intentionally introduced into the food chain. 11(8): 3340

EFSA Journal (2014). Scientific Opinion on the revised exposure assessment of steviol glycosides (E960) for the proposed uses as a food additive. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 12(5): 3639

EFSA Journal (2015). Scientific opinion on the safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive. EFSA Panel on Food Additives and Nutrient Sources added to food (ANS), 13(12): 4316

EFSA/WHO (2015). Threshold of Toxicological Concern Approach: Conclusions and Recommendations of the EFSA/WHO Expert Workshop. Draft for public consultation.

European Commission (1999). Opinion on stevioside as a sweetener. Scientific Committee on Food (CS/ADD/EDUL/167, Final, 17 June 1999). Cited In: GRAS notice GRN No. 667.

FDA Response Letter to GRN No. 355. DuPont.
http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm250909.htm

FDA Response Letter to GRN No. 382. Baolingbao Biology Co. Ltd.
http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm282595.htm

Ferreira AD and Viljoen BC (2003). Yeasts as adjunct starters in matured Cheddar cheese. International Journal of Food Microbiology 86:131-140.

Flamm WG, Blackburn GL, Comer CP, Mayhew DA, Stargel WW (2003). Long-term food consumption and body weight changes in neotame safety studies are consistent with the allometric relationship observed for other sweeteners and during dietary restrictions. Regulatory Toxicology and Pharmacology 38:144-156.

FSANZ (2008). Final Assessment Report, Application A540, Steviol glycosides as intense sweeteners. Food Standards Australia New Zealand.

Gardini F, Suzzi G, Lombardi A, Galgano F, Crudele MA, Andrighetto C, Schirone M, Tofalo R (2001). A survey of yeasts in traditional sausages of southern Italy. FEMS Yeast Research 1:161-167.

Geuns JMC (2003). Stevioside. Phytochemistry 64(5): 913-921. Cited In: GRAS notice GRN. No. 667.

Geuns JMC (2007). Comments to the paper by Nunes *et al.* (2007), Analysis of genotoxic potential of stevioside by comet assay, Food Chemical Toxicol 45:662-666. Food Chem Toxicol 45:2601-2602.

Geuns JMC and Pietta P (2004). Stevioside metabolism by human volunteers. Unpublished report from Laboratory Functional Biology, Kuleuven, Leuven Belgium and ITB-CNR, Segrate (MI); Italy. Submitted to WHO by the Federal Ministry of Social Affairs, Public Health and the Environment; Belgium. Cited In: JECFA, 2006.

Geuns JMC, Augustijns P, Mols R, Buyse JG, Driessen B (2003a). Metabolism of stevioside in pigs and intestinal absorption characteristics of Stevioside, Rebaudioside A and Steviol. Food Chem Toxicol 41:1599-1607.

Geuns JMC, Malheiros RD, Moraes VMB, Decuypere EMP, Compernolle F, Buyse JG (2003b). Metabolism of stevioside by chickens. Journal of Agricultural and Food Chemistry 51: 1095-1101.

Geuns JMC, Buyse J, Vankeirsbilck A, Temme EHM (2007). Metabolism of stevioside by healthy subjects. Experimental Biology and Medicine 232:164-173.

GRN No. 252. GRAS notice for Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni. Whole Earth Sweetener Company (a wholly owned subsidiary of Merisant Company). https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm264109.pdf

GRN No. 253. GRAS notice for Rebaudioside A purified from *Stevia rebaudiana* (Berton) Bertoni. Cargill. https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm269137.pdf

GRN No. 282. GRAS notice for Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni. Sweet Green Fields, LLC. https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm269251.pdf

GRN No. 304. GRAS notice for Purified steviol glycosides with rebaudioside A and stevioside as the principal components. Sunwin and WILD Flavors. https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm269405.pdf

GRN No. 473. GRAS notice for Purified steviol glycosides with rebaudioside X as the principal component. PureCircle Ltd.

https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm359700.pdf

GRN No. 512. GRAS notice for High purity Rebaudioside M. GLG Life Tech Corporation. https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm400646.pdf
GRN No. 667. GRAS notice for Rebaudioside M. GLG Life Tech Corporation. Blue California. https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm524575.pdf

Groenewald M, Boekhout T, Neuvéglise C, Gaillardin C, van Dijck PWM and Wyss M (2013). *Yarrowia lipolytica*: safety assessement of an oleaginous yeast with a great industrial potential. Critical Reviews of Microbiology, 40(3): 187-206

Health Canada (2012). Information and Consultation Document on Health Canada's Proposal to allow the Use of the Food Additive Steviol Glycosides as a Table-Top Sweetener and as a sweetener in Certain Food Categories. Available at http://www.hc-sc.gc.ca/fn-an/consult/steviol/document-consultation-eng.php#a3.

Hutapea AM, Tolskulkao C, Wilairat P, Buddhaukh D (1999). High-performance liquid chromatographic separation and quantitation of stevioside and its metabolites. Journal of Liquid Chromatography and Related Technologies 22:1161-1170.

Ishidate M, Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada M, Matsuoka A (1984) Primary mutagenicity screening of food additives currently used in Japan. Food Chem Toxicol 22(8):623-636. Cited In: GRAS notice GRN. No. 667.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (1999). Safety evaluation of certain food additives. WHO Food Additives Series 42. World Health Organization, Geneva, 119-143.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (2006). Safety evaluation of certain food additives. Prepared by the 63rd meeting of the Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additives Series, No. 54: 117-144.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (2008). Compendium of Food Additive Specifications. Monograph 5. Steviol glycosides. Available at: http://www.fao.org/ag/agn/jecfa-additives/details.html?id=898

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (2009). Safety evaluation of certain food additives. Prepared by the 69th meeting of the Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additives Series, No. 66: 183-220.

Kerr W, Mello M, Bonadio E (1983). Mutagenicity tests on the Stevioside from *Steavia rebaudiana* (Beert) Bertoni. SOC Brasil Genetica Dept Genetica FAC Medicina Ribeirao Preto, 14049-900 Pret, Brazil. Cited In: GRAS notice GRN. No. 667.

Klongpanichpak S, Temcharoen P, Toskulkao C, Apibal S, Glinsukon T (1997). Stevia 1997. J Med Assoc Thai 80(1):S121-128. Cited In: GRAS notice GRN. No. 667.

Koyama E, Ohori Y, Kitazawa K, Izawa O, Kakegawa K, Fujino A, Ui M (2003a). *In vitro* metabolism of the glycosidic sweeteners, Stevia mixture and enzymically modified Stevia in human intestinal microflora. Food Cheml Toxicol 41:359-374.

