ORIGINAL SUBMISSION

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DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

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Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

GRN 000632

February 16, 2015

GRAS Notification:
the use of fermentative Reb A as a general purpose sweetener

Dear Dr. Mattia

On behalf of DSM Food Specialties ("DSM"), I am submitting under cover of this letter one paper copy and one digital copy of DSM's generally recognized as safe ("GRAS") notice for its rebaudioside A produced by a genetically engineered *Yarrowia lipolytica*. The electronic copy is provided on a virus-free CD, scanned using McAfee Virus Scan Enterprise version 8.8, and is an exact copy of the paper submission. DSM has determined through scientific procedures that its rebaudioside A produced by a genetically engineered *Yarrowia lipolytica* is GRAS for use as a general purpose sweetener for use in commercial food products such as beverages, baked goods, confections and dairy products at levels not to exceed the amounts reasonably required to accomplish its intended effect in foods as required by FDA regulation, 21 CFR 182.1 (b)(1).

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, this use of rebaudioside A produced by a genetically engineered *Yarrowia lipolytica* is exempt from premarket approval requirements of the Federal Food, Drug and Cosmetic Act, because the notifier has determined that such use is GRAS.

If you have any questions regarding this notification, or require any additional information to aid in the review of DSM's conclusion, please do not hesitate to contact me via email at james.lamarta@dsm.com or by telephone, (973)257-8347.

Sincerely,

(b) (6)

James La Marta, Ph.D, CFS
Sr. Manager Regulatory Affairs

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James La Marta, Ph.D, CFS
Sr. Manager Regulatory Affairs

Registered as DSM Nutritional Products, LLC

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DSM Reb A

Rebaudioside A produced by a strain of

Yarrowia lipolytica

for use in human food products

SUMMARY OF DATA FOR A GRAS CONCLUSION

By: DSM Food Specialties

James La Marta

DSM Nutritional Products

Parsippany, New Jersey

Melina Rumelhard

DSM Food Specialties

Delft, The Netherlands

DSM3068-001

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1   Executive Summary

The purpose of this document is to provide technical information supporting the determination of safety of DSM's rebaudioside A (Reb A) produced by microbial fermentation for use in human food products and that it is Generally Recognized as Safe (GRAS) based on scientific procedures.

DSM Food Specialties (DFS) developed the product and a sister company, DSM Nutritional Products will manufacture and package the product for distribution. DFS will market the product. Reb A will be used by consumer food and beverage manufactures as a non-caloric sweetener.

DSM's Reb A is produced by a strain of *Yarrowia lipolytica* genetically engineered to contain and express the steviol glycoside metabolic pathway of the stevia plant, *Stevia rebaudiana*, the same plant species that is used as a source of the highly purified stevia leaf extracts and isolated glycosides that have been the subject of over 30 GRAS Notices. DSM's Reb A will be sold as a crystalline powder with a purity of no less than 95% rebaudioside A and the balance of the solids being predominantly the   steviol glycoside Reb B and stevioside.

The safety of *Yarrowia lipolytica* has been established in the published scientific literature. The organism is not known to produce toxins and is considered a biosafety class 1 organism. DSM did not introduce any genes that code for toxin production, antibiotic production or antibiotic resistance. As previously noted, the rebaudioside A production genes are primarily from the same plant that is the source of the current commercial stevia leaf extract derived sweeteners.

DSM has produced several batches of Reb A that are compositionally equivalent to the current commercial products and meet the JECFA and FCC published specifications. Additionally, an independent third party has confirmed that the sensorial properties of DSM's Reb A are equivalent to commercial products derived from the stevia plant. Therefore, DSM believes that its Reb A produced by yeast fermentation is equivalent to other commercial stevia products used in food and beverages for human consumption.

To further support the safety of DSM's Reb A, material from a commercial pilot production batch was evaluated for genotoxicity and subchronic toxicity. The results of the genotoxicty studies were negative. The 90 day study was completed on 15 June 2015 and the final report from the study director noted no adverse observations from the in-life portion of the study other than weight loss in the high dose group, which is consistent with other studies on plant derived Reb A reported in the literature. The No-Observed-Adverse-Effect-Level (NOAEL) for this study was therefore considered to be >2000 mg/kg bw/day for males and females.

1.1   Name and Address of the Notifier

DSM Food Specialties

45 Waterview Blvd.

Parsippany, New Jersey, 07054, USA

Tel: 973-257-8500

Person responsible for the submission:

James La Marta, Ph.D.

45 Waterview Boulevard

Parsippany, New Jersey 07054

Tel: 973-257-8325

1.2   Name and Address of the Manufacturer

DSM Nutritional Products

1416 Williamsburg County Hwy

Kingstree, South Carolina 29556

1.3   Common or Usual Name of the Substance

DSM Food Specialties proposes that the substance that is the subject of this GRAS dossier be identified as Rebaudioside A , Reb A or steviol glycosides with rebaudioside A as its principal component.

1.4 Applicable Condition of Use

DSM's high purity Reb A, rebaudioside A (≥ 95%) as the principal component, manufactured under current good manufacturing procedures will be marketed for use as a flavor and as a general purpose sweetener for use in commercial food products such as beverages, baked goods, confections and dairy products but it will be excluded from use in infant formula and meat and poultry products.   The intended use levels will vary by actual food category, but the actual levels are self-limiting due to organoleptic factors and consumer taste considerations.   The substance will be used at levels that do not exceed the amounts reasonably required to accomplish its intended effect in foods as required by FDA regulation,(21 CFR 182.1 (b)(1)), see FDA 21 CFR 182.

1.6 Basis for The GRAS Conclusion

This GRAS conclusion is based upon scientific procedures.

1.7 GRAS Exemption Claim

DSM Food Specialties provided the appropriate information on the safety and utility of the notified substance to an independent panel of qualified experts, the GRAS Panel for their evaluation. The enclosed dossier contains the information on the identity of the production organism, manufacture of the commercial product and information supporting the safety of its intended use. Also included are copies of the pertinent literature and other supportive data.

DSM Food Specialties concluded that DSM's Reb A meeting appropriate food-grade specifications as described in this dossier and manufactured consistent with current Good Manufacturing Practices (cGMP), is Generally Recognized As Safe (GRAS) based on scientific procedures and is therefore exempt from the requirement for premarket approval noted in Section 201 (s) of the Federal Food Drug and Cosmetic Act.

1.8 Availability of the Information

The complete data and information that are the basis of the GRAS Notification are available to the Food and Drug Administration for review and copying upon request during normal business hours.

(b) (6)

James La Marta, Ph.D., CFS   Date: 12 February 2016

2 Pre-Manufacturing : Description of the production organism

2.1 Classification of the organism: *Yarowia lipolytica*

Kingdom: Fungi

Phylum: Ascomycota

Class: Saccharomycetes

Order: Saccharomycetales

Family: Dipodascaceae

Genus: Yarrowia

Species: lipolytica

2.2 Modifications to the production microorganism

The original strains were obtained from the American Type Culture Collection. The parent strains of *Yarrowia lipolytica* have been modified to over-express the genes responsible for the production of steviol glycosides (rebaudioside A). 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 both random and targeted introduction of DNA sequences will not lead to an increased risk because of unintended pleiotropic effects.

The metabolic pathway for the production of rebaudioside A is shown below.

Genetically Engineered *Yarrowia lipolytica* Biosynthetic Pathway to rebaudiosides

2-Acetyl-CoA ⟶ AA-CoA ⟶ 3-HMG-CoA ⟶ MVA ⟶ MVA-P

GGPP ⟵ FPP ⟵ GPP ⟵ IPP ⟵ MVA-PP

CPP ⟶ kaurene ⟶ kaurenol ⟶ kaurenal ⟶ kaurenoic Acid

steviolbioside ⟵ steviol-13-monoside ⟵ steviol

rebaudioside E ⟵ stevioside   rebaudioside B   stevioside-19-monoside

rubusoside

rebaudioside D ⟵ rebaudioside A

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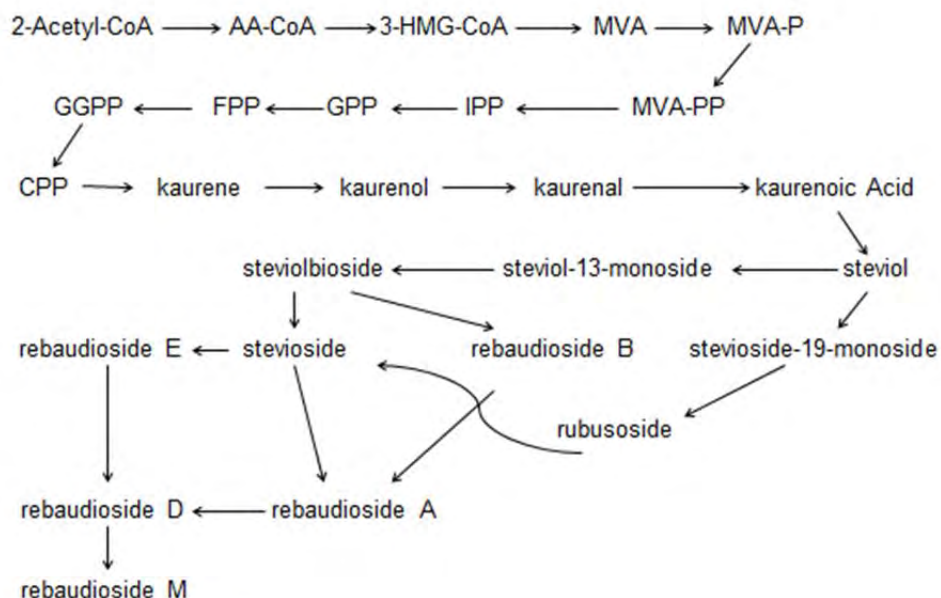
The mevalonate pathway serves as a supply of precursors for the production of steviol glycosides. The biosynthesis pathway is described in more detail in Brandle and Telmer, 2007.

2.3 Parental strains

Three parental strains of *Yarrowia lipolytica* were obtained directly from the ATCC and used to generate two starting strains. Our 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. From one of these spores the production strain was derived.

2.4 Genetic Engineering of the production strain

The genetic engineering of the production organism is covered by several patents and patent applications listed below.

Code	Subject matter	Priority	Filing	Published	Published as
28446	Metabolic engineering of rebaudioside production *in S. cerevisiae* and *Y. lipolitica*	23 Jan 2012 16 Nov 2012	23 Jan 2013	01 Aug 2013	WO2013/110673
29197	Extracellular production of rebaudiosides in metabolically engineered cells	31 May 2013	2 Jun 2014	04 Dec 2014	WO2014/191580
29325	Increased rebaudioside production in 11 different deletion mutants	31 May 2013	2 Jun 2014	04 Dec 2014	WO2014/191581
29915	RebM production in metabolically engineered cells	15 Jul 2013	15 Jul 2014	22 Jan 2015	WO2015/007748
29930	Stable solution of RebA	31 July 2013	31 Jul 2014	05 Feb 2015	WO2015/014958
29932	*Yarrowia* temp effect (high temp to increase rebaudioside production)	23 Jul 2013	23 Jul 2014	29 Jan 2015	WO2015/011209
29940	Down Stream Process using adsorbant-based recovery	31 Jul 2013	31 Jul 2014	05 Feb 2015	WO2015/014959
29943	Use patent coving commercial product applications of Reb A produced by fermentation	31 Jul 2013	31 Jul 2014	05 Feb 2015	WO2015/014969

2.5   Antibiotic resistance

The final production strain does not contain any antibiotic resistance genes. The strain is susceptible to antibiotics and to anti-fungals. 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.

2.6   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*.

Y. lipolytica is an avirulent yeast species historically used for the production of citric acid and the flavor chemical, γ-decalactone. *Y. lipolytica* is approved by the United States FDA as a secondary direct food additive in citric acid production (21 CFR §173.165) and was previously classified as *Candida lipolytica* (van der Walt and von Arx, 1980), the name by which the organism is described in 21 CFR §173.165.

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 000355) In November 2011, FDA agreed with GRAS Notice 000382 for erythritol from Baolingbao Biology Co., Ltd. Of Shangdong, China, where the erythritol was produced from glucose via biotransformation by a strain of *Yarrowia lipolytica*.

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 recent 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.

Yarrowia lipolytica is generally regarded as a biosafety class 1 microorganism (Groenewald et al, 2013). *Yarrowia lipolytica* is an avirulent yeast species historically used for the production of citric acid and the flavor chemical, γ-decalactone. *Y. lipolytica* has been used extensively at manufacturing scale without documented toxic, allergenic, or other harmful effects on humans' or other animals' health. It is

approved by the United States FDA as a secondary direct food additive in citric acid production (21 CFR § 173.165).

A review of the safety of *Yarrowia lipolytica* has recently been completed and concludes 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 (Groenewald et al., 2013). In addition, *Y. lipolytica* has been reported to stimulate the production of biogenic amines when using this yeast for cheese ripening, 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 written by 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 described 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 given tyramine orally at 0, 200, 2 000 or 10 000 mg/kg feed resulted in a NOAEL of 2 000 mg/kg feed (180 mg/kg body weight (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 A by using the ADI of 4 mg steviol equivalents/kg bw/day established by JECFA – equivalent to 12.1 mg DSM's Reb A/kg bw/day. A maximum level of 8 mg biogenic amines per g DSM's Reb A (or 8,000 ppm) is therefore considered acceptable.

For practical reasons we can use the level of nitrogen in Reb A as an indication. Until now, no more than 1,000 ppm nitrogen has been found in samples of Reb A (i.e. 910, 360, 1060 and 320 ppm in batches NBK-5203-0008-1210BPF1-2, NBK-5203-009-2207BPF2-2, NBK-5203-010-310BPF3-1 and NBK-05203-011-076).

Even considering that this nitrogen only comes from biogenic amines – which is highly improbable, the concentration of biogenic amines in Reb A is well below the acceptable level of 8,000 ppm. Nevertheless the maximum level of nitrogen for commercial production of Reb A is set at 100 ppm.

In production batches, the nitrogen content will be monitored as an indicator of the maximum level of biogenic amines that theoretically could be present. As it will be kept below 100 mg/kg (100 ppm) in the product, there is no safety issue.

A comprehensive search of the scientific literature for safety and toxicity information on *Y. lipolytica* was conducted by DSM Food Specialties. The search was limited to data available from 2013 in order to complete the extensive review performed by Groenewald et al. (2013). The search terms were 'lipolytica' / 'lipolytica and *safe', 'lipolytica and *tox' and the data bases searched included PubMed, Toxnet, US FDA GRAS Notices, CDAT, NTP, GESTIS, IPCS INCHEM, TSCATS, US EPA, EFSA, EU Scientific Committees, Health Canada and NICNAS. From the 6 hits that were found, only one was relevant for the safety of the microorganism. In this review of the different food-related applications of *Y. lipolytica*, Zinjarde (2014) reaffirmed the safety of the microorganism. It can also be noted that EFSA has 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 only inserted the genes of the *Stevia rebaudiana* and *Arabidopsis thaliana* plants, both of which have a history of safe use. There have been over 30 GRAS Notices filed with FDA for highly purified *Stevia* leaf extracts, all of them receiving 'no questions' letters. *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 employed the Pariza and Johnson decision tree to determine if that well accepted rubric revealed any questions about the use of the genetically engineered Yarrowia lipolytica, see below.

Since the decision tree did not reveal any concerns and the aforementioned characteristics of the production organism are not unsafe, DSM therefore concludes that the use of the genetically engineered *Yarrowia lipolytica* presents no known safety concerns.