Koyama E, Sakai N, Ohori Y, Kitazawa K, Izawa O, Kakegawa K, Fujino A, Ui M (2003b). Absorption and metabolism of glycosidic sweeteners of stevia mixture and their aglycone, steviol, in rats and humans. Food Chemical Toxicol 41:875-883.

Kraemer T and Maurer HH (1994). On the metabolism of the sweetener stevioside in humans. European Journal of Pharmaceutical Sciences 2:103 [Abstract No. FC12].

Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A, Schilter B, Schlatter J, van Schothorst F, Vos JG and Würtzen G (2004). Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. Food Chem Toxicol, 42: 65-83.

Krsmanovic L and Huston T (2006). Rebaudioside A: Mammalian Erythrocyte Micronucleus Test. BioReliance, Rockville, MD. Unpublished Report (Study Number AB21TG.123.BTL). Cited In: GRAS notice GRN No. 667.

Kumar D and Oommen V (2008). Stevia rebaudiana Bertani does not produce female reproductive toxic effect: Study in Swiss albino mouse. J Endocrinol Reprod 12(1):57-60. Cited In: GRAS notice GRN No. 667.

Lanciotti R, Gianotti A, Baldi D, Agrisani R, Suzzi G, Mastrocola D and Guerzoni ME (2005) Use of *Yarrowia lipolytica* strains for the treatment of olive mill wastewater. BioResource Tech. 96: 317-322.

Madzak C, Gaillardini C and Beckerich JM (2004). Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. Journal of Biotechnology 109 (2004) 63–81

Maki KC, Curry LL, Reeves MS, Toth PD, McKenney JM, Farmer MV, Schwartz SL, Lubin BC, Boileau A, Dicklin MR, Carakostas M, Tarka S (2008a). Chronic consumption of rebaudioside A, a steviol glycoside, in men and women with type 2 diabetes mellitus, Food Chem Toxicol 46:S47-53. Cited In: GRAS notice GRN. No. 667.

Maki KC, Curry LL, Carakostas M, Tarka S, Reeves MS, Farmer MV, McKenney JM, Toth PD, Schwartz SL, Lubin BC, Dicklin MR, Boileau A, Bisognano JD (2008b). The hemodynamic effects of rebaudioside A in healthy adults with normal and low-normal blood pressure. Food Chem Toxicol 46:S40-465. Cited In: GRAS notice GRN. No. 667.

Matsui M, Matsui K, Kawasaki Y, Oda Y, Noguchi T, Kitagawa Y, Sawada M, Hayashi M, Nohmi T, Yoshihira K (1996). Evaluation of the genotoxicity of stevioside and steviol using six *in vitro* and one *in vivo* mutagenicity assays. Mutagenesis 11(6):573-579. Cited In: GRAS notice GRN. No. 667.

McCabe-Sellers BJ, Staggs CG and Bogle ML (2006). Tyramine in foods and monoamine oxidase inhibitor drugs: A crossroad where medicine, nutrition, pharmacy, and food industry converge. Journal of Food Composition and Analysis 19 (2006) S58–S65

Medon PJ, Pezzuto JM, Hovanec-Brown JM, Nanayakkara NP, Soejarto DD, Kamath SK and Kinghorn AD (1982). Safety assessment of some Stevia rebaudiana sweet principles. Federal Procedure, 41: 1568. Cited in: EFSA, 2010.

Melis MS (1999). Effects of chronic administration of Stevia rebaudiana on fertility in rats. J Ethnopharmacol 167: 157-161. Cited In: GRAS notice GRN. No. 282.

Mitsuhashi H (1976). Safety of Stevioside. Tama Biochemical Co. Ltd. Cited In: GRAS notice GRN. No. 667.

Mori N, Sakanoue M, Takeuchi M, Shimpo K, Tanabe T (1981). Effect of stevioside on fertility in rats. Journal of Food Hygiene Society Japan, 22(5):409-414 (in Japanese) Cited In: GRAS notice GRN. No. 667.

Munro IC, Ford RA, Kennepohl E and Sprenger JG (1996). Correlation of a structural class with No-Observed-Effect-Levels: a proposal for establishing a threshold of concern. Food Chem Toxicol. 34, 829 867.

Nakajima M *et al.* (2000a). Chromosome aberration assay of rebaudioside A in cultured mammalian cells. Test number 5001 (079-085). Unpublished report of a study conducted at the Biosafety Research Center, Japan. Submitted to WHO by Ministry of Health and Welfare, Japan. Cited In: GRAS notice GRN. No. 667.

Nakajima M *et al.* (2000b). Micronucleus test of rebaudioside A in mice. Unpublished report of a study conducted at the Biosafety Research Center, Japan. Submitted to WHO by Ministry of Health and Welfare, Japan. Cited In: GRAS notice GRN. No. 667.

Nakayama K, Kasahara D, Yamamoto F (1986). Absorption, Distribution, Metabolism and Excretion of Stevioside in rats. Journal of the Food Hygienic Society of Japan 27:1-8.

Ng SW, Slining MM, Popkin BM (2012). Use of Caloric and Noncaloric Sweeteners in US Consumer Packaged Foods, 2005-2009. Journal of the Academy of Nutrition and Dietetics, 112: 1828-1834

Nikiforov AI and Eapen AK (2008). A 90-day oral (dietary) toxicity study of rebaudioside A in Sprague-Dawley rats. International Journal of Toxicology 27:65-80.

Nikiforov AI, Rihner OM, Eapen, AK, Thomas, JA (2013). Metabolism and Toxicity Studies Supporting the Safety of Rebaudioside D. International Journal of Toxicology 32:261-273.

Nunes APM, Ferreira-Machado SC, Nunes RM, Dantas FJS, De Mattos JCP, Caldeira-de-Araújo A (2007). Analysis of genotoxic potentiality of stevioside by comet assay. Food Chem Toxicol 45:662-666.

Oh HY, Han ES, Choi DW, Kim JW, Eom MO, Kang IH, Kang HJ, Ha KW (1999). *In vitro* and *In vivo* Evaluation of Genotoxicity of Stevioside and Steviol, Natural Sweetner. JOURNAL-PHARMACEUTICAL SOCIETY OF KOREA 43:614-622. Cited In: GRAS notice GRN. No. 667.

Oliveira-Filho RM, Uehara OA, Minetti CA, Valle LB (1989). Chronic administration of aqueous extract of Stevia rebaudiana (Bert.) Bertoni in rats: endocrine effects. General Pharmacology: The Vascular System 20(2):187-191. Cited In: GRAS notice GRN. No. 667.

Pariza MW and Johnson EA (2001). Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Regulatory Toxicology and Pharmacology 33:173-186.

Pezzuto JM, Compadre CM, Swanson SM, Nanayakkara NPD, Kinghorn AD (1985). Metabolically activated steviol, the aglycone of stevioside is mutagenic. Proceedings of the National Academy of Science 82:2478-2482.

Pezzuto JM, Nanayakkara NP, Compadre CM, Swanson SM, Kinghorn AD, Guenthner TM, Sparnins VL, Lam LK (1986). Characterization of bacterial mutagenicity mediated by 13-hydroxy-ent-kaurenoic acid (steviol) and several structurally-related derivatives and evaluation of potential to induce glutathione S-transferase in mice. Mutation Research 169(3):93-103.

Planas GM and Kuć J (1968). Contraceptive properties of Stevia rebaudiana. Science 162(3857):1007-1007. Cited In: GRAS notice GRN. No. 667.

Prakash I, Markosyan A, Bunders C (2014). Development of Next Generation Stevia Sweetener: Rebaudioside M. Foods 3:162-175.

Prillinger H, Molnar O, Eliskases-Lechner F and Lopandic K (1999). Phenotypic and genotypic identification of yeasts from cheese. *Antonie van Leeuwenhoek* **75:** 267–283, 1999.

Purkayastha S, Bhusari S, Pugh G, Teng X, Kwok D, Tarka SM (2015). *In vitro* metabolism of rebaudioside E under anaerobic conditions: Comparison with rebaudioside A. Regul Toxicol Pharmacol 72(3):646-657.

Purkayastha S, Markosyan A, Prakash I, Bhusari S, Pugh G, Lynch B, Roberts A (2016). Steviol glycosides in purified stevia leaf extract sharing the same metabolic fate. Reg Tox Pharm 77:125-133.

Renwick AG (2008). The use of a sweetener substitution method to predict dietary exposures for the intense sweetener rebaudioside A. Food Chem Toxicol 46: S61-69.

Roberts A and Renwick AG (2008). Comparative toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol in rats. Food Chem Toxicol 46:S31-S39.

Rumelhard M, Hosako H, Eurlings IM, Westerink WM, Staska LM, van de Wiel JA, La Marta J (2016). Safety evaluation of rebaudioside A produced by fermentation. Food Chem Toxicol 89: 73-84. Cited In: GRAS notice GRN. No. 667.

Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K, Taniguchi K, Tsuda S (2002). The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 519(1):103-119. Cited In: GRAS notice GRN. No. 667.

SCF (Scientific Committee on Food), 1984. Reports of the Scientific Committee for Food Concerning Sweeteners (Opinion Expressed by the SCF on 14 September 1984). In: Food Science and Techniques. Brussels, Belgium: Commission of the European Communities (EEC), Health & Consumer Protection Directorate-General, Scientific Committee on Food, 1985. (Reports of the Scientific Committee for Food (16th series).

SCF (Scientific Committee on Food), 1989. Reports of the Scientific Committee for Food on Sweeteners. 21th series. Opinion expressed 11 December 1987 and 10 November 1988, and adopted 10 November 1988.

SCF (Scientific Committee on Food), 1999. Opinion on Stevioside as a Sweetener (Adopted on 17/6/99). Belgium: European Commission, Health & Consumer Protection Directorate-General, Scientific Committee on Food. [CS/ADD/EDUL/167 Final]

Sekihashi K, Saitoh H, Sasaki YF (2002). Genotoxicity studies of stevia extract and steviol by the comet assay. Journal of toxicological sciences 27(Suppl 1):1-10. Cited In: GRAS notice GRN. No. 667.

Smith JJ, Szilard RK, Marelli M and Rachubinski RA (1997). The Peroxin Pex17p of the Yeast *Yarrowia lipolytica* Is Associated Peripherally with the Peroxisomal Membrane and Is Required for the Import of a Subset of Matrix Proteins. MOLECULAR AND CELLULAR BIOLOGY 17:2511–2520.

Suttajit M, Vinitketkaumnuen U, Meevatee U, Buddhasukh D (1993), Mutagenicity and human chromosomal effect of stevioside, a sweetener from Stevia rebaudiana Bertoni. Environmental Health Perspectives 101(Suppl 3):53-56. Cited In: GRAS notice GRN. No. 667.

Temcharoen P, Klongpanichpak S, Glinsukon T, Suwannatrai M, Apibal S, Toskulkao C (2000). Evaluation of the effect of steviol on chromosomal damage using micronucleus test in three laboratory animal species. Journal of the Medical Association of Thailand 83:S101-108.

Temme E, Vankeirsblick,A, Buyse J (2004). A short term study of stevioside in healthy subjects. Safety of Stevioside:Proceedings of the First Symposium. Sponsored by KULeuven 63-74. Cited In: GRAS notice GRN. No. 667.

Terai T, Ren H, Mori G, Yamaguchi Y, Hayashi T (2002). Mutagenicity of steviol and its oxidative derivatives in *Salmonella typhimurium* TM677. Chemical and Pharmaceutical Bulletin 50:1007-1010.

Til HP, Falke HE, Prinsen MK and Willems MI (1997). Acute and Subacute Toxicity of Tyramine, Spermidine, Spermine, Putrescine and Cadaverine in Rats. Food Chem Toxicol 35: 337-348.

Toskulkao C, Chaturat L, Temcharoen P and Glinsukon T (1997). Acute toxicity of stevioside, a natural sweetener, and its metabolite, steviol, in several animal species. Drug and Chemical Toxicology 20: 31-44.

Toyoda K, Matsui H, Shoda T, Uneyama C, Takada K, Takahashi M (1997). Assessment of the carcinogenicity of stevioside in F344 rats. Food Chem Toxicol 35:597-603.

Usami M, Sakemi K, Kawashima K, Tsuda M, Ohno Y (1995). Teratogenicity study of stevioside in rats. Bulletin of the National Institute of Hygienic Sciences 113:31-35 (in Japanese). Cited In: GRAS notice GRN. No. 667.

USDA Table 50, USDA ERS Sugar and Sweeteners Outlook. June 4, 2015. Table 50 U.S. per capita caloric sweeteners estimated deliveries for domestic food and beverage use, by calendar year. http://www.ers.usda.gov/data-products/sugar-and-sweeteners-yearbook-tables.aspx

USDA Table 51, USDA ERS Sugar and Sweeteners Outlook. June 4, 2015. Table 51 Refined cane and beet sugar: estimated number of per capita calories consumed daily, by calendar year. http://www.ers.usda.gov/data-products/sugar-and-sweeteners-yearbook-tables.aspx

van der Walt JP and von Arx JA (1980). The yeast genus *Yarrowia* gen. nov. Antonie von Leeuwenhoek 46 (1980) 517-521

Viljoen BC, Dykes GA, Callis M and von Holy A (1993). Yeasts associated with Vienna sausage packaging. International Journal of Food Microbiology 18:53-62.