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 A). 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. Another gene was obtained from *Arabidopsis thaliana* (an edible species of cress). Also inserted was a gene from *Giberella fujikuroi.* 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 A*

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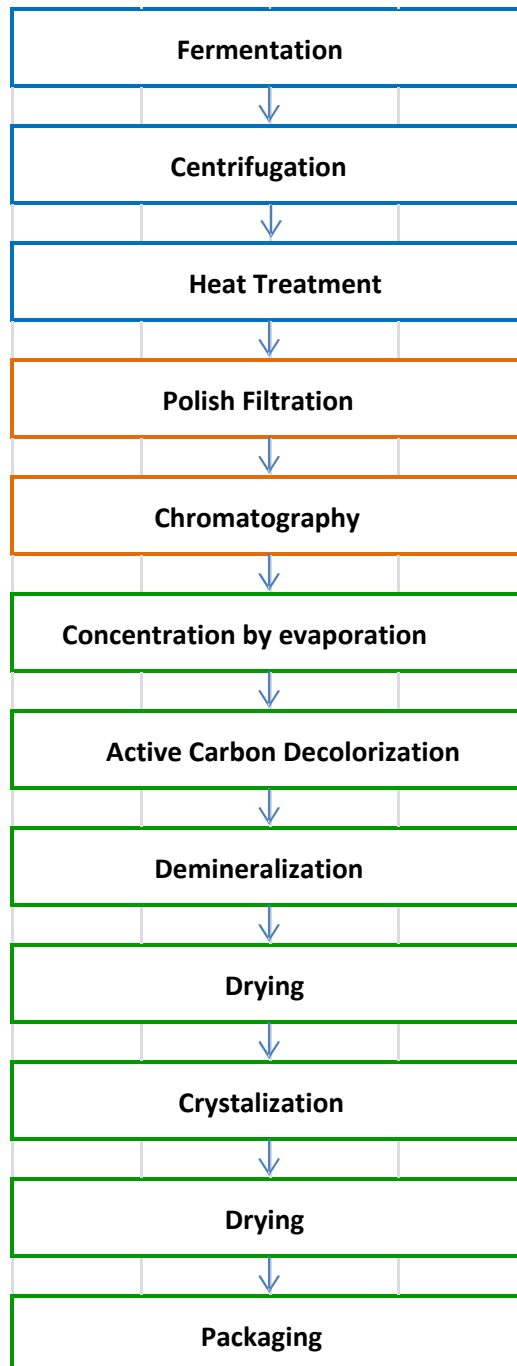
If yes, the test article is **ACCEPTED**.

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.

3 Manufacturing Process for Rebaudioside A

The manufacturing process is composed of the following steps: fermentation, isolation, purification, and finally, quality control of the finished product. The process is described below. All equipment is made of stainless steel or other materials suitable for food contact.

Figure 3-1   Flow chart of manufacturing process

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┌─────────────────────────────────┐
│         Fermentation            │
└─────────────────────────────────┘
               ↓
┌─────────────────────────────────┐
│         Centrifugation          │
└─────────────────────────────────┘
               ↓
┌─────────────────────────────────┐
│         Heat Treatment          │
└─────────────────────────────────┘
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┌─────────────────────────────────┐
│         Polish Filtration       │
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┌─────────────────────────────────┐
│         Chromatography          │
└─────────────────────────────────┘
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│    Concentration by evaporation │
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│         Demineralization        │
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│            Drying               │
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│            Drying               │
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│            Packaging            │
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3.1   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. There are no allergenic proteins in the media.

The fermentation medium composition has been developed for optimum production of Reb A. The list of the media components is in Annex 1.

3.2   Fermentation Process

Reb A is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *Yarrowia lipolytica* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are incorporated and microbiological analyses are done 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 Reb A 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 A production are checked at the end of the main fermentation by analysis of aseptically collected samples. After the fermentation is stopped, the recovery process begins.

3.3   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, and subsequently clarified by centrifugation or filtration. The steviol glycosides are recovered from the fluid stream by adsorption chromatography, eluted by aqueous alcohol, decolorized and further purified by the use of active carbon and demineralization resins from the aqueous phase that comply with 21 CFR 173.25, followed by concentration to dry powder. Purified Reb A is isolated by crystallization followed by drying.

3.5 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 specification noted in Section 4 following the method indicated for each material characteristic.

3.6 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 that 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 ^{0}C) and media, by enrichment and viewing morphology (colony shape and microscopy) before release. The general overview of the strain control process is in Annex 2.

3.7 Global capabilities

DSM has multiple fermentation facilities located in the major industrial markets which each have the ability to manufacture Reb A following the above noted process under cGMP.

4 Compositional analysis and specifications

4.1 Identity

IUPAC Name: 19-O-beta-glucopyranosyl-13-O-(beta-glucopyranosyl(1-2)-beta-glucopyranosyl(1-3))-beta-glucopyranosyl-13-xhydroxykaur-16-en-19-oic acid

Synonyms: 19-O-beta-glucopyranosyl-13-O-(beta-glucopyranosyl(1-2)-beta-glucopyranosyl(1-3))-beta-glucopyranosyl-13-xhydroxykaur-16-en-19-oic acid

CAS Number: 58543-16-1

Chemical Formula: $C_{44}H_{70}O_{23}$

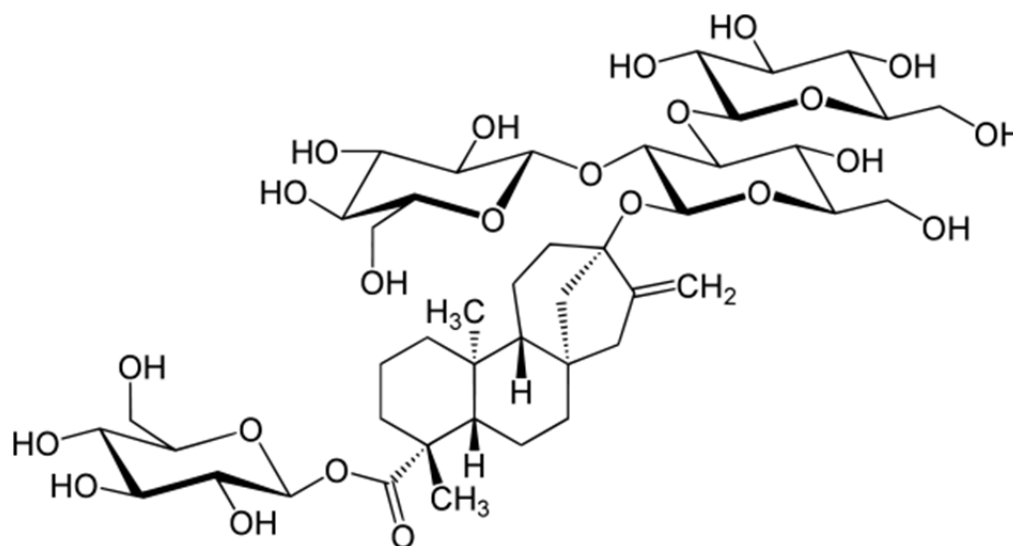**Molar Mass:** 967.01 g/mol

Molecular Structure:

Figure 4-1

4.2   Specifications

Physical properties

Appearance	Off-white to white powder
Odor	sweet, aromatic
Moisture content by loss on drying	≤ 6%
Optical rotation	-29 to -37 degrees
Ash	≤1%
Particle size #80 Mesh	>99%
Solubility in purified water at room temperature (20°C)	Freely soluble in water

Chemical properties*

Rebaudioside A (on dry basis)	≥ 95 %
Total steviol glycosides (on dry basis)	> 95 %
Residual solvents (Ethanol)	<5000 ppm
pH (1 gram dissolved in 100 ml of water)	4.5 – 7.0
Lead	< 1 ppm
Mercury	< 1 ppm
Cadmium	< 1 ppm
Arsenic	< 1 ppm
Nitrogen (internal specification)	<100 ppm
Steviol (internal specification)	<50 ppm
Kaurenoic Acid & glycosides (internal specification)	<10 ppm

Microbiological properties

Total plate count	≤ 1000 CFU in 1 g
Yeast and Mold	≤ 100 CFU in 1 g
Coliform	≤ 10 CFU in 1 g
Escherichia coli	< 3 MPN in 1 g

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

*A Specification Sheet is in Annex 3.

4.3   Batch Analysis

DSM produced 3 pilot batches in its Netherlands facility, two in the USA and one production batch at its Capua, Italy facility. The three pilot plant batches were made for analytic purposes whereas the Capua batch was used for the toxicology studies and sensory evaluation. The results of the analysis of those batches are presented in the table below and compared to the tentative DSM, FCC and JECFA specifications. The analysis was done in-house and then by WIL Research following GLP. The DSM analysis is provided for the Capua batch, see Annex 4. WIL Research also performed a spectrophotometric analysis and found the three pilot plant batches to be similar to that of the standard.

With respect to the first batch made in the DSM pilot lab the low pH value may be due to it being the first attempt to scale-up the process and the use of acidified solvent in the crystallization step. The pH in subsequent batches was within specification and is expected to remain there.

The third batch from the pilot lab originally had a high ethanol concentration detected by WIL Research whereas the DSM R&D lab had the ethanol within specification. Re-drying the Reb A brought the ethanol to within specification. More importantly, the large scale run at Capua was within specification, see lot NBK-5203-011-076 in the table below.

DSM does not use methanol in its process, the three labs batches were analyzed for residual methanol because that parameter is a component of the JECFA and FCC specifications. DSM does not plan to analyze for methanol going forward which is why it was not measured for the Capua pilot batch or the two additional pilot runs.

The results of the recombinant DNA analysis for the three pilot lab batches can be found in Annex 5.

Table 4-1 Batch Data for DSM Reb A

Parameter	Method	DSM Tentative Specs	JECFA Spec	FCC Spec	Reb A Lot ID NBK-5203-0008-1210BPF1-2	Reb A Lot ID NBK-5203-009-2207BPF2-2	Reb A Lot ID NBK-5203-010-310BPF3-1	Capua lot ID NBK-5203-011-076	Reb A Lot VVA 1501-453	Reb A Lot VVA 1501-451
Product characteristics										
Appearance	visual	Off-white to white powder	White to light yellow powder	Off-white to white crystal, granule or powder	Off-white powder	Off-white powder	Off-white powder	Off-white powder	Off-white powder	Off-white powder
Odor	smell	sweet aromatics		NS						
Moisture content	Karl Fisher	≤ 6%	NMT 6%	≤ 6%	1.5%	1.6%	2.8%	1.0%	1.8%	1.8%
Optical rotation	polarimetry	-29 to -37 degrees	NS	NS	-33.15	31.41	32.41	30.5	32.0	31.3
Ash	JECFA	≤ 1%	NMT 1%	≤ 1%	0.02%	0.0019%	0.016%	0.9%	0.5%	0.5%
Particle size #80 Mesh	to be determined	>99%			-	-	-	-	-	
Solubility in purified water at room temperature (20°C)		Soluble in water at a level > 3000 ppm (>0.3%)	Freely soluble in water:ETOH (50:50)	Freely soluble in water:ETOH (50:50)	NT	NT	NT	yes	yes	Yes
Rebaudioside A (on dry basis)	FCC (LC-UV) validated	≥ 95 %	NA	≥ 95 %	96.9%	96.3%	98.4%	96.9%	96.3%	96.5%
Total steviol glycosides (on dry basis)	FCC (LC-UV) validated	> 95 %	NLT 95%	NS	98.0%	97.9%	99.1%	99.3%	98.4%	98.6%
Stevioside (on dry basis)	FCC (LC-UV) validated	< 2 %		NS	ND	0.21	ND	0.15%	<0.2%	0.02%

Parameter	Method	DSM Tentative Specs	JECFA Spec	FCC Specification	Reb A Lot ID NBK-5203-0008-1210BPF1-2	Reb A Lot ID NBK-5203-009-2207BPF2-2	Reb A Lot ID NBK-5203-010-310BPF3-1	Capua lot ID NBK-5203-011-076	Reb A Lot VVA 1501-453	Reb A Lot VVA 1501-451
Residual solvents (Ethanol)	FCC (GC_FID), JECFA GC-head space	<5000 ppm	NMT 0.5%	NMT 0.5%	1276 ppm	250 ppm	4840 ppm	1400 ppm	3120 ppm	3500 ppm
Residual solvents (Methanol)	JECFA GC-head space		NMT 0.02%	NMT 0.02%	<150 ppm	<150 ppm	<150 ppm	ND	ND	ND
pH (1 gram dissolved in 100 ml of water)	FCC	4.5 – 7.0	4.5 – 7.0	4.5 - 7.0	4.4	4.8	4.7	6.4	7.0	7.0
Lead	SLD A1603 NEN-EN-ISO 11885 (ICP-AES) validated	<1 ppm	<1 ppm	≤1 ppm	<1 ppm	<1 ppm	<1 ppm	0.2 ppm	0.3 ppm	0.3 ppm
Mercury	SLD A1603 NEN-EN-ISO 11885 (ICP-AES) validated	<1 ppm	NS	NS	<1 ppm	<1 ppm	<1 ppm	0.05 ppm	0.03 ppm	0.03 ppm
Cadmium	SLD A1603 NEN-EN-ISO 11885 (ICP-AES) validated	<1 ppm	NS	NS	<1 ppm	<1 ppm	<1 ppm	0.05 ppm	0.02 ppm	0.02 ppm
Arsenic	SLD A1603 NEN-EN-ISO 11885 (ICP-AES) validated	<1 ppm	<1 ppm	≤1 ppm	<1 ppm	<1 ppm	<1 ppm	0.02 ppm	0.03 ppm	0.03 ppm
Recombinant DNA	PCR	absent by test			<1 ng/g	<1 ng/g	<1 ng/g	<1 ng/g	ND	ND

Parameter	Method	DSM Tentative Specs	JECFA Spec	FCC Specification	Reb A Lot ID NBK-5203-0008-1210BPF1-2	Reb A Lot ID NBK-5203-009-2207BPF2-2	Reb A Lot ID NBK-5203-010-310BPF3-1	Capua lot ID NBK-5203-011-076	Reb A Lot VVA 1501-453	Reb A Lot VVA 1501-451
Microbiology										
Total Plate Count	European and US Pharmacopeias, membrane filtration	≤1000 cfu in 1g	NA	NA	5	1	16	10	<1000	60
Yeast and mold	European and US Pharmacopeias, membrane filtration	≤ 100 CFU in 1 g	NA	NA	< 1	< 1	< 1	< 10	<100	<10
Coliforms	SLD M9849 ISO 21528-1 2004	≤ 10 CFU in 1 g	NA	NA	NA	NA	NA	< 3	<3	<3
Escherichia coli	SLD M9807 ISO 21528	< 3 MPN in 1 g	NA	NA	NA	NA	NA	negative	< 3	<3

5   Stability

The following information has been provided in several previous GRAS Notices.

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 after 2 months of storage at 37°C was detected. However, no significant change at room temperature or below following 5 months of storage of stevioside and 3 months of storage of rebaudioside A was noted. 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 000252) conducted stability testing on rebaudioside A (1) as a powder, (2) as a pure sweetener in solution, and (3) on both cola-type and citrus carbonated beverages. In these investigations 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 (Merisant, GRN 000252).

Cargill (GRN 000253) 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 the degradation products were found to share the same steviol aglycone backbone structure as found in stevioside and rebaudioside A, but they differed by virtue of 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 (GRN 000253).

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 above described stability reports for purified rebaudioside A, in a GRAS notification by Sunwin and WILD Flavors (GRN 000304) 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.

The stability data in the scientific literature for stevioside, the JECFA report, and the extensive stability testing presented by Merisant, Cargill and Sunwin and WILD Flavors indicate that Reb A is stable in storage and application. Since the DSM Reb A is materially identical to the Reb A utilized in the aforementioned citations, there is no reason to believe that the DSM Reb A would behave differently under these conditions.

None the less, DSM has initiated a stability trial with their Reb A produced by fermentation and did not find any statistically significant reduction during the first 6 months of storage at 25 and 40 °C. See table below and

Table 5-1 Stability

Parameters/ methods no.	Limits Release / end of shelf specs.	Condition	Temp	Time 0	1 Month	2 Months	3 Months	6 Months
				%	%	%	%	%
		D-number		D33369	D33477	D33565	D33660	
Reb A analyses *	≥95%	1	25 C	98.1	97.0	100.3	98.8	96.2
		2	40C	97.7	98.0	99.4	95.9	94.8
				%	%	%	%	%
		C-number		c35250	c35308	c35387	c35432	C35608
Moisture **	<6%	1	25 C	0.21	3.9	3.6	4.0	5.7
		2	40C	0.44	0.7	6.1	6.1	6.6
X-rite ***	a/b/L	1	25 C	-1.3/2.5/86.7				
A1882 v3	a/b/L	2	40C	-1.2/2.6/85.8				
AW****	< 50%	1	25 C	17.5				
A10054 v1		2	40C	17.6				
		1	25 C	5.99	5.44	5.18	5.34	5.16
pH*****	4.5 – 7.0	2	40C	5.80	5.25	5.14	5.25	5.08

The data indicates that the DSM Reb A is stable at 25 °C for at least six months and is capable of withstanding short term exposure to excessive storage conditions typically encountered during transportation and distribution.