Wagner V O and Van Dyke MR (2006). Bacterial Reverse Mutation Assay of Rebaudioside A. BioReliance, Rockville, MD. Study Number AB21TG.503.BTL. Cited In: GRAS notice GRN. No. 667.

Wasuntarawat C, Temcharoen P, Toskulkao C, Mungkornkarn P, Suttajit M, Glinsukon T (1998). Developmental toxicity of steviol, a metabolite of stevioside, in the hamster. Drug Chem Toxicol 21(2):207-222. Cited In: GRAS notice GRN. No. 667.

Wheeler A, Boileau A, Winkler P, Compton J, Prakash I, Jiang X, Mandarino D and Jenkins J (2008). Pharmacokinetics of rebaudioside A and stevioside after single oral doses in healthy men. Food Chem Toxicol 46:S54-60.

Williams GM (2007). Letter to the editor, Food Chem Toxicol 45:2597-2598.

Williams LD and Burdock GA (2009). Genotoxicity studies on a high-purity rebaudioside A preparation. Food Chem Toxicol 47(8):1831-1836. Cited In: GRAS notice GRN. No. 667.

Wingard RE, Brown JP, Enderlin FE, Dale JA, Hale RL, Seitz CT (1980). Intestinal degradation and absorption of the glycosidic sweeteners stevioside and rebaudioside A. Experientia 36(5):519-520.

Xili L, Chengjiany B, Eryi X, Reiming S, Yuengming W, Haodong S, Zhiyian H (1992). Chronic oral toxicity and carcinogenicity study of stevioside in rats. Food Chem Toxicol 30:957-965.

Yamada A, Ohgaki S, Noda T, Shimizu M (1985). Chronic toxicity study of dietary stevia extracts in F344 rats. Journal of the Food Hygienic Society of Japan (Japan). Cited In: GRAS notice GRN No. 667.

Yodyingyuad V and Bunyawong S (1991). Effect of stevioside on growth and reproduction. Human Reproduction 6(1):158-165.

Zinjarde SS (2014). Food-related applications of *Yarrowia lipolytica*. Food Chemistry, 152: 1-10

7.3 Annexes

Annex 1

Specifications for DSM Steviol Glycosides (Rebaudioside M) produced by *Yarrowia lipolytica*

Product Specification Sheet

For application development purposes

STEVIOL GLYCOSIDES, REB-M 95%

Product number:

Issue date: 19-12-2017

Physical properties

Description	Steviol glycosides, fermentative Rebaudioside-M (Reb-M) 95% is a dry crystalline powder and used as food additive sweetener.
Appearance	Off-white to white powder
Odor	Odourless or a slight characteristic odour
Moisture content by loss on drying	≤ 10%
Ash	≤ 1%
Solubility in purified water at room temperature (20°C)	Freely soluble to slightly soluble

Chemical properties

Rebaudioside M (on dry basis)	≥ 95 %
Total steviol glycosides (on dry basis)	> 95 %
pH (1 gram dissolved in 1l of water)	4.5 – 7.0
Lead	< 1 ppm
Mercury	< 1 ppm
Cadmium	< 1 ppm
Arsenic	< 1 ppm

Microbiological properties

Total plate count	≤ 1000 CFU in 1 g
Yeast	≤ 100 CFU in 1 g
Mold	≤ 100 CFU in 1 g
Coliforms	≤ 10 CFU in 1 g

Storage

Steviol glycoside, Reb M 95% must be stored in the original sealed containers in ambient (10°C - 32°C), dark and dry place with a humidity of <60%. If kept under these conditions the recommended shelf life is 12 months.

DSM Food Specialties B.V. | A. Fleminglaan 1 | 2613 AX Delft | The Netherlands | info.food@dsm.com | Trade Register Number 27235314

Regulatory information

Steviol glycosides, including Reb M 95% (E960 and INS960) manufactured from Stevia leaves are approved in most countries as a food additive (sweetener). The approval processes for the fermentative manufacturing is ongoing. A No Objection letter from FDA for fermentative Reb A was received in June 2016. The Codex Committee on Food Additives confirmed that the fermentatively produced Steviol glycosides (Reb A) have the same level of safety as the plant derived Steviol glycosides. An additive dossier was submitted to the European Commission in 2016 for evaluation of the fermentative manufacturing of Steviol glycosides and update on the Steviol glycosides specification to include production by fermentation.

DSM Food Specialties B.V. | A. Fleminglaan 1 | 2613 AX Delft | The Netherlands | info.food@dsm.com | Trade Register Number 27235314

Annex 2

Analysis based on Pariza and Johnson (2001) Decision Tree

This analysis is based on the Decision Tree of MW Pariza and EA Johnson (2001): *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology, 33:173-186.

1. *Is the production strain genetically modified?*

The production organism used is a genetically modified *Yarrowia lipolytica.* According to the decision tree, the production strain should be "nonpathogenic, non-toxigenic, and thoroughly characterized." *Yarrowia lipolytica* is a well-known yeast that that has been used to produce select food ingredients such as eicosapentanoic acid rich oil which was the subject of GRAS notice to the FDA. *Yarrowia lipolytica* has also been found in cheeses, and meat and dairy products. A review of the safety of the organism was published by Groenewald *et al*. in 2014. While the production organism is derived from a parent line that is nonpathogenic, non- toxigenic, and is well characterized, the production organism is genetically modified, hence, according to the decision tree, if yes, go to 2.

2. *Is the production strain modified using rDNA techniques?*

The parent strain was modified using recombinant DNA techniques as described in the GRAS document. According to the decision tree, if yes go to 3.

3. *Issues relating to the introduced DNA are addressed in 3a–3e.*

3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food or feed?

The parent strains of *Yarrowia lipolytica* have been modified to over-express the genes responsible for the production of steviol glycosides (rebaudioside M). Most of the genes originate from the plant *Stevia rebaudiana* (but were produced synthetically and are adapted with respect to codon usage for optimal expression in the yeast). *Stevia rebaudiana* is the current botanical source of the steviol glycosides. Equivalent alternative genes were obtained from *Arabidopsis thaliana* (an edible species of cress) , or other edible plant sources e.g. red pepper (*Solanum lycopersicum*) or lettuce (*Lactuca sativa*). Also inserted was a gene from *Giberella fujikuroi* (produced synthetically and adapted with respect to codon usage for optimal expression in the yeast). The genes introduced are under the genetic control of host-own promoter and terminating sequences. The introduced DNA sequences are integrated in the genome of the host-organism, partly in pre-defined loci (targeted integration) but mostly randomly. As the yeast *Yarrowia lipolytica* is not known to harbor any genes encoding for toxins or otherwise harmful sequences both random and targeted introduction of DNA sequences will not lead to an increased risk because of unintended pleiotropic effects (see also questions 4 and 5).

If yes, go to 3c. If no, go to 12. YES, assuming that the test article is *Rebaudioside M*

3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?

The lowest published NOAEL is 2000 mg/ Kg BW/ day, when a 100 x safety factor is used for interspecies differences there is additional safety margin compared to the conservative highest anticipated exposure of 7.9 mg/Kg BW/day. Therefore the answer is YES.

3c. *Is the test article free of transferable antibiotic resistance gene DNA?*

The final production strain does not contain any antibiotic resistance genes, which was confirmed by genotyping the final strain. The strain is susceptible to antibiotics and to anti-fungals. When tested, the genetic changes introduced into the *Yarrowia lipolytica* strain do not affect antifungal susceptibility.

If yes, go to 3e. If no, go to 3d. YES

3d. Does the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animal? If yes, go to 12. If no, go to 3e.

There are no antibiotic resistance genes in the production organism, answer is NO.

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?

It would appear that the DNA differences between the wild parent strains and the production organism are restricted to the enzymes of interest and it is well characterized.

If yes, go to 4. If no, go to 12. YES

4. Is the introduced DNA randomly integrated into the chromosome?

Method of insertion was mostly random. As the yeast *Yarrowia lipolytica* is not known to harbor any genes encoding for toxins or otherwise harmful sequences both random and targeted introduction of DNA sequences will not lead to an increased risk because of unintended pleiotropic effects.

If yes, go to 5. If no, go to 6. YES

5. *Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?*

As the yeast *Yarrowia lipolytica* is not known to harbor any genes encoding for toxins or otherwise harmful sequences both random and targeted introduction of DNA sequences will not lead to an increased risk because of unintended pleiotropic effects.
Therefore the production strain is safe.

If yes, go to 6. If no, go to 7. YES

6. *Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?*

The strain of *Yarrowia lipolytica* used is from a safe lineage.

If yes, the test article is ACCEPTED. If no, go to 7. **YES, The test article is accepted**

7. Is the organism nonpathogenic?

If yes, go to 8. If no, go to 12.

8. Is the test article free of antibiotics?

If yes, go to 9. If no, go to 12.

9. Is the test article free of oral toxins known to be produced by other members of the same species?

If yes, go to 11. If no, go to 10.

10. Are the amounts of such toxins in the test article below levels of concern?

If yes, go to 11. If no, go to 12.

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety?

If yes, the test article is **ACCEPTED**.

12. An undesirable trait or substance may be present and the test article is not acceptable for feed use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.

Annex 3

Fermentation Media Components

Fermentation Media Ingredient List

Raw Material	Grade
Dextrose	FG
$MnSO_4.1H_2O$	FG
$ZnSO_4.7H_2O$	EP / USP
$FeSO_4.7H_2O$	EP / USP
$CaCl_2.0H_2O$	EP
KH_2PO_4	EP
$MgSO_4.7H_2O$	EP / USP
$(NH_4)_2SO_4$	FG
Thiamine chloride hydrochloride (vitamin B1 hydrochloride)	FG
$CuSO_4.0H_2O$	EP
Citric Acid	FG
Glycerol (85%)	FG
Yeast extract	FG
Titrant H_2SO_4 [98%]	FG
Titrant NaOH [25%]	FG
Titrant Ammonia [25%]	FG
Glucosidases	FG
Antifoams	FG
Potable water	-

FG = Food Grade, FCC
USP = US Pharmacopeia
EP = European Pharmacopeia

Annex 4

Production Strain Control

Production Strain Control

Technical measures:

The batches of **primary seed material**, called the WCB (working Cell bank) are always prepared from the MCB (Master Cell Bank) in Laminar Air-flow (down-flow) safety cabinets to ensure the absence of contamination. The batches are divided into a large number of vials for use in production over a long period of years without any changes in strain- and production properties. In theory, a batch is large enough to last for about 10 years, depending on the strain viability and the fermentation frequency and thus the market demand.

The WCB is preserved by deep-freezing using glycerol as protective agent and slow freezing (1°C per min.) to reduce cell damage to a minimum. The deep-frozen vials are stored at minus 75°C or in the vapour phase of liquid nitrogen.
The above procedures for preparation, preservation and storage are chosen to avoid degeneration and to secure genetic stability. All vials are clearly labelled and in revival of the culture, strict aseptic techniques are applied.

Control measures:

A new WCB is prepared from the MCB as soon as the previous batch becomes depleted or the concentration of viable cells decreases.
After preparation of a new WCB, samples are checked for identity, viability and microbial purity, using different temperatures (25, 30 and 37°C) and media, by enrichment and viewing morphology (colony shape and microscopy). If all these parameters are correct, the strain is tested for production capacity, first on laboratory scale and later on large scale production level. Only if the productivity and the product quality meet the required standards, the new WCB is accepted for further production runs.

The viability of the WCB is checked at least once a year.

Annex 5

Certificates of Analysis

DSM Food Specialties

Print date 11-May-17

Certificate of Analysis

Material: Our: / Your reference

N/A - Steviol Glycosides Reb-M 95%

Batch: NBK-017589-005-1012 / Production date: Jun-2016 / Expiry date Jun-2018

Characteristic	Unit	Value	Specification
Appearance	Visual	Off-white to white powder	Off-white to white powder
Odour	Smell	Odourless	Odourless or slight characteristic
Moisture by loss on drying	%	2.6	≤ 10
Ash	%	< 1	≤ 1
Solubility in water at RT	g/l	> 1	> 1
Rebaudioside M (on dry basis)	%	97.7	≥ 95
Total steviol glycosides (on dry basis)	%	98.6	> 95
pH	-	6.58	4.5 - 7.0
Lead	ppm	< 0.3	< 1
Mercury	ppm	< 0.02	< 1
Cadmium	ppm	< 0.02	< 1
Arsenic	ppm	< 0.02	< 1
Total plate count	CFU/g	10	≤ 1000
Yeast	CFU/g	< 10	≤ 100
Mold	CFU/g	< 10	≤ 100
Coliforms	CFU/g	< 0.3	≤ 10