6   Intended uses

6.1   Intended Food Uses

DSM's Reb A is intended to be used as a general purpose non-nutritive sweetener[1] for use in various foods but it will be excluded from use in infant formulas and meat and poultry products. Because DSM's Reb A has a sweetness value between 200 and 300 that of sucrose depending upon the food product, DSM anticipates that its Reb A will be used in a manner similar to that of other non-nutritive sweeteners such as Aspartame. DSM envisages that its Reb A will be used in consumer products such as beverages, dairy products, baked goods and confections. DSM Reb A could be used in other food categories within the limits of cGMP. Due to the solubility and sensorial properties of Reb A, there are technical and consumer acceptance barriers to utilization in some food products and for full replacement of nutritive sweeteners.

GRAS Notices by Merisant GRN 000252, Cargill GRN 000253, McNeil GRN 000275, Blue California GRN 000278, Sunwin/Wild flavors GRN 000304, Pyure Brands GRN 000318, Pure Circle GRN 000323 and GLG Life Tech GRN 000348 , all address use levels in various food and beverage categories where Reb A could be utilized and each of them has received 'No Questions' letters from FDA. The above cited GRAS Notices can be found in the references.

6.2   Estimated Daily Intake

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

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[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

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; and 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 ADI of 4.0 mg/kg bw/day.

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 beverage, particularly soda typically contains 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 beverage consumed as much as the sugar sweetened beverage consumer. Four 12 oz. cans is approximately one kilogram in weight and at 12% sugar the amount of sugar in the kilogram of beverage would be 120 grams. At 200 times the sweetness of sucrose, the quantity of Reb A consumed at the 95th percentile would be 600 mg. For a 60 Kg adult the exposure due to carbonated beverages would be 10 mg Reb A/Kg bw/day, or 3.3 mg steviol equivalent/Kg bw/day[2]. This is below the Acceptable Daily Intake of 0-4 mg steviol equivalent/Kg bw/day established in 2008 by JECFA for steviol glycosides (JECFA, 2008).

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 equivalent) 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 the toddlers respectively (EFSA, 2014). At the exception of 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 Reb A would replace all steviol glycosides currently used on the market – which is highly unlikely – the intake of DSM's Reb A will still not exceed the ADI established by JECFA.

6.3   Estimated exposure based upon caloric sweetener consumption

In order to estimate human exposure to steviol glycosides, Renwick (2008) used a different approach, basing his calculation on the observed exposure data for aspartame and considering relative sweetness potencies in relation to sucrose, of 180 for aspartame and 200 for rebaudioside A. By using this approach, Renwick predicted average dietary exposure to rebaudioside A, expressed as steviol equivalents for children (aged 1-14 years), including diabetics, ranging from 0.4 to 1.3 mg/kg bw/day, and from 1.5 to 4.2 mg/kg bw/day at the high percentile (90th/97.5th). For adults, the mean dietary

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[2] Conversion from Reb A and steviol of 0.33% calculated based on the ratio of molecular weights of rebaudioside A (967.01 g/mol) and steviol (318.45 g/mol)

exposure to rebaudioside A, expressed as steviol equivalents, including diabetics, ranged from 0.3 to 0.7 mg/kg bw/day, and from 1.5 to 3.1 mg/kg bw/day at the high percentile (90th/97.5th).

The relative sweetness intensity of rebaudioside A generally ranges from 200-300 that of sucrose, , which is also the case for DSM's Reb A, as confirmed by sweetness profile comparisons of DSM's Reb A and rebaudioside A from competitors where the relative sweetness of both the commercial plant derived Reb A and that of DSM was found to be approximately 250 times sweeter than sucrose. See Annex 7.

The USDA reported in their publication titled 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). That means the actual consumption is approximately 95.7 Lbs./person/yr. or 43.4 Kg/person/yr. This is equivalent to 0.119 Kg/person/day, or 119 g/person/day. For a 60 Kg person the consumption would be approximately 1.983 g/Kg BW/day.

The DSM sensory study indicates that DSM's Reb A is about 250 times sweeter than sucrose, Annex 7. Thus, based on this sweetness equivalence the estimated consumption of Reb A as the sweetener in all foods would be approximately 7.9 mg / Kg BW/day, below the ADI calculated by FSANZ of 12 mg/Kg BW/day, see section 7.1. In GRAS Notice 253 (GRN 000253), Cargill calculated an 18 mg/ Kg BW/day exposure for their rebiana product; possibly due to the use of the wholesale production quantity of caloric sweeteners rather than the consumption quantity and a lower sweetness value for rebiana. Even if DSM's Reb A had a lower sweetness equivalence of 200, this would result in an increase in consumption to 9.88 mg/Kg BW/day which is still less than the ADI established by JECFA. As noted by Cargill, these exposure values are greatly exaggerated because Reb A is not going to replace all the sweeteners used in food and beverages due to both technical and sensorial barriers.

Because DSM's microbiologically produced Reb A is equivalent in chemical and physical characteristics and sensorial properties to that of commercially available plant-derived high purity Reb A products, it can be used in various foods and beverages at the same level and the resulting exposure from DSM's Reb A will be no different than what has been reported and presented to the U.S. FDA in the previous twenty two GRAS Notices.

7 Safety

The safety of the production organism was addressed in section 2.6 of this dossier.

The safety of DSM'S Reb A is based upon a number of factors; the safety of the yeast *Yarrowia lipolytica*, used to make products already on the market and whose safety has been demonstrated, on the safety evaluation of the strain modifications, see section 2.6 of this dossier, and most importantly on the extensive safety literature available on rebaudioside A and other steviol glycosides that have been consumed via multiple food products for several years. The safety of DSM's Reb A is further supported by analytical studies demonstating the high purity and full compliance of DSM's Reb A with specifications established by JECFA for all steviol glycosides, as well as by toxicological studies confirming the absence of genotoxicity, reproductive and chronic toxicity/carcinogenicity all support the safety of DSM's Reb A.

7.1 Safety data of steviol glycosides

Overview

Steviol glycosides extracted from the plant *Stevia rebaudiana* Bertoni have been commercialized and used safely as a sweetener since the 1970's. See Carakostas et al. 2008.

Their safety has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for many years, and in older studies adverse effects were found in preparations that were not sufficiently purified and characterized. As late as the year 2000, JECFA was not convinced of the safety of steviol glycosides, and the European Scientific Committee for Food (SCF), the predecessor of EFSA, concluded that stevioside as a sweetener was "toxicologically not acceptable due to insufficient available data to assess its safety" as reported by the Scientific Committee on Foods in 1984, 1989 and 1999 (SCF 1984, SCF 1989 and SCF 1999). The JECFA had notably concerns about the mutagenic effect observed *in vitro* Reverse Bacterial Mutagenesis Assay (Ames Test) in the presence of metabolic activation and the paucity of *in vivo* data available at that time. Further study of the stevia leaf identified the two major sweetening steviol glycosides as stevioside and rebaudioside A, along with several rebaudiosides and minor glycosides. These findings allowed for subsequent production of high purity materials that could be tested in toxicology and clinical safety studies. Based on these additional safety studies, performed with highly purified extract, the JECFA concluded that stevioside and rebaudioside A are not genotoxic *in vitro* and *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives *in vitro* is not expressed *in vivo*, see section 7.3.3.1. In addition, results of a recent study have shown the absence of adverse effects of steviol glycosides when taken at doses of about 4 mg/kg bw/day, expressed as steviol, for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal and low-normal blood pressure for 4 weeks (Maki et al. 2008a and 2008b). In 2008, JECFA established an Acceptable Daily Intake for steviol glycosides of 0-4 mg/kg bw/day expressed as steviol, based on the No Observed Effect Level of 970 mg stevioside/kg bw/day derived from a 2 year study (Toyoda et al, 1997).

JECFA has also established specifications to ensure that the material tested is representative of the material of the commerce.

As of 6 July 2015, FDA has reviewed 36 GRAS Notices for stevia extracts and purified glycosides since 2008, and issued "no questions" letters for all of them, see Table 7-1 below. These safety assessments are based on the JECFA (re-)evaluation of steviol glycosides and on the ADI that was established. GRAS notices have notably been submitted and received letters of no objection for purified mixed steviol glycosides (min. 95% pure on a dry weight basis), but also more recently for a use of Rebaudioside A, Rebaudioside D (GRN 000456), Rebaudioside X (now known as Reb M)(GRN 000473 and GRN 000512) or enzyme-modified steviol glycoside preparations as a general purpose sweetener. Other regulatory bodies (e.g. European Food Safety Authority, Food Standards Australia New Zealand (FSANZ) and Health Canada) have also evaluated the safety of steviol glycosides and reached the same conclusions as JECFA (EFSA, 2010; FSANZ, 2008; Health Canada, 2012).

Table 7-1 Generally Recognized As Safe Notices to FDA concerning Stevia Based products

GRN No.	Substance	Date of NO Questions Letter
252	Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni	17-Dec-08
253	Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni	17-Dec-08
275	Purified steviol glycosides with rebaudioside A as the principal component	11-Jun-09
278	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	20-Jul-09
282	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	11-Aug-09
287	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	28-Aug-09
303	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	22-Mar-10
318	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	15-May-10
329	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	9-Sep-10
323	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	9-Jul-10
337	Enzyme-modified steviol glycosides preparation (EMSGP)	17-Jun-11

GRN No.	Substance	Date of NO Questions Letter
348	Stevioside purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (stevioside)	14-Jul-11
349	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	14-Jul-11
354	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	15-Jul-11
365	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	18-Aug-11
367	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	8-Jul-11
369	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	11-Oct-11
375	Enzyme-modified steviol glycosides	2-Sep-11
380	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	28-Nov-11
388	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	9-Jan-12
389	Steviol glycosides with stevioside as the principal component	18-Jan-12
393	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	23-Jan-12
395	Steviol glycosides with rebaudioside A and stevioside as the principal components	24-Jan-12
418	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	7-Jun-12
448	Enzyme-modified steviol glycosides	3-May-13
452	Enzyme-modified steviol glycosides	1-Jul-13
456	Rebaudioside D purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside D)	1-Jul-13
461	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	14-Aug-13

GRN No.	Substance	Date of NO Questions Letter
467	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	25-Nov-13
473	Purified steviol glycosides with rebaudioside X as the principal component	17-Dec-13
493	High purity steviol glycosides (minimum purity 95%)	30-May-14
512	High purity Rebaudioside M	24-Oct-14
516	Steviol glycosides with rebaudioside A and stevioside as the principal components	30-Oct-14
536	High Purity Rebaudioside C	10-Feb-15
548	High purity Rebaudioside D	20-Apr-15
555	High purity steviol glycosides (minimum purity 95%) consisting primarily of rebaudioside A	20-Apr-15

7.2 Absorption, distribution, metabolism, excretion

The absorption and hepatic metabolism of either a Stevia mixture – containing mainly rebaudioside A, stevioside, rebaudioside C, dulcoside A – or steviol was studied in rats (Koyama et al., 2003b). While *ex vivo* no absorption of steviol glycosides was noted, a significant absorption of steviol was reported. In addition, the oral administration of steviol led to a peak of steviol concentration in plasma after only 15 minutes, demonstrating its rapid absorption, while the administration of the Stevia mixture led to a steady increase of steviol in the plasma over 8 hours (Koyama et al., 2003b).). In addition, while no absorption of steviol glycosides was noted *ex vivo*, a significant absorption of steviol was observed. In another study, *in vivo* plasma steviol concentrations were noted to increase over the course of 24 hours after oral stevioside administration (Wang et al., 2004). This suggests that the steviol glycosides first need to be metabolized before being absorbed as steviol in the rat intestine. *In vitro* studies have confirmed that digestive enzymes or fluids such as salivary or pancreatic α-amylase or pepsin, pancreatin solutions, or intestinal brush border membrane enzymes from humans, mice, rats, hamsters or pigs are not able to digest stevioside by hydrolyzing its β-glycosidic bonds (Hutapea et al., 1997). However, the caecal microflora of all species tested was able to metabolize stevioside to steviol. This has been further confirmed by the results of several *in vitro* studies using Stevia mixture, stevioside, rebaudioside A , rebaudioside B, rebaudioside D, rebaudioside M and rebaudioside E incubated with pooled human faecal homogenates or isolated bacterial strains (Koyama et al., 2003a; Gardana et al., 2003; Renwick and Tarka, 2008, Purkayastha et al. 2014, Purkayastha et al. 2015). These steviol glycosides were completely metabolized to steviol within 24 hours, whereas no metabolism of steviol was observed during this period. **These data support the use of toxicology data available on steviol,**

and on steviol glycosides metabolized to steviol (i.e.,Stevioside and Reb A) to underpin the safety of Reb A. The complete conversion of stevioside into steviol by the colon bacteria was also shown in pigs (Geuns et al., 2003a), while stevioside administered to chickens and roosters has been shown to be eliminated largely untransformed (Geuns et al., 2003b). Microbes of the *Bacteriodaceae* family (predominantly Bacteroides) have been identified as responsible for this metabolism. The intestinal transport of stevioside, rebaudiose A and steviol was also studied in Caco-2 cells (Geuns et al., 2003b). The apparent permeability value of steviol was found to be 200 to 300-times higher than that of stevioside or rebaudioside A.

As observed *in vitro* and *ex vivo*, steviol is rapidly absorbed from the gastrointestinal tract (Wingard et al., 1980; Geuns et al., 2003b; Koyama et al., 2003a). After steviol has been absorbed, it 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 or 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).

As observed after an oral administration of either steviol glycosides or steviol to rats, steviol is primarily excreted in the faeces 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 behavior of labelled ^{3}H-stevioside administered orally to Wistar rats was studied (Nakayama et al., 1986). A slow increase of radioactivity in 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 caecum. After 4 hours, the level in the caecum 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 faeces, 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 caecal flora to steviol and sugars, which are thereafter absorbed from the caecum, distributed throughout the whole body and excreted mainly into faeces and expired air. A recent study in rats by 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 at 2,000 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. Although these low levels of parent compound were reported, this is considered to have no safety consequence as all the toxicological studies conducted with rebaudioside A and D showed no adverse toxicological findings.

In human volunteers having ingested stevioside or rebaudioside A, no free steviol was detected in the blood, 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 that were identified in rats or hamsters particularly those that may

be mutagenic, were detected in human plasma. Similarly 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 faeces of humans having ingested stevioside or rebaudioside A (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.