In addition to the results listed on the certificate of analysis, we confirm that each individual batch meets the specification limits as listed in the applicable product data sheet

This document is a non-signed computer form generated after release by the QA manager Hans Vloet

DSM Food Specialties

Print date 24-July-17

Certificate of Analysis

Material: Our: / Your reference

N/A - Steviol Glycosides Reb-M 95%

Batch: NBK-017589-005-1035 / Production date: Jun-2016 / Expiry date Jun-2018

Characteristic	Unit	Value	Specification
Appearance	Visual	Off-white to white powder	Off-white to white powder
Odour	Smell	Odourless	Odourless or slight characteristic
Moisture by loss on drying	%	3.0	≤ 10
Ash	%	< 1	≤ 1
Solubility in water at RT	g/l	> 1	> 1
Rebaudioside M (on dry basis)	%	99.1	≥ 95
Total steviol glycosides (on dry basis)	%	99.7	> 95
pH	-	6.59	4.5 - 7.0
Lead	ppm	< 0.3	< 1
Mercury	ppm	< 0.02	< 1
Cadmium	ppm	< 0.02	< 1
Arsenic	ppm	< 0.02	< 1
Total plate count	CFU/g	200	≤ 1000
Yeast	CFU/g	< 10	≤ 100
Mold	CFU/g	20	≤ 100
Coliforms	CFU/g	< 0.3	≤ 10

In addition to the results listed on the certificate of analysis, we confirm that each individual batch meets the specification limits as listed in the applicable product data sheet

This document is a non-signed computer form generated after release by the QA manager Hans Vloet

DSM Food Specialties

Print date 11-May-17

Certificate of Analysis

Material: Our: / Your reference

N/A - Steviol Glycosides Reb-M 95%

Batch: NBK-017589-005-113 / Production date: Jul-2016 / Expiry date Jul-2018

Characteristic	Unit	Value	Specification
Appearance	Visual	Off-white to white powder	Off-white to white powder
Odour	Smell	Odourless	Odourless or slight characteristic
Moisture by loss on drying	%	4.4	≤ 10
Ash	%	< 1	≤ 1
Solubility in water at RT	g/l	> 1	> 1
Rebaudioside M (on dry basis)	%	99.8	≥ 95
Total steviol glycosides (on dry basis)	%	100	> 95
pH	-	6.61	4.5 - 7.0
Lead	ppm	< 0.3	< 1
Mercury	ppm	< 0.02	< 1
Cadmium	ppm	< 0.02	< 1
Arsenic	ppm	< 0.02	< 1
Total plate count	CFU/g	20	≤ 1000
Yeast	CFU/g	< 10	≤ 100
Mold	CFU/g	< 10	≤ 100
Coliforms	CFU/g	< 0.3	≤ 10

In addition to the results listed on the certificate of analysis, we confirm that each individual batch meets the specification limits as listed in the applicable product data sheet

This document is a non-signed computer form generated after release by the QA manager Hans Vloet

DSM Food Specialties

Print date 11-May-17

Certificate of Analysis

Material: Our: / Your reference

N/A - Steviol Glycosides Reb-M 95%

Batch: NBK-017589-008-001 / Production date: Dec-2016 / Expiry date Dec-2018

Characteristic	Unit	Value	Specification
Appearance	Visual	Off-white to white powder	Off-white to white powder
Odour	Smell	Odourless	Odourless or slight characteristic
Moisture by loss on drying	%	2.3	≤ 10
Ash	%	< 1	≤ 1
Solubility in water at RT	g/l	1.1	> 1
Rebaudioside M (on dry basis)	%	98	≥ 95
Total steviol glycosides (on dry basis)	%	99.0	> 95
pH	-	6.85	4.5 - 7.0
Lead	ppm	< 0.3	< 1
Mercury	ppm	< 0.02	< 1
Cadmium	ppm	< 0.02	< 1
Arsenic	ppm	< 0.02	< 1
Total plate count	CFU/g	< 5	≤ 1000
Yeast	CFU/g	< 10	≤ 100
Mold	CFU/g	< 10	≤ 100
Coliforms	CFU/g	< 0.3	≤ 10

In addition to the results listed on the certificate of analysis, we confirm that each individual batch meets the specification limits as listed in the applicable product data sheet

This document is a non-signed computer form generated after release by the QA manager Hans Vloet

DSM Food Specialties

Print date 11-May-17

Certificate of Analysis

Material: Our: / Your reference

N/A - Steviol Glycosides Reb-M 95%

Batch: NBK-017589-010-001 / Production date: Dec-2016 / Expiry date Dec-2018

Characteristic	Unit	Value	Specification
Appearance	Visual	Off-white to white powder	Off-white to white powder
Odour	Smell	Odourless	Odourless or slight characteristic
Moisture by loss on drying	%	6.5	≤ 10
Ash	%	< 0.3	≤ 1
Solubility in water at RT	g/l	1.0	> 1
Rebaudioside M (on dry basis)	%	98	≥ 95
Total steviol glycosides (on dry basis)	%	100	> 95
pH	-	6.9	4.5 - 7.0
Lead	ppm	< 0.3	< 1
Mercury	ppm	< 0.02	< 1
Cadmium	ppm	< 0.01	< 1
Arsenic	ppm	< 0.02	< 1
Total plate count	CFU/g	65	≤ 1000
Yeast	CFU/g	< 10	≤ 100
Mold	CFU/g	< 10	≤ 100
Coliforms	CFU/g	< 3	≤ 10

In addition to the results listed on the certificate of analysis, we confirm that each individual batch meets the specification limits as listed in the applicable product data sheet

This document is a non-signed computer form generated after release by the QA manager Hans Vloet

Annex 6

rDNA detection in pilot batches of DSM Steviol Glycosides (Rebaudioside M) produced by *Yarrowia lipolytica*

Proof of absence of rDNA in Rebaudioside M (RebM) produced with a genetically modified strain of *Yarrowia lipolytica* VRM

Samples derived from several pre-production batches as well as one sample from the tox batch (also a pre-production batch) were used to analyse on the presence of recombinant DNA (rDNA). Fermentation and recovery were performed according to the production process described in the dossier. An overview of the samples is provided in the table below.

	RebM sample
Pre-production tox batch	NBK-017589-010-001
Pre-production batch	NBK-017589-005-1012
Pre-production batch	NBK-017589-005-1035
Pre-production batch	NBK-017589-005-113
Pre-production batch	NBK-0017589-008-001 (VVJ1602A)

The absence of rDNA was determined using the method from the guidelines provided by the European Food Safety Authority (EFSA, 2011)[1].