Comparison stevioside – rebaudioside A

The toxicokinetics and metabolism of rebaudioside A, stevioside and steviol were compared in rats in order to determine whether toxicological studies previously conducted with stevioside would be applicable to rebaudioside A (Roberts and Renwick , 2008). Gavage treatment of male and female Sprague-Dawley rats with radiolabeled rebaudioside A or stevioside resulted in peak plasma concentrations for both steviol glycosides after 2 to 8 hours of dosing (Roberts and Renwick, 2008). The predominant radioactive component in all samples was steviol, with 5 to 17-times lower amounts of steviol glucuronide. However, peak plasma radioactivity levels were slightly higher for rats treated with stevioside than for those treated with rebaudioside A. This is consistent with the fact that stevioside is expected to be more easily degraded to steviol than rebaudioside A due to the presence of one less glucose unit. This will result in a faster metabolism of stevioside to steviol followed by the systemic absorption of steviol, in comparision with rebaudioside A. Previous studies have also shown that the hydrolysis of rebaudioside A to steviol is slower than the one of stevioside to steviol (Wingard et al., 1980; Koyama et al., 2003a). It was suggested by Koyama that rebaudioside A is first converted to stevioside (mainly) or to rebaudioside B (minor pathway) before being metabolized to steviol. After gavage treatment of male and female Sprague-Dawley rats with radiolabeled rebaudioside A or stevioside, the steviol glycosides were metabolized and excreted rapidly, as 83 to 98% of the radioactivity was eliminated in the faeces within 48 hours (Roberts & Renwick , 2008). A very small proportion of the administered dose was excreted in the urine, while most of the dose was excreted via the bile both for rebaudioside A and stevioside. The predominant radioactive compound detected in the bile of rats was steviol glucuronide. Similarly to what was observed in the rat, the T_{max} of steviol glucuronide in humans was shorter following the ingestion of stevioside as compared to rebaudioside A due to the faster bacterial degradation of stevioside (Wheeler et al., 2008). In addition, the maximum plasma concentration for steviol glucuronide was lower following a single dose of rebaudioside A as compared to a single oral dose of stevioside. Overall, rebaudioside A and stevioside follow similar patterns of toxicokinetics and metabolism. Overall, the data demonstrate that rebaudioside A and stevioside have similar metabolism and pharmacokinetics in the rat. With the exception of having different numbers and types of sugar moieties, steviol glycosides share the same structural backbone, steviol. As such, all steviol glycosides are expected to follow the same metabolic pathway as

demonstrated for rebaudioside A and stevioside. Therefore, the results of toxicology studies on either stevioside or rebaudioside A are applicable to the safety of all steviol glycosides.

Thus, these results support the use of toxicological safety studies performed with stevioside for the safety assessment of rebaudioside A.

It should be noted that the inter-species difference in the route of elimination of systemically absorbed steviol as steviol glucuronide (*via* the bile in rats and in the urine in humans) referenced here occurs as a result of the lower molecular weight threshold for biliary excretion in rats (325 Da) as compared to humans (500 to 600 Da; molecular weight of steviol glucuronide is 495 Da) (Renwick, 2007). Notably, in bile-duct ligated rats, excretion of steviol glucuronide occurred primarily in the urine (Wingard *et al.*, 1980). While the primary routes of elimination of steviol glucuronide differ between rats and humans, the metabolism and pharmacokinetics of steviol glycosides are quite similar, which confirms the rat as an acceptable model for risk assessment in humans. The difference in the route of elimination is considered to be of no toxicological significance due to the fact that the water-soluble phase II metabolites are rapidly cleared in both species. Therefore, toxicology data generated in rats are applicable to assess the safety of steviol glycosides in humans given the similarities in metabolic fate.

7.3 Toxicity studies

7.3.1 Acute Toxicity
An LD50 of >15 g/kg bw was obtained in acute oral toxicity studies of mice, rats and hamsters administered stevioside (purity 96%) (Toskulkao, 1997). Other acute toxicity studies were conducted with steviol glycosides not complying with JECFA specifications. For example, Rebaudioside A, but also rebaudioside B, stevioside and steviolbioside (purity not specified), administered as a single gavage dose of 2 g/kg bw to male Swiss-Webster mice, were reported to produce no toxic effects (Medon et al., 1982).

7.3.2 Short term and Subchronic Toxicity
Several short-term and subchronic toxicity studies were conducted in animals with rebaudioside A of high purity (Curry and Roberts, 2008; Nikiforov and Eapen, 2008) or stevioside of high purity (Aze et al., 1991; Geuns et al., 2003b). Other studies were conducted earlier with steviol glycosides not meeting JECFA specifications. Due to their lower purity, these studies are not considered useful for the safety assessment of DSM's RebA, and they are therefore, not described below.

Several sub-chronic studies were conducted in animals with rebaudioside A of purity higher than 95% (Curry and Roberts, 2008; Nikiforov and Eapen, 2008). Rebaudioside A (purity > 97%) was administered to Wistar rats at concentrations up to 100,000 ppm diet in a 4-week dose-range finding study and at concentrations up to 50,000 ppm diet in a 13-week toxicity study (Curry and Roberts, 2008). No deaths,

adverse clinical signs, changes in clinical chemistry and haematology parameters, and no pathological findings related to treatment were reported in these studies. The only observations that could be linked to treatment were effects on body weight, feed intake and feed conversion efficiency. Indeed, in the 4-week study and in the 13-week study, body weight gains were statistically significantly lower in both sexes receiving 50,000 and 100,000 mg rebaudioside A/kg diet and 25,000 and 50,000 mg rebaudioside A/kg diet, respectively, 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 of toxicological significance (JECFA, 2009). Similarly, JECFA considered that the decrease in body weight gain observed in rats given 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 reduced concentrations of bile acids were observed. The increases in mean plasma urea and creatinine being small and falling within the historical control range were not considered as a sign of renal toxicity as no changes were seen in macroscopic and microscopic observations of the kidneys. The decreases in bile acids were all within the normal range of historical controls except for the high dose male group. They were 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 and therefore, not considered as adverse. Overall, the NOAEL of this study was determined to be 50,000 ppm rebaudioside in feed, corresponding with 4161 and 4645 mg/kg bw/day for males and females, or 1370 mg and 1530 mg steviol equivalents/kg bw/day in males and females (Curry and Roberts, 2008; JECFA, 2009). In another study, rebaudioside A (purity 99.5%) 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 were reported on body weight gains, terminal body weights, clinical and functional observations, haematology, serum chemistry or urinalysis. 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 the high-dose group for males only, 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 treated diets. Other observations included a tendency to reduced serum bile acids, decreased urine volumes and slight changes in serum electrolytes in the treated groups were also observed, which also corroborates results obtained by Curry and Roberts (2008). In the absence of toxicity effects, the NOAEL was concluded to be 2000 mg/kg bw/day, corresponding to 660 mg steviol equivalents/kg bw/day (Nikiforov and Eapen, 2008).

7.3.2.1   13 week oral toxicity conducted with DSM Reb A

A 13 week oral toxicity study was conducted with DSM Reb A at WIL Research, Ashland, Ohio, U.S.A, in accordance with the following guidelines:

- U.S. Food and Drug Administration (FDA). Redbook 2000: Toxicological Principles for the Safety Assessment of Food Ingredients, chapter IV.C.4a Subchronic Toxicity Studies with Rodents, adopted in July 2000, updated in November 2003.
- OECD Guideline for the Testing of Chemicals 408. Repeated Dose 90-day Oral Toxicity Study in Rodents, adopted 21st September 1998.

The study consisted of four groups of 20 male and 20 female Crl:CD(SD) rats. The three test groups were offered DSM Reb A *ad libitum* in the diet at dosages of 500, 1000 and 2000 mg/kg bw/day for at least 91 consecutive days. The teat group diet was not adjusted for caloric density. A concurrent control group was offered the basal diet on a comparable regimen. Following a minimum of 91 days of control or test diet exposure, all animals were euthanized.

All animals were evaluated with respect to general clinical observations, body weight, food consumption, functional observational battery (FOB) and motor activity (MA) assessment and ophthalmic examination. In addition, clinical pathology parameters were analyzed for 10 animals/sex/group during study weeks 1 and 6 (hematology and serum chemistry), and on the days of the scheduled necropsy (study week 13; hematology, coagulation, serum chemistry, and urinalysis). Complete necropsies were conducted on all animals, and selected organs were weighed at the scheduled necropsy. Selected tissues were examined microscopically from all control and high-dose group animals. In addition, gross lesions were examined from all animals.

Mean achieved consumption of DSM Reb A in the control, 500, 1000, and 2000 mg/kg/day groups were 0, 516, 1026, and 2057 mg/kg/day, respectively, for males and 0, 509, 1016, and 2023 mg/kg/day, respectively, for females.

All animals survived up to the scheduled necropsy. There were no test article-related clinical observations or effects on food consumption or clinical pathology parameters. There were no test article-related ophthalmic, macroscopic, or microscopic findings or changes in organ weights. FOB and MA assessments were unaffected by test article administration.
Test article-related lower mean body weight, body weight gains, and cumulative body weight gains were noted in the 2000 mg/kg/day group males generally throughout the dosing period, which were not associated with a decrease in food intake. During study week 13, the mean body weight in the 2000 mg/kg/day group males was 5.9% lower than the control group. The lower body weights were not considered to be adverse. Because of the proportion of basal diet that was replaced with the test article containing little caloric value, the lower body weight gains may have been the result of the animals not consuming an equivalent number of calories as in the control group. Additionally, the changes were not considered adverse due to the small magnitude of the difference from the control group values.

The dietary administration of DSM Reb A to Crl:CD(SD) rats ad libitum for a minimum of 91 consecutive days did not result in treatment-related effects up to dosages of 2057 and 2023 mg /kg bw/day for males and females, respectively. The lower mean body weight and body weight gains that were observed with the high dose male group was not considered to be of toxicological significance and was

consistent with the results observed in the 13 week toxicity study described by Nikiforov and Eapen (2008).

The No-Observed-Adverse-Effect-Level (NOAEL) for this study is therefore considered to be >2000 mg/kg bw/day for males and females. This NOAEL provides a margin of safety greater than 500 times the anticipated human exposure based upon the JECFA ADI of 4 mg/Kg BW/day (JECFA 2009). See the toxicology manuscript in Annex 8.

Studies performed with steviosides of high purity fed to animals led to similar results as the ones obtained with rebaudioside A (Aze et al., 1991). Stevioside (95.6% purity) was administered to Fischer 344 rats up to 5% of their diet for 13 weeks (Aze et al., 1991). The treatment did not lead to any deaths. No statistically significant difference in body weight gain or food intake was reported between the control and treated groups. However, the terminal body weights were statistically significantly decreased in the female 2.5%-dose group and male and female 5%-dose group in comparison to the controls. In addition, a few sporadic, but statistically significant changes at some doses were observed for some biochemical parameters. JECFA Panel agreed with the authors' conclusion that the effects observed were nonspecific and not treatment related (Aze et al., 1991; JECFA, 1999). 5% stevioside in the diet was therefore considered as the NOAEL of the study, which is equivalent to 2500 mg/kg bw/day or approximately 942 mg steviol equivalents/kg bw/day.

Studies were conducted with broiler chickens and laying hens given diets containing stevioside (purity > 96%) at a concentration of 667 mg/kg of feed for 14 and 10 days, respectively. No significant differences in feed intake, body-weight gain and feed conversion were observed (Geuns et al., 2003b).

7.3.3   Genotoxicity

Steviol glycosides, including rebaudioside A, have been extensively tested for their genotoxicity, both *in vitro* and *in vivo* evaluations. Overall, rebaudioside A and stevioside do not show evidence of genotoxicity. A critical review of the genetic toxicity of steviol glycosides and steviol has also been published (Brusick, 2008).

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 were administered stevioside (purity 88.6%) in the drinking water at concentrations of 0 or 4 g/l 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) have both 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.

The absence of genotoxicity of rebaudioside A is corroborated by the results of a reverse mutation study and an *in vitro* micronucleus test conducted with DSM's Reb A.

7.3.3.1 Bacterial reverse mutation (Ames) test

A bacterial reverse mutation (Ames) test was conducted with the test substance at WIL Research Europe B.V., 's Hertogenbosch, the Netherlands, in accordance with the following guidelines:

- Organization for Economic Co-operation and Development (OECD) guideline no. 471, Genetic toxicology: Bacterial Reverse Mutation Test, adopted 21 July 1997.
- European Community (EC) Commission regulation No. 440/2008, Part B: Methods for the Determination of Toxicity and other health effects, Guideline B.13/14: "Mutagenicity: Reverse Mutation Test using Bacteria". Official Journal of the European Union No. L142, 31 May 2008.

The test substance was assessed for mutagenic activity in four histidine-requiring strains of *Salmonella typhimurium,* TA1535, TA1537, TA98 and TA100, as well as in the tryptophan-requiring strain of *Escherichia coli,* WP2uvrA. The test was performed in two independent experiments in the presence and absence of S9-mix (rat liver S9-mix induced by Aroclor 1254).

The concentrations analysed in the samples prepared for use during the second mutation experiment (follow-up experiment) were in agreement with the nominal concentrations (i.e. mean accuracies were 102% to 106%).

The test substance was tested in the first mutation assay at a concentration range of 52 to 5000 µg/plate in the absence and presence of 5% (v/v) S9-mix in all five tester strains. In a follow-up experiment of the assay with additional parameters, the test substance was tested at a concentration range of 492 to 5000 µg/plate in the absence and presence of 10% (v/v) S9-mix in the tester strains TA1535, TA1537, TA98, TA100 and WP2uvrA.

The test substance did not precipitate on the plates at this dose levels. The bacterial background lawn was not reduced at any of the concentrations tested and no biologically relevant decrease in the number of revertants was observed.

Negative (i.e. the vehicle, Milli-Q water or dimethyl sulfoxide) and positive controls were run simultaneously with the tested batch.

The test substance did not induce a significant dose-related increase in the number of revertant (His+) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp+) colonies in the tester strain WP2uvrA both in the absence and presence of S9-metabolic activation. These results were confirmed in a follow-up experiment.

The negative control values were within the laboratory historical control data ranges.

The strain-specific positive control values were at least three times the concurrent vehicle control group mean indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that the test substance is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

7.3.3.2 Micronucleus test *in vitro*

An *in vitro* micronucleus test in cultured peripheral human lymphocytes was performed with the tested batch at WIL Research Europe B.V., 's Hertogenbosch, the Netherlands in accordance with the following guidelines:

- Organization for Economic Co-operation and Development (OECD) Guideline for the Testing of Chemicals, Guideline no. 487: In Vitro Mammalian Cell Micronucleus Test (adopted 26 September 2014).
- European Community (EC) Commission regulation No. 440/2008, Part B: Methods for the Determination of Toxicity and other health effects, Guideline B.49 "In Vitro Mammalian Cell Micronucleus Test". Official Journal of the European Union No. L142; Amended by EC No. 640/2012 OJ No. L193, 20 July 2012.

The test substance was examined for its effect on the number of micronuclei formed in cultured peripheral human lymphocytes in the presence and absence of a metabolic activation system (phenobarbital and ß-naphthoflavone induced rat liver S9-mix). The possible clastogenicity and aneugenicity of the test substance was tested in two independent experiments.
Negative (i.e. the vehicle, dimethylsulfoxide) and positive controls were run simultaneously with the tested batch.

The concentrations analysed in the samples prepared for use during the second cytogenetic assay were in agreement with the nominal concentrations (i.e. mean accuracies were 94% to 98%).
No test substance was detected in the vehicle.

(i) first cytogenetic assay
The test substance was tested up to the recommended dose level of 5000 µg/ml for a 3 hours exposure time with a 27 hours harvest time in the absence and presence of S9-fraction.

(ii) Second cytogenetic assay
The test substance was again tested up to 5000 µg/ml for a 24 hours exposure time with a 24 hours harvest time in the absence of S9-mix.

The number of mono- and binucleated cells with micronuclei found in the solvent control cultures was within the laboratory historical control data range. The positive control chemicals, mitomycin C and cyclophosphamide both produced a statistically significant increase in the number of binucleated cells with micronuclei. The positive control chemical colchicine produced a statistically significant increase in the number of mononucleated cells with micronuclei. In addition colchicine also showed a statistically significant increase in the number of binucleated cells with micronuclei in the first cytogenetic assay. It was therefore concluded that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly.

The test substance did not induce a statistically significant or biologically relevant increase in the number of mono- and binucleated cells with micronuclei in the absence and presence of S9-mix, in either of the two experiments.

Based on the results of this study it is concluded that this test is valid and that the test substance is not clastogenic or aneugenic in human lymphocytes under the experimental conditions described in this report.

7.3.3.3 *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). Finally, the available toxicokinetic data indicate the absence of free steviol from the systemic circulation of humans. Any concern raised by the few genotoxic results of steviol observed *in vitro* is therefore 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.

7.3.4   Chronic toxicity

Two chronic studies were performed with stevioside (Xili et al., 1992; Toyoda et al., 1997). Since rebaudioside A and stevioside are both converted to steviol in the gut via the same metabolic pathway, the results of these studies are relevant for the safety evaluation of DSM's Reb A. Both studies were conducted with rats exposed for 2 years to dietary concentrations of stevioside. Neither of the two studies showed any evidence of adverse effects or carcinogenicity. The first study led to a No Observed Adverse Effect Level (NOAEL) of 1.2% of the diet, equivalent to 600 mg stevioside/kg bw/day (Xili et al.,, 1992). The second study, which was more recent and 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). Based on the NOAEL obtained in this last study and using a 100-fold uncertainty factor, JECFA established an Acceptable Daily Intake (ADI) for steviol glycosides of 4 mg steviol equivalents/kg bw/day (JECFA, 2008, 2009).

7.3.5   Developmental and Reproductive Toxicity

Reproduction studies conducted in rats and hamsters have shown the absence of effects of purified stevioside preparations on fertility and fetal development (Mori et al., 1981; Usami et al., 1995). The absence of adverse effects of rebaudioside A on reproductive function or reproductive organs was also demonstrated in a 2-generation reproductive toxicity study conducted with rebaudioside A administered up to 25,000 ppm, or approximately 2,048 and 2,273 mg/kg bw/day respectively for males and females (Curry et al., 2008).

7.3.6   Human Studies

As already described on section 7.2, studies were conducted in humans to evaluate the metabolism and pharmacokinetics of steviol glycosides.

Specific human studies were also conducted to evaluate the safety and tolerability of purified steviol glycosides and *Stevia* extracts on glucose homeostasis, following single or repeated administrations to healthy subjects and those with type-2 *diabetes mellitus*. Many of the studies also included endpoints to assess effects on blood pressure, and some of the studies were specifically performed in hypertensive subjects.

In randomized, double-blind, placebo-controlled trials, subjects with type-2 diabetes characterized by insulin resistance, were assessed for their post-meal response to steviol glycoside-induced glucose stimulation. The administration of single doses of rebaudioside A (purity 97%) to men or women with normal glucose tolerance or with type-2 *diabetes mellitus* at levels up to 1000 mg/person/day did not acutely affect glucose homeostasis or blood pressure levels in individuals (Maki et al., 2009).

Similarly, the repeated administration of rebaudioside A (purity 97%) at 250 mg/person/day, 4 times a day to type-2 diabetes mellitus-afflicted adult men and women over the course of 16 weeks revealed no change in glucose homeostasis to the exposed individuals(Maki et al, 2008a).

In addition, blood pressure endpoints were examined in individuals with low-normal and low systolic blood pressure after the oral administration of steviol glycosides. The oral intake of 250 mg rebaudioside A (purity 97%) per person 4 times a day during 4 weeks did not lead to alterations of blood pressure in healthy adults with normal or low-normal blood pressure, and no changes in resting, seated SBP, DBP, Mean Arterial Pressure, Heart Rate or 24-hour ambulatory blood pressures responses were observed (Maki et al., 2008b).

7.4 Other safety considerations

7.4.1   Reb A metabolites

Steviol and kaurenoic acid are intermediates in the biosynthetic pathway of steviol glycosides and may therefore constitute impurities in DSM's Reb A. 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.

As indicated section 7.3.3.1, among the many genotoxicity data available on steviol and its oxidative derivatives, a few *in vitro* studies have reported genotoxicity of steviol. However, due to the fact that these observations were reported in very specific bacteria tests only and that this genotoxic potential is not expressed *in vivo*, Brusick (2008) concluded that the single positive *in vivo* study measuring single-strand DNA breaks in Wistar rat tissues by stevioside, was not confirmed in experiments in mice and appears to be measuring processes other than direct DNA damage. Further, neither stevioside nor

steviol-induced clastogenic effects at extremely high dose levels *in vivo*. He concluded that the application of a Weight-of-Evidence approach to assess the genetic toxicology database concludes that these substances do not pose a risk of genetic damage following human consumption. Indications of genotoxicity have also been reported for kaurenoic acid (Cavalcanti et al., 2006; 2010). These findings prompted a third party review using the expertise of an independent laboratory, Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO), who was requested to assess the genotoxic and carcinogenic potential of kaurenoic acid. A literature search was performed, which indicated contradictory outcomes of genotoxicity studies done with kaurenoic acid. On the first hand, an absence of genotoxicity was reported *in vitro* with *Salmonella typhimurium* strain TM677 treated with kaurenoic acid up to 5 mg/ml in the absence and presence of a metabolic activation system (Pezzuto et al., 1985; 1986). On the other hand, Comet and Micronucleus assays performed *in vitro* with Chinese hamster lung fibroblasts led to DNA damage at 30 and 60 µg/ml, the two highest concentrations of kaurenoic acid tested (Cavalcanti et al., 2006). Kaurenoic acid was also reported to be genotoxic and mutagenic *in vitro* in human peripheral blood leukocytes (PBLs) and in yeast (*S. cerevisiae*), and *in vivo* in mice (bone marrow, liver and kidney) probably due to the generation of DNA double-strand breaks and/or inhibition of topoisomerase (Cavalcanti et al., 2010). However, DNA damage observed was induced at concentrations reported to be cytotoxic through an apoptotic pathway (Cavalcanti et al., 2010; Lizarte Neto et al., 2013). Taken into account that apoptosis can induce DNA-strand breaks, chromosome and chromatid breaks and micronuclei as a result of defragmentation of the DNA, and considering that the apoptosis induced by kaurenoic acid is related to apoptotic pathways covering the regulation of several genes, TNO concluded that the DNA damage observed in presence of kaurenoic acid is likely the result of artifacts due to DNA fragmentation as a result of apoptosis and should therefore not be considered the result of a mutagenic mode of action. These effects were therefore considered to be a threshold-related effect. In addition, no indication of carcinogenicity of kaurenoic acid could be found in literature. Despite the presence at a low level of steviol and kaurenoic acid in DSM's Reb A (5 to 12 ppm kaurenoic acid and 3 to 15 ppm steviol), a reverse mutation study and an *in vitro* micronucleus test conducted with DSM's Reb A have shown the absence of genotoxicity of DSM Reb A as reported in section 7.3.3, which further corroborates the absence of genotoxicity of these two impurities.

Besides these publicly available studies on the genotoxicity of steviol and kaurenoic acid, only limited data are available assessing the safety of these potential impurities. TNO also performed a search for safety data of structurally related compounds of kaurenoic acid, but was not able to retrieve relevant toxicity data of these read-across candidates. The threshold of toxicological concern (TTC) approach, commonly used by FDA to assess the risk of migrants from food packing materials, and by JECFA to assess the safety of flavors (Munro, 1996) can be used in this case. The use of this approach is also recommended by WHO and EFSA to assess substances of unknown toxicity present at low levels in the diet (EFSA/WHO, 2015) and was therefore used to determine acceptable levels of these two impurities in DSM's Reb A. The first step of the TTC approach consists in determining whether the substance to be assessed has structural alerts or chemical-specific genotoxicity data indicating the chemical may be a DNA-reactive carcinogen. As concluded above, neither steviol, nor kaurenoic acid should be considered

as being genotoxic. In addition, the chemical structure of these two compounds was 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, for which a TTC of 1.5 µg/kg bw/day applies (Kroes et al., 2004; EFSA/WHO, 2015). By considering the ADI of 4 mg steviol equivalent/kg bw/day established by JECFA – equivalent to 12.1 mg DSM Reb A/kg bw/day[3], the maximum level of kaurenoic acid acceptable in DSM's Reb A was determined by TNO as being 124 mg kaurenoic acid/kg DSM's Reb A (124 ppm). A similar level can be defined for steviol: 124 mg steviol/kg DSM's Reb A. The results of analyses performed on three pilot batches of DSM Reb A have revealed levels well below these acceptable limits, as levels of 5 to 12 ppm kaurenoic acid were detected, and levels of 3 to 15 ppm steviol were reported. The batch from the Capua facility had a steviol concentration of 100 ppm, which was 20% below the acceptable maximum. For comparison, the analysis of commercially available samples of stevia extracts has also revealed the presence of steviol at levels of 1 to 40 ppm, and of kaurenoic acid below 0.3 ppm. See Annex 10 for the analytic results for both the DSM and commercial samples and Annex 9 for the TNO report supporting the safety of the concentrations of kaurenoic acid found in the DSM samples.

For safety reasons, an acceptable limit of 124 ppm (KA + KA glycosides) in Reb A is therefore defined. A similar acceptable limit of 124 ppm in Reb A can be defined for steviol. DSM monitors the production batches by using Liquid Chromatography-Mass Spectrometry (LC-MS) to ensure that these metabolites remain below the threshold of concern.

7.4.2   Biogenic amines

As noted in the review of the production organism, there have been reports of the formation of biogenic amines by *Yarrowia lypolytica*. Although the production of these toxic compounds are unlikely under the controlled fermentation conditions utilized to produce Rebaudiodise A, DSM monitors the finished material for nitrogen content and uses this measurement as an internal control for the presence of these metabolites.

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[3] Conversion from Reb A and steviol of 0.33% calculated based on the ratio of molecular weights of rebaudioside A (967.01 g/mol) and steviol (318.45 g/mol)

8 List of Annexes

Annex 1- Fermentation Media

Annex 2- Production Strain Control

Annex 3 – Specification Sheet Reb A

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Annex 7 – DSM Reb A Sweetness Analysis

Annex 8 – Toxicology studies manuscript

Annex 9 –Secondary Metabolite report

Annex 10 – DSM and Commercial Stevia Products Composition Report

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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

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10. GRAS Panel Statement -

EXPERT PANEL OPINION ON
THE SAFETY AND GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF
DSM's REB A, REBAUDIOSIDE A PRODUCED BY A STRAIN OF
Yarrowia lipolytica
FOR USE IN HUMAN FOOD PRODUCTS

Introduction

The undersigned, an independent panel of experts, qualified by their scientific training and national and international experience to evaluate the safety of food and food ingredients (the "GRAS Panel"), was specially convened by DSM Nutritional Products, and asked to evaluate the safety and Generally Recognized as Safe (GRAS) status of the proposed uses of DSM's Reb A, Rebaudioside A, for use as a general purpose non-nutritive sweetener[1] in various foods excluding infant formulas and meat and poultry products, based on scientific procedures as described in Title 21 of the Code of Federal Regulations (21CFR§170.30) (U.S. FDA, 2007).

Rebaudioside A is a glycoside of steviol containing four glucose units esterified to the steviol molecule [CAS number 58543-16-1]. It's sweetness potency is reported to be between 200 and 300 times the sweetness of sucrose.

Steviol glycosides extracted from the plant *Stevia rebaudiana* Bertoni have been commercialized and used safely as a sweetener since the 1970's. See Carakostas et al. 2008.

As of 6 July 2015, FDA has reviewed 36 GRAS Notices, see table below, for both purified stevia extracts and high purity steviol glycosides since 2008, and issued "no questions" letters for all of them. These safety assessments are based both on the safety and clinical data considered most recently by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) in their (re-)evaluation of the safety of steviol glycosides for use as a sweetener in food and on the final Acceptable Daily Intake (ADI) that was established. GRAS notices have notably been submitted and received letters of no objection for purified mixtures of steviol glycosides (min. 95% pure on a dry weight basis and meeting the established JECFA specification for nine (9) recognized steviol glycosides), and also, more recently, for the use of Rebaudioside A and Rebaudioside D (GRN 000456), Rebaudioside X (now known as Reb M)(GRN 000473 and GRN 000512) or enzyme-modified steviol glycoside preparations as a general purpose sweetener. Other regulatory bodies (e.g. European Food Safety Authority, Food Standards Australia New Zealand (FSANZ) and Health Canada) have also evaluated the safety of steviol glycosides and reached the same conclusions as JECFA (EFSA, 2010; FSANZ, 2008; Health Canada, 2012).

Generally Recognized As Safe Notices to FDA concerning Steviol Glycoside-Based products

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[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

GRN No.	Substance	Date of NO Questions Letter
252	Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni	17-Dec-08
253	Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni	17-Dec-08
275	Purified steviol glycosides with rebaudioside A as the principal component	11-Jun-09
278	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	20-Jul-09
282	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	11-Aug-09
287	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	28-Aug-09
303	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	22-Mar-10
318	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	15-May-10
329	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	9-Sep-10
323	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	9-Jul-10
337	Enzyme-modified steviol glycosides preparation (EMSGP)	17-Jun-11
348	Stevioside purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (stevioside)	14-Jul-11
349	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	14-Jul-11
354	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	15-Jul-11
365	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	18-Aug-11
367	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	8-Jul-11
369	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	11-Oct-11

GRN No.	Substance	Date of NO Questions Letter
375	Enzyme-modified steviol glycosides	2-Sep-11
380	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	28-Nov-11
388	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	9-Jan-12
389	Steviol glycosides with stevioside as the principal component	18-Jan-12
393	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	23-Jan-12
395	Steviol glycosides with rebaudioside A and stevioside as the principal components	24-Jan-12
418	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	7-Jun-12
448	Enzyme-modified steviol glycosides	3-May-13
452	Enzyme-modified steviol glycosides	1-Jul-13
456	Rebaudioside D purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside D)	1-Jul-13
461	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	14-Aug-13
467	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	25-Nov-13
473	Purified steviol glycosides with rebaudioside X as the principal component	17-Dec-13
493	High purity steviol glycosides (minimum purity 95%)	30-May-14
512	High purity Rebaudioside M	24-Oct-14
516	Steviol glycosides with rebaudioside A and stevioside as the principal components	30-Oct-14
536	High Purity Rebaudioside C	10-Feb-15
548	High purity Rebaudioside D	20-Apr-15
555	High purity steviol glycosides (minimum purity 95%) consisting primarily of rebaudioside A	20-Apr-15

A comprehensive search of the scientific literature for safety and toxicity information on rebaudioside A and stevia extracts was performed by DSM Nutritional Products and included both a general Google literature search, as well as the use of the SciVerse-Scopus abstract and citation database of peer-reviewed literature and also the internal library sources of DSM. Using the search terms stevia, steviol, steviol glycosides and rebaudioside.

All relevant publications were reviewed, summarized and incorporated into a GRAS dossier, **"THE SAFETY AND GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF DSM's REB A, REBAUDIOSIDE A PRODUCED BY A STRAIN OF** *Yarrowia lipolytica* **FOR USE IN HUMAN FOOD PRODUCTS"** and submitted to the GRAS Panel. Copies of the literature were available for review by the GRAS Panel.

The GRAS Panel evaluated information pertaining to the method of manufacture, product specification, analytical data, intended use levels, potential exposure estimates from consumption of foods from all intended uses, safety studies conducted with rebaudioside A, and other information on safety and tolerance deemed relevant. The members of the GRAS Panel were Stanley M. Tarka, Jr., PhD (Chairman), John Thomas, Ph.D, DABT, Palma Ann Marone, Ph.D., ERT, and José Avalos, Ph.D. Following independent and collective critical evaluation of the information summarized in the Dossier, the GRAS Panel conferred and unanimously agreed to the decision described herein.

The wealth of literature and previous reviews of GRAS Notices by FDA indicate that rebaudioside A (Reb A) is safe under the condition of use proposed by the various sponsors, primarily as a general purpose sweetener. EFSA has also reviewed the safety of steviol glycosides in 2010, 2014 (EFSA 2010, EFSA 2014) and established an ADI of 4 mg/kg bw/day. Their most recent review of exposure in June of 2015 found that the ADI was not being exceeded except in toddlers where the conservative calculation for the high-dose group (95th percentile) was 4.3 mg/kg bw/day (EFSA 2015). DSM does not anticipate that its product will be used in a manner that is dissimilar from that of other Reb A products.

The GRAS Panel noted that DSM's Reb A, with a purity of greater than 95% rebaudioside A, is essentially identical to other Reb A products currently in the marketplace and available for use in human food products. Results from physical chemistry analysis, genotoxicity studies and the in-life portion of a 90-day subchronic rat study all collectively provide additional support for the safety of DSM's Reb A.

The GRAS Panel convened via telephone conference calls on July 7 and November 17, 2015 and unanimously concluded that DSM's Reb A, rebaudioside A, produced consistent with current good manufacturing practice (cGMP) and meeting appropriate specifications, is safe for its intended use as listed in paragraph one above and under "Intended Use" below. The GRAS Panel further concluded that these intended uses are GRAS based on scientific procedures. It is also the opinion of this GRAS Panel that other qualified experts would concur with these conclusions.