Of each RebM sample, 100 mg was weighed on an analytical scale into a 50 ml tube (Greiner), in triplicate. The samples were dissolved in 8 ml milli-Q water, to completely dissolve the RebM (which is less soluble in water when compared to RebA). Next, the mixture was heated to 80 ^{0}C for 10 minutes, vortexed vigorously and instantly added to the genomic DNA solution or milli-Q. To 50 µl of the dissolved RebM sample or milli-Q in a 1.5 ml DNA LoBind Tube (Eppendorf), 50 µl of an undiluted, 10, 100, 1000, 10.000, 100.000 and 1.000.000 fold dilution of the 1.25 ng/µl solution of Y. lipolytica VRM[i] genomic DNA was added. 16 µl of the solution was used as template in a 25 µl PCR reaction.

The possible presence of recombinant DNA was assessed by performing highly sensitive PCR techniques on the DNA from the RebA samples. Two primer sets were designed, one targeting UGT2 and the other CPS from ATG to STOP. This results in PCR products of 1.4 kb for UGT2 and 2.2 kb for CPS. Specifications of the PCR reaction were:

Description	**Primer code**	**Sequence**	**PCR product**
UGT2 start	DBC 12780	ATGGCCACCTCCGACTCC	1.4 kb
UGT2 stop	DBC 12781	TTAGCTTTCGTGGTCAATGG	
CPS start	DBC 12774	ATGTGCAAGGCTGTTTCCAAG	2.2 kb
CPS stop	DBC 12775	TTAAATCACAATCTCAAAGACCTTGG	

PCR reactions were performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, M0530L) according to the supplier's instructions in a S1000 Thermal cycler (BioRad Laboratories):

PCR reaction components	UGT2	CPS
DNA template	16 µl	16 µl
HF buffer 5x	5 µl	5 µl
Primer start	1.25 µl	1.25 µl

[1] EFSA (2011). Scientific Opinion of the EFSA Panel on Genetically Modified Organisms (GMO) on "Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use". EFSA J., 9(6), 2193.http://www.efsa.europa.eu/de/efsajournal/doc/2193.pdf

Primer end	1.25 µl	1.25 µl
Phusion polymerase (2 U/µl)	0.25 µl	0.25 µl
dNTP's (10 mM)	0.5 µl	0.5 µl
Milli Q water	0.75 µl	0.75 µl

CR reaction program	**UGT2**	**CPS**
Denaturation	2 min 98 °C	2 min 98 °C
	10 sec 98 °C 20 sec 65 °C 60 sec 72 °C 35 cycles	10 sec 98 °C 20 sec 65 °C 60 sec 72 °C 40 cycles
Reaction end	10 min 72 °C	10 min 72 °C

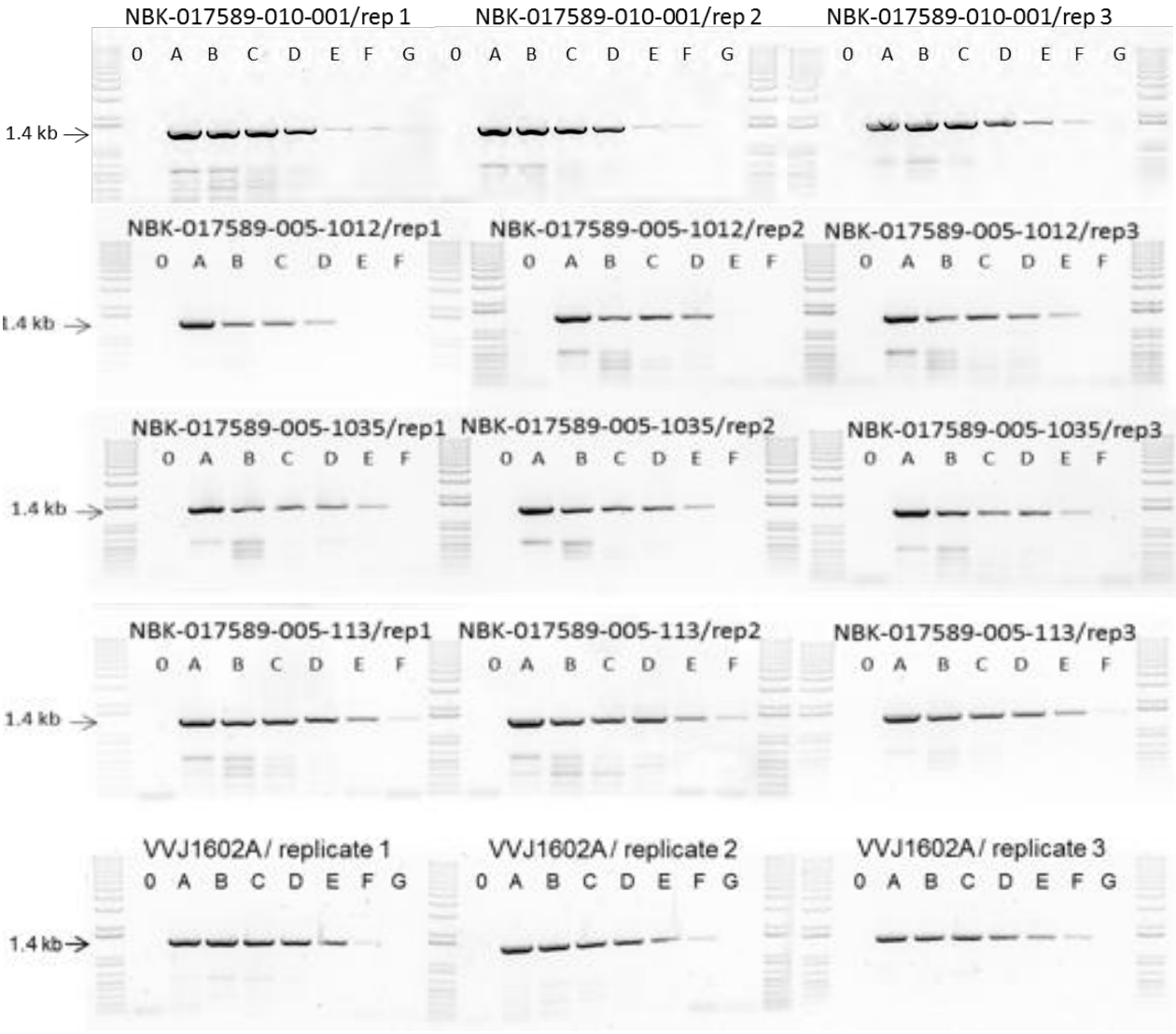
The results of the experiments to detect rDNA in the RebM batches are shown in Figures 1 (UGT2) and 2 (CPS).