The scientific analysis supporting our conclusions is presented below.

Description

The substance that is the subject of this GRAS determination is rebaudioside A.

CAS Number: 58543-16-1

Molecular formula: $C_{44}H_{70}O_{23}$

Molar Mass: 967.01 g/mol

IUPAC Name: 19-O-beta-glucopyranosyl-13-O-(beta-glucopyranosyl(1-2)-beta-glucopyranosyl(1-3))-beta-glucopyranosyl-13-xhydroxykaur-16-en-19-oic acid

Synonyms: Reb A, steviol glycosides, 19-O-beta-glucopyranosyl-13-O-(beta-glucopyranosyl(1-2)-beta-glucopyranosyl(1-3))-beta-glucopyranosyl-13-xhydroxykaur-16-en-19-oic acid

Manufacture

DSM's Reb A is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain 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*.

Y. lipolytica is an avirulent yeast species historically used for the production of citric acid and the flavor chemical, γ-decalactone. *Y. lipolytica* is approved by the United States FDA as a secondary direct food additive in citric acid production (21 CFR §173.165) and was previously classified as *Candida lipolytica* (van der Walt and von Arx, 1980), the name by which the organism is described in 21 CFR §173.165.

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 000355) In November 2011, FDA agreed with GRAS Notice 000382 for erythritol from Baolingbao Biology Co., Ltd. Of Shangdong, China, where the erythritol was produced from glucose via biotransformation by a strain of *Yarrowia lipolytica*.

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 production of the commodity chemical citric acid, the peach aroma γ-decalactone, and for specific lipase enzymes.

Y. lipolytica is one of the more intensively studied yeast species and has been the subject of recent in-depth scholarly reviews. Barth and Gaillardin (1997) published a history of *Y. lipolytica* research, including a review of the physiology, biochemistry and cell structure with details on occurrence in nature, life cycle, and genetic and molecular data. Barth and Gaillardin (1997) also provided 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.

Yarrowia lipolytica is generally regarded as a biosafety class 1 microorganism (Groenewald et al, 2013). *Yarrowia lipolytica* is an avirulent yeast species historically used for the production of citric acid and the flavor chemical, γ-decalactone. *Y. lipolytica* has been used extensively at manufacturing scale levels without any documented toxic, allergenic, or other harmful effects on humans' or other animals' health. *Yarrowia lipolytica* is also approved by the United States FDA as a secondary direct food additive in citric acid production (21 CFR § 173.165).

A recent review of the safety of *Yarrowia lipolytica* 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 (Groenewald et al., 2013). In addition, *Y. lipolytica* has been reported to stimulate the production of biogenic amines when using this yeast for cheese ripening, 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 written by 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 was stated 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 given tyramine orally at 0, 200, 2 000 or 10 000 mg/kg feed resulted in a NOAEL of 2 000 mg/kg feed (180 mg/kg body weight (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 for what may be present in DSM's Reb A by using the ADI maximum of 4 mg steviol equivalents/kg bw/day established by JECFA – equivalent to 12.1 mg DSM's Reb A/kg bw/day. Thus, a maximum level of 8 mg biogenic amines present per g DSM's Reb A (or 8,000 ppm) would be below this equivalence threshold and be considered acceptable.

A comprehensive search of the scientific literature for safety and toxicity information on *Y. lipolytica* was conducted by DSM Food Specialties. The search was limited to data available from 2013 in order to complete the extensive review performed by Groenewald et al. (2014). The search terms were 'lipolytica' / 'lipolytica and *safe', 'lipolytica and *tox' and the data bases searched included PubMed, Toxnet, US FDA GRAS Notices, CDAT, NTP, GESTIS, IPCS INCHEM, TSCATS, US EPA, EFSA, EU Scientific Committees, Health Canada and NICNAS. From the 6 hits that were found, only one was relevant to the safety of the microorganism. In this review of the different food-related applications of *Y. lipolytica*, Zinjarde (2014) reaffirmed the safety of the microorganism. It can also be noted that EFSA has 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 only inserted the genes of the *Stevia rebaudiana* and *Arabidopsis thaliana* plants, both of which have a history of safe use. *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.

There have been over 36 GRAS Notices filed with FDA for highly purified *Stevia* leaf extracts, all of them receiving 'no questions' letters..

DSM also employed the Pariza and Johnson decision tree (2001) to determine if that well accepted rubric revealed any questions about the use of the genetically engineered Yarrowia lipolytica, see below.

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?* YES

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 a GRAS notice to the FDA (GRN 000355). *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 2013. 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?* YES

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 A). 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. Another gene was obtained from *Arabidopsis thaliana* (an edible species of cress) CPR_3 (a cytochrome P450 reductase). Also inserted was the gene for kaurene oxidase from *Giberella fujikuroi.* 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 A*

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. The strain is susceptible to antibiotics and to anti-fungals. When tested, the genetic changes introduced into the *Yarrowia lipolytica* STV2050 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 gene 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**

Since the decision tree did not reveal any concerns and the aforementioned characteristics of the production organism are not unsafe, DSM therefore concludes that the use of the genetically engineered *Yarrowia lipolytica* presents no known safety concerns.

Antibiotic resistance

The final production strain does not contain any Antibiotic Resistance genes. The strain is susceptible to antibiotics and to anti-fungals. When tested, the genetic changes introduced into the *Yarrowia lipolytica* ML350 do not affect antifungal susceptibility.

Fermentation and product recovery

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.

All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are incorporated and microbiological analyses are done 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 Reb A 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.

The major part of the production organism is removed by centrifugation and the supernatant is heat-treated to kill-off any remaining microorganism, and subsequently clarified by centrifugation or filtration. The steviol glycosides are recovered from the fluid stream by adsorption chromatography, eluted by aqueous alcohol, decolorized and further purified by the use of active carbon and demineralization resins that comply with 21 CFR 173.25 followed by concentration to dry powder.

DSM's purified Reb A is isolated by crystallization followed by drying.

Current Regulatory Approvals for Rebaudioside A

As of 6 July 2015, FDA has reviewed 36 GRAS Notices, see table above for purified stevia leaf extracts and purified glycosides submitted since 2008, and issued "no questions" letters for all of them. These safety assessments are based both on the safety and clinical data considered most recently by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) in their (re-)evaluation of the safety of high purity steviol glycosides for use as a sweetener in food and on the final Acceptable Daily Intake (ADI) that was established. GRAS notices have notably been submitted and received letters of no objection for purified mixtures of steviol glycosides (min. 95% pure on a dry weight basis and meeting the established JECFA specification for nine (9) recognized steviol glycosides), and also, more recently, for the use of use of Rebaudioside A and Rebaudioside D (GRN 000456), Rebaudioside X (now known as Reb M)(GRN 000473 and GRN 000512) or enzyme-modified steviol glycoside preparations as a general purpose sweetener. Other regulatory bodies (e.g. European Food Safety Authority, Food Standards Australia New Zealand (FSANZ) and Health Canada) have also evaluated the safety of steviol glycosides and reached the same conclusions as JECFA (EFSA, 2010; FSANZ, 2008; Health Canada, 2012).

Their safety has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for many years, and in older studies adverse effects were found in preparations that were not sufficiently purified and characterized. As late as the year 2000, JECFA was not convinced of the safety of steviol glycosides, and the European Scientific Committee for Food (SCF), the predecessor of EFSA, concluded that stevioside as a sweetener was "toxicologically not acceptable due to insufficient available data to assess its safety" as reported by the Scientific Committee on Foods in 1984, 1989 and 1999 (SCF 1984, SCF 1989 and SCF 1999). The JECFA had notably concerns about the mutagenic effect observed *in vitro* Reverse Bacterial Mutagenesis Assay (Ames Test) in the presence of metabolic activation and the paucity of *in vivo* data available at that time. Further study of the stevia leaf identified the two major sweetening steviol glycosides as stevioside and rebaudioside A, along with several rebaudiosides and minor glycosides. These findings allowed for subsequent production of high purity materials that could be tested in toxicology and clinical safety studies. Based on these additional safety studies performed with highly purified extracts, the JECFA concluded that stevioside and rebaudioside A are not genotoxic *in vitro* and *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives *in vitro* is not expressed *in vivo*, see section 7.3.3.1 of the GRAS dossier. In addition, results of clinical studies have shown the absence of adverse effects of steviol glycosides when taken at doses of about 4 mg/kg bw/day, expressed as steviol, for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal and low-normal blood pressure for 4 weeks (Maki et al. 2008a and 2008b). In 2008, JECFA established an Acceptable Daily Intake for steviol glycosides of 0-4 mg/kg bw/day expressed as steviol, based on the No Observed Effect Level of 970 mg stevioside/kg bw/day derived from a 2 year study (Toyoda et al, 1997). JECFA has also established specifications to ensure that the material tested is representative of the material of the commerce.

Intended Uses

DSM Nutritional Products intends to market their rebaudioside A as a general purpose non-nutritive sweetener[2] for use in various foods but it will be excluded from use in infant formulas and meat and poultry products. Because DSM's Reb A has a sweetness equivalence value between 200 and 300 that of sucrose depending upon the food product, DSM anticipates that its Reb A will be used in a manner similar to that of other non-nutritive sweeteners such as Aspartame. DSM envisages that its Reb A will be used in consumer products such as beverages, dairy products, baked goods and confections. DSM Reb A could be used in other food categories within the limits of cGMP. Due to the solubility and sensorial properties of Reb A, there are technical and consumer acceptance barriers to utilization in some food products and for full replacement of nutritive sweeteners.

DSM's Reb A will be sold as a free-flowing powder, off-white in color and with a purity greater than 95%.

Exposure

In order to estimate human exposure to steviol glycosides, Renwick (2008) based his calculation on the observed exposure data for aspartame and considering relative sweetness potencies in relation to sucrose, of 180 for aspartame and 200 for rebaudioside A. By using this approach, Renwick predicted average dietary exposure to rebaudioside A, expressed as steviol equivalents for children (aged 1-14 years), including diabetics, ranging from 0.4 to 1.3 mg/kg bw/day, and from 1.5 to 4.2 mg/kg bw/day at the high percentile (90th/97.5th). For adults, the mean dietary exposure to rebaudioside A, expressed as steviol equivalents, including diabetics, ranged from 0.3 to 0.7 mg/kg bw/day, and from 1.5 to 3.1 mg/kg bw/day at the high percentile (90th/97.5th).

The relative sweetness intensity of rebaudioside A generally ranges from 200-300X that of sucrose, which is also the case for DSM's Reb A, as confirmed by sweetness profile comparisons of DSM's Reb A and rebaudioside A from competitors where the relative sweetness of both the commercial plant-derived Reb A and that of DSM's product was found to be approximately 250 times sweeter than sucrose.

The USDA reported in their publication titled 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). That means the actual consumption is approximately 95.7 Lbs./person/yr. or 43.4 Kg/person/yr. This is equivalent to 0.119 Kg/person/day, or 119 g/person/day. For a 60 Kg person the consumption would be approximately 1.983 g/Kg BW/day.

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[2] 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

The DSM sensory study indicates that DSM's Reb A is about 250 times sweeter than sucrose, Annex 9. Thus, based on this sweetness equivalence, the estimated consumption of Reb A as the sweetener in all foods would be approximately 7.9 mg /Kg BW/day, below the ADI calculated by JECFA of 12 mg/Kg BW/day, (see section 7.1 in GRAS dossier). In GRAS Notice 253 (GRN 000253), Cargill calculated an 18 mg/Kg BW/day exposure for their rebiana product; possibly due to the use of the wholesale production quantity of caloric sweeteners rather than the consumption quantity and a lower sweetness value for rebiana. Even if DSM's Reb A had a lower sweetness equivalence of 200, this would result in an increase in consumption to 9.88 mg/Kg BW/day which is still less than the ADI established by JECFA. As noted by Cargill, these exposure values are greatly exaggerated because Reb A is not going to replace all the sweeteners used in food and beverages due to both technical and sensorial barriers.

Because DSM's microbiologically produced Reb A is equivalent in chemical and physical characteristics and sensorial properties to that of commercially available plant-derived high purity Reb A products, it can be used in various foods and beverages at the same level and the resulting exposure from DSM's Reb A will be no different than what has been reported and presented to the U.S. FDA in the previous twenty two GRAS Notices for Reb A.

Safety Data

As part of their safety assessment for the intended use of DSM's Reb A as a general purpose sweetener, the GRAS Panel critically evaluated available safety information on Reb A and other steviol glycosides. This included an evaluation of all available and published toxicological studies on mutagenicity, cytogenetic effects and subchronic toxicity.

Genotoxicity

The absence of genotoxicity for rebaudioside A was supported by the results of a reverse mutation study and an *in vitro* micronucleus test conducted with DSM's Reb A. Mutagenic activity of DSM Reb A was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* (WP_2uvrA), up to concentrations of 5000 µg/plate in the absence and presence of S9-mix (Ames et al. 1973). DSM's Reb A did not induce a significant dose-related increase in the number of revertant (His^+) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp^+) colonies in the tester strain WP_2uvrA both in the absence and presence of S9-metabolic activation, which was also confirmed in a follow-up experiment. It was therefore concluded that DSM's Reb A is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

Acute Toxicity

An LD50 of >15 g/kg bw was obtained in acute oral toxicity studies of mice, rats and hamsters administered stevioside (purity 96%) (Toskulkao, 1997). Other acute toxicity studies were conducted with steviol glycosides not complying with JECFA specifications. For example, Rebaudioside A, but also rebaudioside B, stevioside and steviolbioside (purity not specified), administered as a single gavage dose of 2 g/kg bw to male Swiss-Webster mice, were reported to produce no toxic effects (Medon et al., 1982).

Short-term and Subchronic Oral Toxicity in Rodents

Several short-term and subchronic toxicity studies were conducted in animals with rebaudioside A of high purity (Curry and Roberts, 2008; Nikiforov and Eapen, 2008) or stevioside of high purity (Aze et al., 1991; Geuns et al., 2003b). Other studies were conducted earlier with steviol glycosides not meeting JECFA specifications. Due to their lower purity, these studies are not considered useful for the safety assessment of DSM's Reb A, and they, therefore, were not reviewed.

Several sub-chronic studies were conducted in animals with rebaudioside A of purity higher than 95% (Curry and Roberts, 2008; Nikiforov and Eapen, 2008). Rebaudioside A (purity > 97%) was administered to Wistar rats at concentrations up to 100,000 ppm in the diet in a 4-week dose-range finding study and at concentrations up to 50,000 ppm in the diet in a 13-week toxicity study (Curry and Roberts, 2008). No deaths, adverse clinical signs, changes in clinical chemistry and haematology parameters, and no pathological findings related to treatment were reported in these studies. The only observations that could be linked to treatment were effects on body weight, feed intake and feed conversion efficiency. Indeed, in the 4-week study and in the 13-week study, body weight gains were statistically significantly lower in both sexes receiving 50,000 and 100,000 ppm rebaudioside A in the diet and in the 25,000 and 50,000 ppm rebaudioside A in the diet, respectively, 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 of toxicological significance (JECFA, 2009). Similarly, JECFA considered that the decrease in body weight gain observed in rats given rebaudioside A for 13 weeks could 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 reduced concentrations of bile acids were observed. The increases in mean plasma urea and creatinine being small and falling within the historical control range were not considered as a sign of renal toxicity as no changes were seen in macroscopic and microscopic observations of the kidneys. The decreases in bile acids were all within the normal range of historical controls except for the high dose male group. They were 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 and therefore, not considered as adverse.

Overall, the NOAEL of this study was determined to be 50,000 mg rebaudioside A/kg feed, corresponding with 4161 and 4645 mg/kg bw/day for males and females, or 1370 mg and 1530 mg steviol equivalents/kg bw/day in males and females (Curry and Roberts, 2008; JECFA, 2009). In another study, rebaudioside A (purity 99.5%) 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 were reported on body weight gains, terminal body weights, clinical and functional observations, haematology, serum chemistry or urinalysis. 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 the high-dose group for males only, 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 treated diets. Other observations included a tendency to reduced serum bile acids, decreased urine volumes and slight changes in serum electrolytes in the treated groups were also observed, which also corroborates results obtained by Curry and Roberts (2008). In the absence of toxicity effects, the NOAEL was concluded to be 2000 mg/kg bw/day, corresponding to 660 mg steviol equivalents/kg bw/day (Nikiforov and Eapen, 2008).

A 13 week oral toxicity study was performed with DSM's Reb A under GLP following the FDA Red Book 2000 and OECD guidelines for Testing Chemicals, Health Effects Test Guidelines, Section 408, September 1998 , in order to comply with requirements of EU registration (study number WIL-825009)Annex 10. The study began on 17 March 2015 and ended on 14 June. DSM's Reb A was administered orally in the diet to CD rats for 13 weeks at target concentrations of 500, 1000 and 2000 mg/kg bw/day. Initial data corroborate the results obtained with comparable studies performed with high purity rebaudioside A obtained from stevia leaf extraction (Curry and Roberts, 2008; Nikiforov and Eapen, 2008). No deaths were reported during the current study. A small decrease in body weight gain was observed in the high-dose group for males only, which was not associated with a decrease in food intake, as was reported in the 13 week toxicity study described by Nikiforov and Eapen (2008). The No-Observed-Adverse-Effect-Level (NOAEL) for this study is therefore considered to be 2057 and 2023 mg/kg bw/day for males and females, respectively. This NOAEL provides a margin of safety greater than 500 times the anticipated human exposure based upon the JECFA ADI of 4 mg/Kg BW/day (JECFA 2009). Based on the high homology of DSM's Reb A with conventional stevia plant leaf derived high purity rebaudioside A and other steviol glycosides previously studied in toxicological evaluations (i.e., stevioside) as well as the results from the 13-week subchronic toxicity evaluation of DSM's microbiologically produced Reb A, DSM is confident that the results for their 13 week study support the safety of DSM's Reb A for its intended uses.

Studies performed with stevioside of high purity where it was fed to animals led to similar results as the ones obtained with rebaudioside A (Aze et al., 1991). Stevioside (95.6% purity) was administered to Fischer 344 rats at levels up to 5% of their diet for 13 weeks (Aze et al., 1991). The treatment did not lead to any deaths. No statistically significant difference in body weight gain or food intake was reported between the control and treated groups. However, the terminal body weights were statistically significantly decreased in the female 2.5%-dose group and male and female 5%-dose group in

comparison to the controls. In addition, a few sporadic, but statistically significant changes at some doses were observed for some biochemical parameters. The JECFA Panel agreed with the authors' conclusion that the effects observed were nonspecific and not treatment related (Aze et al., 1991; JECFA, 1999). 5% stevioside in the diet was therefore considered as the NOAEL of the study, which is equivalent to 2500 mg/kg bw/day or approximately 942 mg steviol equivalents/kg bw/day.

Studies were also conducted with broiler chickens and laying hens given diets containing stevioside (purity > 96%) at a concentration of 667 mg/kg of feed for 14 and 10 days, respectively. No significant differences in feed intake, body-weight gain and feed conversion were observed (Geuns et al., 2003b).

Chronic Toxicity

Two chronic studies were performed with stevioside (Xili et al., 1992; Toyoda et al., 1997). Since rebaudioside A and stevioside are both converted to steviol in the gut via the same metabolic pathway, the results of these studies are relevant for the safety evaluation of DSM's Reb A. Both studies were conducted with rats exposed for 2 years to dietary concentrations of stevioside. Neither of the two studies showed any evidence of adverse effects or carcinogenicity. The first study led to a No Observed Adverse Effect Level (NOAEL) of 1.2% of the diet, equivalent to 600 mg stevioside/kg bw/day (Xili et al.,, 1992). The second study, which was more recent and 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). Based on the NOAEL obtained in this last study and using a 100-fold uncertainty factor, JECFA established an Acceptable Daily Intake (ADI) for steviol glycosides of 4 mg steviol equivalents/kg bw/day (JECFA, 2008, 2009).

Developmental Toxicity in Rodents

Reproduction studies conducted in rats and hamsters have shown the absence of effects of purified stevioside preparations on fertility and fetal development (Mori et al., 1981; Usami et al., 1995). The absence of adverse effects of rebaudioside A on reproductive function or reproductive organs was also demonstrated in a 2-generation reproductive toxicity study conducted with rebaudioside A administered in the diet up to 25,000 ppm, or approximately 2,048 and 2,273 mg/kg bw/day respectively for males and females (Curry et al., 2008).

Human Studies

Specific human studies were also conducted to evaluate the safety and tolerability of purified steviol glycosides and *Stevia* extracts on glucose homeostasis, following single or repeated administrations to healthy subjects and those with type-2 *diabetes mellitus*. Many of the studies also included endpoints to assess effects on blood pressure, and some of the studies were specifically performed in hypertensive subjects.

In randomized, double-blind, placebo-controlled trials, subjects with type-2 diabetes characterized by insulin resistance, were assessed for their post-meal response to steviol glycoside-induced glucose stimulation. The administration of single doses of rebaudioside A (purity 97%) to men or women with normal glucose tolerance or with type-2 *diabetes mellitus* at levels up to 1000 mg/person/day did not acutely affect glucose homeostasis or blood pressure levels in individuals (Maki et al., 2009).

Similarly, the repeated administration of rebaudioside A (purity 97%) at 250 mg/person/day, 4 times a day to type-2 diabetes mellitus-afflicted adult men and women over the course of 16 weeks revealed no change in glucose homeostasis in these individuals (Maki et al, 2008a).

In addition, blood pressure endpoints were examined in individuals with low-normal and low systolic blood pressure after the oral administration of steviol glycosides. The oral intake of 250 mg rebaudioside A (purity 97%) per person 4 times a day during 4 weeks did not lead to alterations of blood pressure in healthy adults with normal or low-normal blood pressure, and no changes in resting, seated SBP, DBP, Mean Arterial Pressure, Heart Rate or 24-hour ambulatory blood pressures responses were observed (Maki et al., 2008b).

Summary

Steviol glycosides extracted from the plant *Stevia rebaudiana* Bertoni have been commercialized and used safely as a sweetener since the 1970's. See Carakostas et al. 2008.

Further study of the stevia leaf identified the two major sweetening steviol glycosides as stevioside and rebaudioside A, along with several rebaudiosides and minor glycosides. These findings allowed for subsequent production of high purity materials that could be tested in toxicology and clinical safety studies. Based on these additional safety studies, performed with highly purified extracts, the JECFA concluded that stevioside and rebaudioside A are not genotoxic *in vitro* and *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives *in vitro* is not expressed *in vivo*. In addition, results of a recent study have shown the absence of adverse effects of steviol glycosides when taken at doses of about 4 mg/kg bw/day, expressed as steviol, for up to 16 weeks by individuals with type 2 diabetes mellitus and in a study in individuals with normal and low-normal blood pressure for 4 weeks (Maki et al. 2008a and 2008b). In 2008, JECFA established an Acceptable Daily Intake for steviol glycosides of 0-4 mg/kg bw/day expressed as steviol, based on the No Observed Effect Level of 970 mg stevioside/kg bw/day derived from a 2 year study (Toyoda et al, 1997). JECFA has also established specifications to ensure that the material tested is representative of the material of commerce.

As of 6 July 2015, FDA has reviewed 36 GRAS Notices for stevia extracts and purified glycosides submitted since 2008, and issued "no questions" letters for all of them. These safety assessments are based both on the safety and clinical data considered most recently by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) in their (re-)evaluation of the safety of steviol glycosides for use as a sweetener in food and on the final Acceptable Daily Intake (ADI) that was established. GRAS notices

have notably been submitted and received letters of no objection for purified mixed steviol glycosides (min. 95% pure on a dry weight basis), but also more recently for a use of Rebaudioside A, Rebaudioside D (GRN 000456), Rebaudioside X (now known as Reb M)(GRN 000473 and GRN 000512) or enzyme-modified steviol glycoside preparations as a general purpose sweetener. Other regulatory bodies (e.g. European Food Safety Authority, Food Standards Australia New Zealand (FSANZ) and Health Canada) have also evaluated the safety of steviol glycosides and reached the same conclusions as JECFA (EFSA, 2010; FSANZ, 2008; Health Canada, 2012).

The absence of genotoxicity of rebaudioside A is corroborated by the results of a reverse mutation study and an *in vitro* micronucleus test conducted with DSM's Reb A. Mutagenic activity of DSM Reb A was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* (WP$_2$uvrA), up to concentrations of 5000 µg/plate in the absence and presence of S9-mix (Ames et al. 1973). DSM's Reb A did not induce a significant dose-related increase in the number of revertant (His$^+$) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp$^+$) colonies in the tester strain WP$_2$uvrA both in the absence and presence of S9-metabolic activation, which was also confirmed in a follow-up experiment. It was therefore concluded that DSM's Reb A is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

An *in vitro* micronucleus assay with DSM's Reb A was also conducted in cultured peripheral human lymphocytes in the presence and absence of a metabolic activation system. The possible clastogenicity and aneugenicity of DSM's Reb A was tested in two independent experiments. In the first cytogenetic assay, it was tested up to the recommended dose level of 5000 µg/ml for a 3 hours exposure time with a 27 hours harvest time in the absence and presence of S9-fraction, while in the second cytogenetic assay, it was again tested up to 5000 µg/ml for a 24 hours exposure time with a 24 hours harvest time in the absence of S9-mix. The number of mono- and binucleated cells with micronuclei found in the solvent control cultures was within the laboratory historical control data range, and the number of mono- or binucleated cells with micronuclei was statistically significantly increased in the positive control chemicals used during the assay. It was therefore concluded that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly. DSM's Reb A did not induce a statistically significant or biologically relevant increase in the number of mono- and binucleated cells with micronuclei in the absence and presence of S9-mix, in either of the two experiments. It was concluded that this test is valid and that DSM's Reb A is not clastogenic or aneugenic in human lymphocytes under the experimental conditions tested.

Steviol and kaurenoic acid are intermediates in the biosynthetic pathway of steviol glycosides and may therefore constitute impurities in DSM's Reb A. 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 requested a third party review using the expertise of an independent laboratory, Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO), to assess the genotoxic and

carcinogenic potential of kaurenoic acid. TNO concluded that the DNA damage observed in presence of kaurenoic acid is likely the result of artifacts due to DNA fragmentation as a result of apoptosis and should therefore not be considered the result of a mutagenic mode of action. These effects were therefore considered to be a threshold-related effect. In addition, no indication of carcinogenicity of kaurenoic acid could be found in literature. Despite the presence at a low level of steviol and kaurenoic acid in DSM's Reb A (5 to 12 ppm kaurenoic acid and 3 to 15 ppm steviol), a reverse mutation study and an *in vitro* micronucleus test conducted with DSM's Reb A have shown the absence of genotoxicity of DSM Reb A, which further corroborates the absence of genotoxicity. By considering the ADI of 4 mg steviol equivalents/kg bw/day established by JECFA – equivalent to 12.1 mg DSM Reb A/kg bw/day[3], the maximum level of kaurenoic acid acceptable in DSM's Reb A was determined by TNO as being 124 mg kaurenoic acid/kg DSM's Reb A (124 ppm). A similar level can be defined for steviol: 124 mg steviol/kg DSM's Reb A. The results of analyses performed on three pilot batches of DSM Reb A have revealed levels well below these acceptable limits, as levels of 5 to 12 ppm kaurenoic acid were detected, and levels of 3 to 15 ppm steviol were reported. The batch from the Capua facility had a steviol concentration of 100 ppm, which was 20% below the acceptable maximum. For comparison, the analysis of commercially available samples of stevia extracts has also revealed the presence of steviol at levels of 1 to 40 ppm, and of kaurenoic acid below 0.3 ppm.

The safety of DSM's Reb A is supported by the both the published literature, FDA's review of 36 steviol glycoside GRAS Notices and the safety studies of DSM. Specifically, DSM's Reb A that is the subject of this safety assessment is manufactured following cGMP to a purity that meets both the FCC and JECFA specifications and therefore would not be expected to present a risk different from other commercial Reb A products. DSM's Reb A elicits no genotoxicity or chronic toxicity concerns, is 200 – 300 times sweeter than sucrose and the use of which would not alter the exposure of the consuming public. Therefore DSM's Reb A is safe for use as a general purpose sweetener.

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[3] Conversion from Reb A and steviol of 0.33% calculated based on the ratio of molecular weights of rebaudioside A (967.01 g/mol) and steviol (318.45 g/mol)

Conclusion of the Expert Panel

We, the members of the Expert Panel, have independently and collectively, critically evaluated the data and information summarized above and conclude that the proposed uses of DSM'S Reb A, rebaudioside A (Reb A) used as a general purpose sweetener in food, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food grade specifications as described in the GRAS dossier, are safe.

We further conclude that the proposed uses of rebaudioside A (Reb A), manufactured consistent with current Good Manufacturing Practice and meeting appropriate food specifications as described in the dossier, are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

(b) (6)

07 December 2015
Date

Stanley M. Tarka, Jr., Ph.D.
Panel Chairman

(b) (6)

December 3, 2015
Date

Palma Marone, Ph.D., E.R.T.

(b) (6)

Dec 4, 2015
Date

John Thomas, Ph.D, D.A.B.T.

(b) (6)

12/2/2015
Date

José Avalos, Ph.D.
Princeton University

Annexes -

Annex 1

ATCC Strain Data

Stevia Fermentation Ingredients List

Raw material	Grade
Glucose syrup @70 and 55% w/w*	FG
Glycerol Kosher	USP/EP
Yeast extract Expressa 2200S	FG
KH_2PO_2	FG
$MgSO_4 . 7H_2O$	FG
$(NH_4)_2SO_4$	FG
Thiamine chloride hydrochloride (vitamin B1 hydrochloride)	FG
Citric acid	FG
$ZnSO_4.7H_2O$	RG
$MnSO_4.H_2O$	FG
$CuSO_4.5H_2O$	USP
$Na_2MoO_4.2H_2O$	EP
$CaCl_2.2H_2O$	FG
$FeSO_4.7H_2O$	EP
KI	USP
Antifoam	FG
Titrant H_2SO_4 [96%/98%]	FG
Titrant Ammonia [28%]	FG
Titrant NaOH [25%]	FG
Potable water	-

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

Annex 2

Fermentation Media

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 ^{0}C per min.) to reduce cell damage to a minimum. The deep-frozen vials are stored at minus 75 ^{0}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 ^{0}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 3

Specification Sheet Reb A

Product Specification Sheet

STEVIOL GLYCOSIDES, REB-A 95%

Product number:

Issue date: 21-01-2016

Physical properties

Description	Steviol glycosides, fermentative Rebaudioside-A (Reb-A) 95% is a dry crystalline powder and used as food additive sweetener.

Appearance	Off-white to white powder
Odor	sweet aromatics
Moisture content by loss on drying	≤ 6%
Optical rotation	-29 to -37 degrees
Ash	≤ 1%
Particle size #80 Mesh	>99%
Solubility in purified water at room temperature (20°C)	Freely soluble in water

Chemical properties

Rebaudioside A (on dry basis)	≥ 95 %
Total steviol glycosides (on dry basis)	> 95 %
Residual solvents (Ethanol)	<5000 ppm
Residual solvents (Methanol)	<200 ppm
pH (1 gram dissolved in 100 ml 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
Enterobacteriaceae	≤ 100 CFU in 1 g

Storage

Steviol glycoside, Reb A 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 2 years.

Regulatory information

Steviol glycosides, Reb A 95% (E960 and INS960) manufactured from Stevia leaves are approved in most countries as a food additive (sweetener). DSM has started the approval processes for the fermentative manufacturing. GRAS self-affirmation was completed in 2015. Also a dossier was submitted to Codex-JECFA in 2015. Submission of a GRAS notice (USA) and an additive dossier (EU) are planned for Q1 2016.