The sensitivity of the UGT2 assay is significantly higher than that of the CPS assay. For both UGT2 and CPS the sensitivity of the PCR for the RebM samples is approximately equal to the milli-Q control.

The RebM samples were analyzed in triplicate for the presence of rDNA using UGT2 and CPS as a PCR target according to EFSA guidelines as outlined above. As can be seen in Figures 1 and 2, no rDNA could be detected in the tested RebM batches (lanes 0). In addition, the different genomic DNA dilutions show extinction of the signal with increasing dilution ((A) to (G)).

Figure 1:

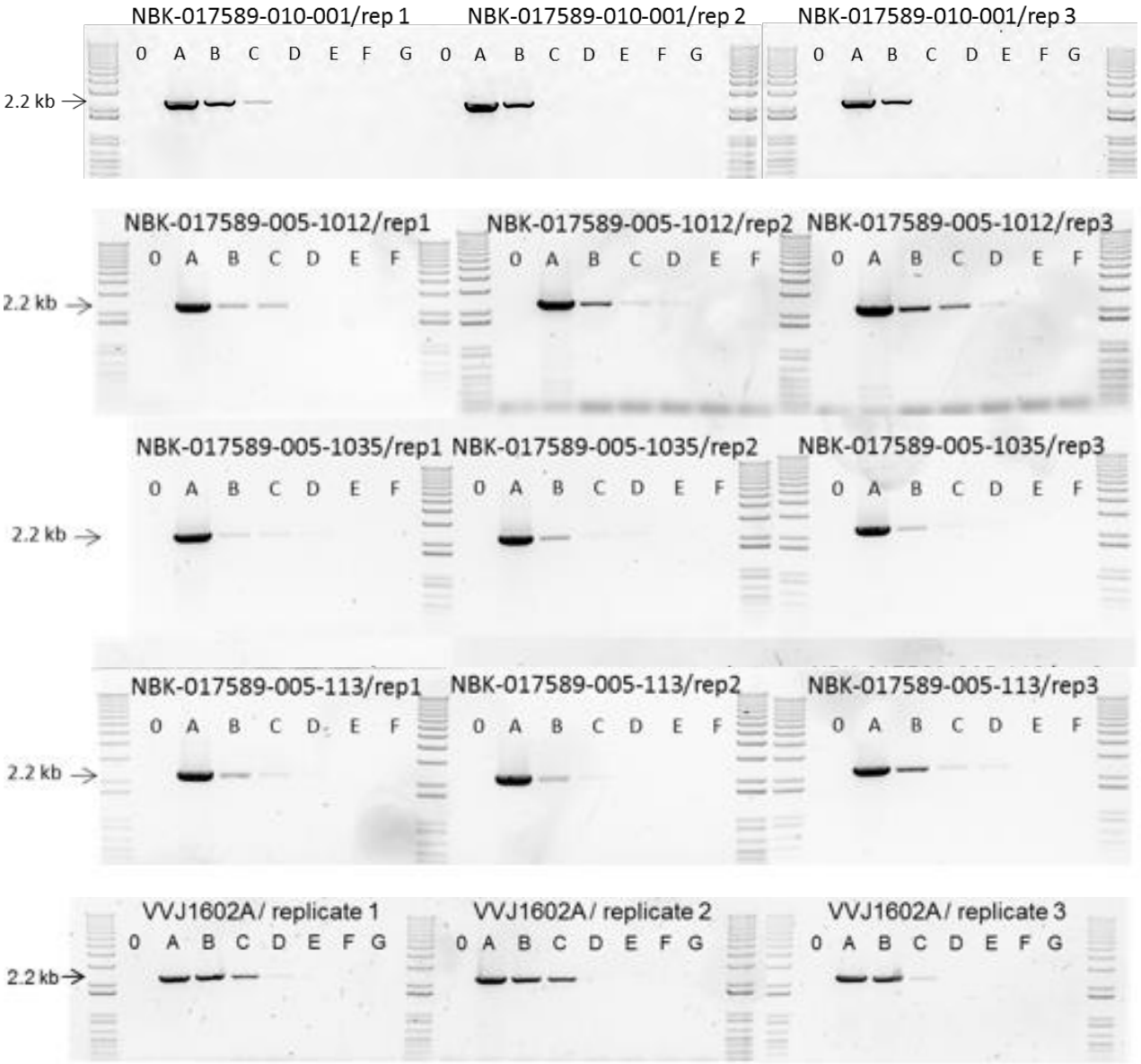
a) UGT2 PCR for detection of rDNA in NBK-017589-010-001, NBK-017589-005-1012, NBK-017589-005-1035, NBK-017589-005-112, and VVJ1602A spiked with 0 (0), no dilution (A), 10 fold dilution (B), 100 fold dilution (C), 1000 fold dilution (D), 10.000 fold dilution (E), 100.000 fold dilution (F) and 1.000.000 fold dilution (G) of *Y. lipolytica* VRM genomic DNA.



b) UGT2 control PCR for the detection of rDNA in milli-Q spiked with 1 fold dilution (A), 10 fold dilution (B), 100 fold dilution (C), 1000 fold dilution (D), 10.000 fold dilution (E) 100.000 fold dilution (F) and 1.000.000 fold dilution (G) of *Y. lipolytica* VRM genomic DNA.

Figure 2:

a) CPS PCR for detection of rDNA in NBK-017589-010-001, NBK-017589-005-1012, NBK-017589-005-1035, NBK-017589-005-112, and VVJ1602A spiked with 0 (0), no dilution (A), 10 fold dilution (B), 100 fold dilution (C), 1000 fold dilution (D), 10.000 fold dilution (E), 100.000 fold dilution (F) and 1.000.000 fold dilution (G) of *Y. lipolytica* VRM genomic DNA.



b) CPS control PCR for the detection of rDNA in milli-Q spiked with 1 fold dilution (A), 10 fold dilution (B), 100 fold dilution (C), 1000 fold dilution (D), 10.000 fold dilution (E) 100.000 fold dilution (F) and 1.000.000 fold dilution (G) of *Y. lipolytica* VRM genomic DNA.

[i] To avoid mistakes the production strain for RebA and RebM are coded differently. For RebA this is STV while for RebM this is VRM. In some reports still the outdated straincode STVM is used.