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Annex 4

Capua Batch Data

Capua Batch Data Lot NBK-5203-011-076

Parameter	Method	Specification	Result	Pass?
purity > 95% (on dry basis)	FCC LC(C18)/UV	" => 95%	96.90%	y
total steviol glycosides (on dry basis)	FCC LC(C18)/UV	> 95%	99.30%	y
< 2% stevioside (on dry basis)	FCC LC(C18)/UV	max 2 %	<2	y
<10 ppm KA(on dry basis)	LC-MS	10 ppm	<10 ppm	y
moisture by loss on drying	TGA	max 6%	<6%	y
optical rotation	Geleen	"-29 to -37 degrees	ND	-
pH	FCC	4.5-7.0	6.4	Y
arsenic	ICP optisch	max 1 mg/kg	0.02 mg/kg	Y
lead	ICP optisch	max 1 mg/kg	0.2 mg/kg	Y
mercury	ICP optisch	max 1 mg/kg	0.02 mg/kg	Y
cadmium	ICP optisch	max 1 mg/kg	0.01 mg/kg	Y
aerobic plate	DSM SLD method	max 1000CFU/g	<5	y
aerobic mold	will not be performed	max 100CFU/g	10	y
aerobic yeast	DSM SLD method	max 100CFU/g	<10	y
coloform	DSM SLD method	max 10CFU/g	<3	y
E.coli	DSM SLD method	< 3 MPN/g	absent	y
solubility	3 g/l	0.3%	3	y
Ash	TGA	max 1 %	0.9	y
residual solvents	GC-FID	etoh < 5000ppm	1400 ppm	Y
DNA	qPCR		NA	-

Annex 5

rDNA detection in

Reb A batches

Proof of absence of rDNA in Rebaudioside A (RebA) produced with a genetically modified strain of *Yarrowia lipolytica* STV2050

Four samples derived from separate pilot fermentations (samples FG, BPF1, BPF2 and BPF3) as well as one sample from the tox batch (sample TOX) were used to analyse the presence of recombinant DNA. Fermentation and recovery were performed according to the production process described in the dossier.

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

Of each RebA sample, 100 mg was weighed on an analytical scale into a DNA LoBind Tube 1.5 ml (Eppendorf). The samples were dissolved in 1 ml milli-Q water. To 50 µl of the dissolved RebA sample or milli-Q in a DNA LoBind Tube 1.5 ml (Eppendorf), 50 µl of a 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0 ng/µl solution of *Yarrowia lipolytica* STV2050 genomic DNA was added, resulting in a proportion of 100, 10, 1, 0.1, 0.01, 0.001 and 0 ng genomic DNA per g RebA. 2 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 extracted 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 size
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	2 µl	2 µl
GC buffer 5x	5 µl	
HF buffer 5x		5 µl
Primer start	1.25 µl	1.25 µl
Primer end	1.25 µl	1.25 µl
DMSO	0.75 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	14 µl	14.75 µl

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Proof of absence of rDNA in Rebaudioside A (RebA) produced with a genetically modified strain of *Yarrowia lipolytica* STV2050

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

The results of the experiments to detect recombinant DNA in the actual RebA batches FG, BPF1, BPF2 and BPF3 are shown in Figures 1 (UGT2) and 2 (CPS).

As is shown in the milli-Q panels of Figures 1 and 2 below, the sensitivity is significantly higher for the UGT2 assay than for the CPS assay. The UGT2 assay is able to detect DNA concentrations as low as 0.01 ng/g RebA, while the CPS assay is able to detect DNA concentrations of 1 ng/g RebA.

As can also be seen in Figures 1 and 2, no rDNA was detected in any of the RebA batches tested. The UGT2 assay, which is more sensitive and has a detection limit of around 0.01 ng/g RebA, does not detect any rDNA in any of the five RebA samples. This implies that rDNA is present, if at all, at a concentration of <0.01 ng/g RebA in all five RebA samples.

Proof of absence of rDNA in Rebaudioside A (RebA) produced with a genetically modified strain of *Yarrowia lipolytica* STV2050

Figure 1: PCR analysis for UGT2 rDNA. Samples or milli-Q water were spiked with:

- Lanes A   100 ng/ml spiked DNA
- Lanes B   10 ng/ml spiked DNA
- Lanes C   1 ng/ml spiked DNA
- Lanes D   0.1 ng/ml spiked DNA
- Lanes E   0.01 ng/ml spiked DNA
- Lanes F   0.001 ng/ml spiked DNA
- Lanes G   RebA sample only (no DNA spiking)

M = DNA size marker

Expected UGT2 product size = 1.4 kb

Proof of absence of rDNA in Rebaudioside A (RebA) produced with a genetically modified strain of *Yarrowia lipolytica* STV2050

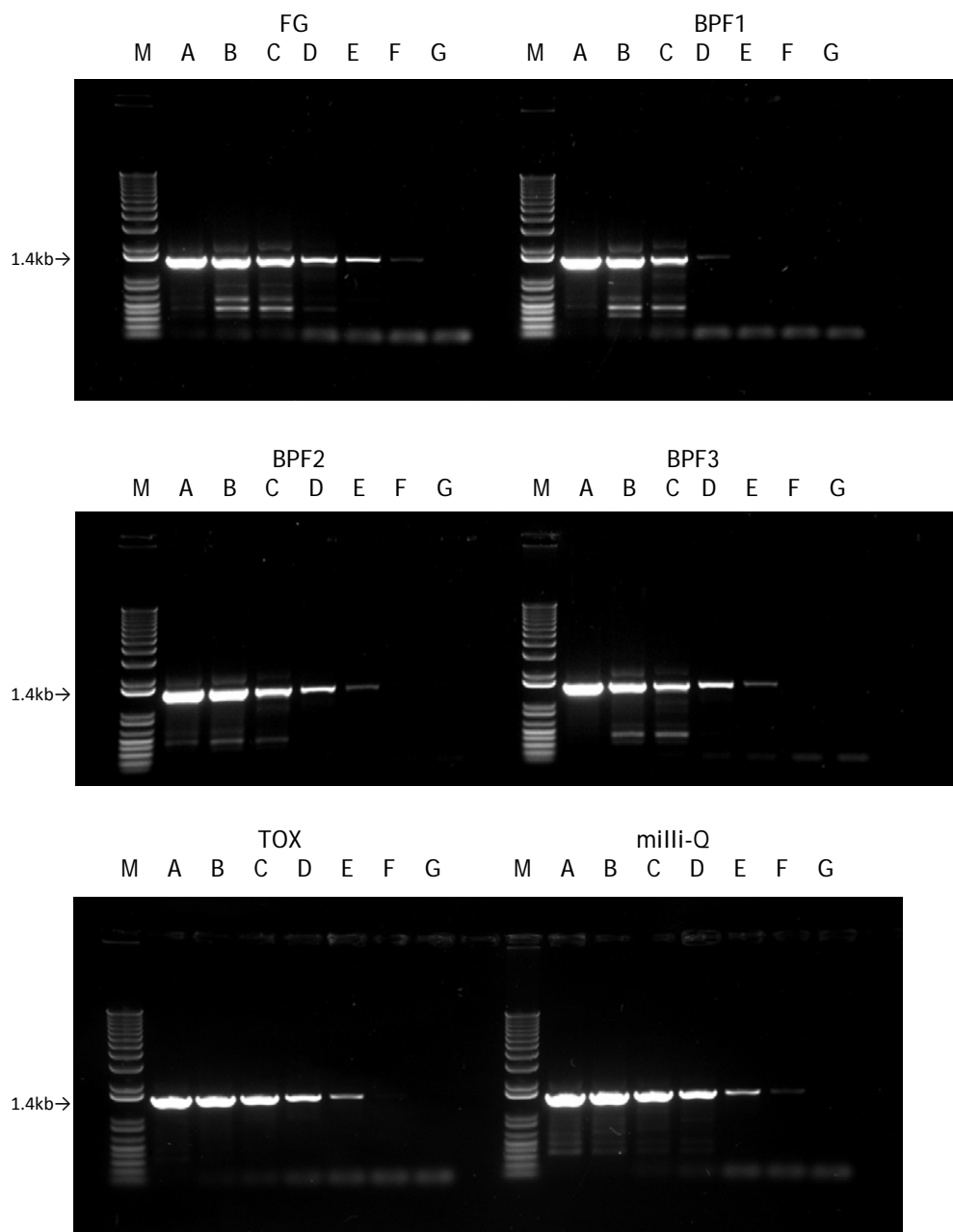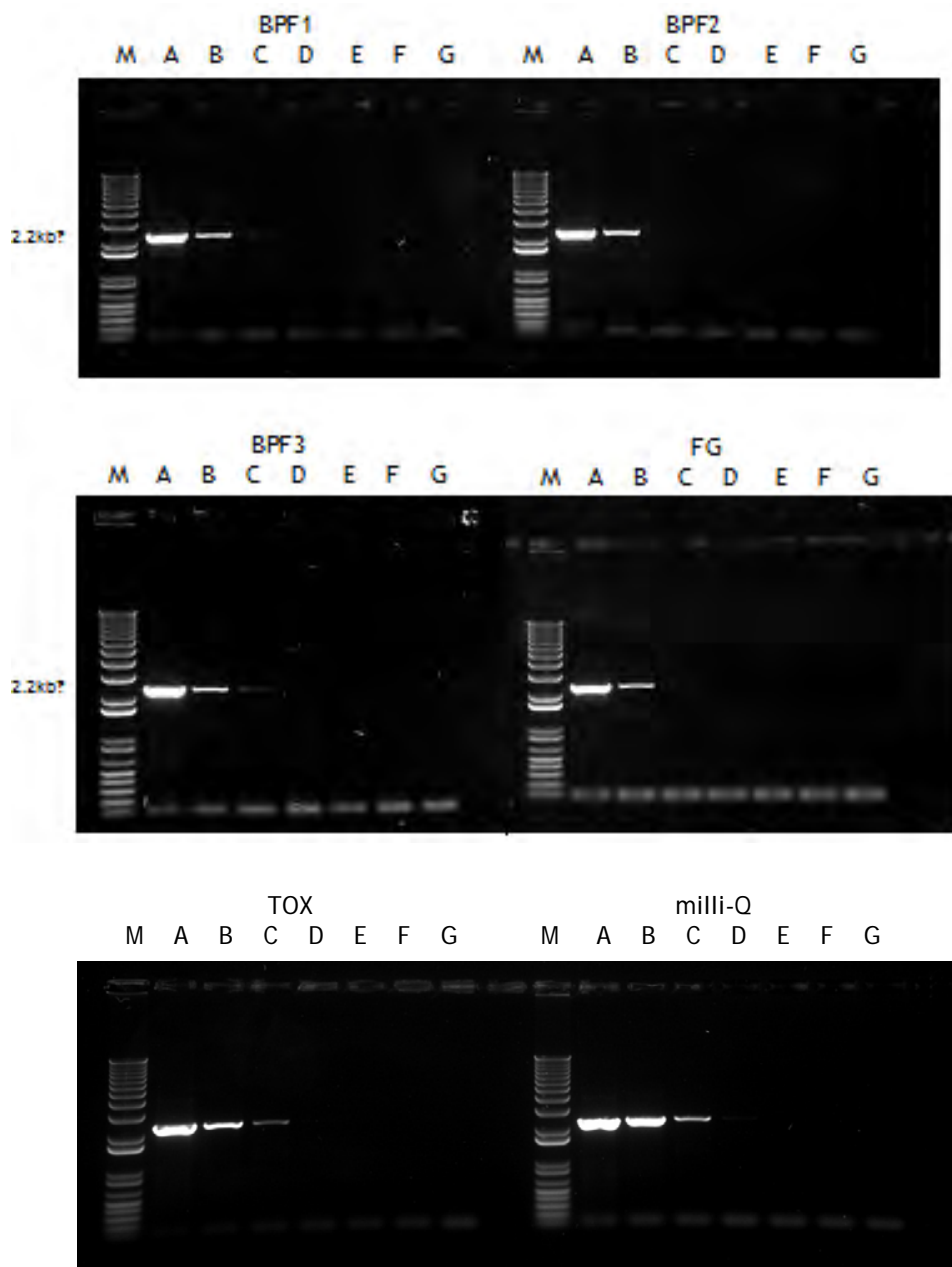

Figure 2: PCR analysis for CPS rDNA. Samples or milli-Q water were spiked with:

- Lanes A   100 ng/ml spiked DNA
- Lanes B   10 ng/ml spiked DNA
- Lanes C   1 ng/ml spiked DNA
- Lanes D   0.1 ng/ml spiked DNA
- Lanes E   0.01 ng/ml spiked DNA
- Lanes F   0.001 ng/ml spiked DNA
- Lanes G   RebA sample only (no DNA spiking)

M = DNA size marker

Expected CPS product size = 2.2 kb

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References

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

Annex 6

Shelf life test Reb A

Shelf life Study Rebaudioisde A: started 25-03-2015

Parameters/ methods no.	Limits Release / end of shelf specs.	Condition	Temperature	0	1 Month	2 Months	3 Months	6 Months
				%	%	%	%	%
		D-number		D33369	D33477	D33565	D33660	
Reb A analyse *	≥95%	1	20 C	98.1	97.0	100.3	98.8	96.2
		2	40C	97.7	98.0	99.4	95.9	94.8
				%	%	%	%	%
		C-number		c35250	c35308	c35387	c35432	C35608
Moisture **	<6%	1	20 C	0.21	3.9	3.6	4.0	5.7
		2	40C	0.44	0.7	6.1	6.1	6.6
X-rite ***	a/b/L	1	20 C	-1.3/2.5/86.7				
A1882 v3	a/b/L	2	40C	-1.2/2.6/85.8				
AW****	< 50%	1	20 C	17.5				
A10054 v1		2	40C	17.6				
		1	20 C	5.99	5.44	5.18	5.34	5.16
pH*****	4.5 – 7.0	2	40C	5.80	5.25	5.14	5.25	5.08

Annex 7

DSM Reb A

Sweetness Analysis

DSM
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Memo

DSM Nutritional Products, LLC

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United States of America

phone +1 800 526 0189
fax +1 973 257 8675

Date
June 15, 2015

From   To   cc
Todd Katz   Jim La Marta

Sensory Spectrum Reb A Sensory Analysis

Samples of unflavored acidified water, lemon tea, orange and pomegranate still beverages and a cola flavored, carbonated beverage were prepared in the DSM Application Labs located in Parsippany, NJ and sent to Sensory Spectrum, Inc. for sensory analysis.

The still beverages were prepared assuming that the DSM Reb A had a sweetness of 250 times that of sucrose.

The cola beverage was a reduced calorie formulation that contained sugar and was designed to have a flavor and sweetness profile equivalent to a commercial 6 brix product, sweetened with high fructose corn syrup or sugar. Although the test beverage was developed based upon sweetness, when we calculated how much DSM Reb A was used, it was at a level equivalent to a 250 X sweetness when compared to sucrose.

Kind regards

High level results sensory application test

Vouvray project
Marieke Nijmeijer

April 30, 2015

HEALTH · NUTRITION · MATERIALS

Sensory results – Stevia in application

April 2015

- *Objective:* Understand the sweetness profile of DSM's high intensity sweetener versus two competitors – description of the flavor profile of 6 applications and their temporal sweetness profile

- *Results:*
  - In general, similar aromatic and mouthfeel profiles were revealed
  - DSM showed similar or slightly lower sweetness in less complex applications (acidified water and near water) and similar or slightly higher sweetness than competitors in more complex applications (except for lemon tea)
  - DSM had a slightly faster decline in sweetness intensity and a slightly less lingering sweetness than competitors, which can be regarded as positive
  - DSM is comparable or higher in sweetness quality* relative to competitors
  - Individual remarks:
    - DSM more mouth drying in acidified water (0.24 g/l) than both competitors
    - Competitor 2 (P) was slightly more bitter than DSM in acidified water (0.4 g/l) and had a hint of liquorice in near water
    - In both juices DSM and Competitor 2 (P) showed similar results, whereas Competitor 1 (T) had a different aroma balance

Sweetness in-mouth

- *Conclusion:* Although differences are found between DSM and its competitors, flavor profiles of the samples are relatively comparable
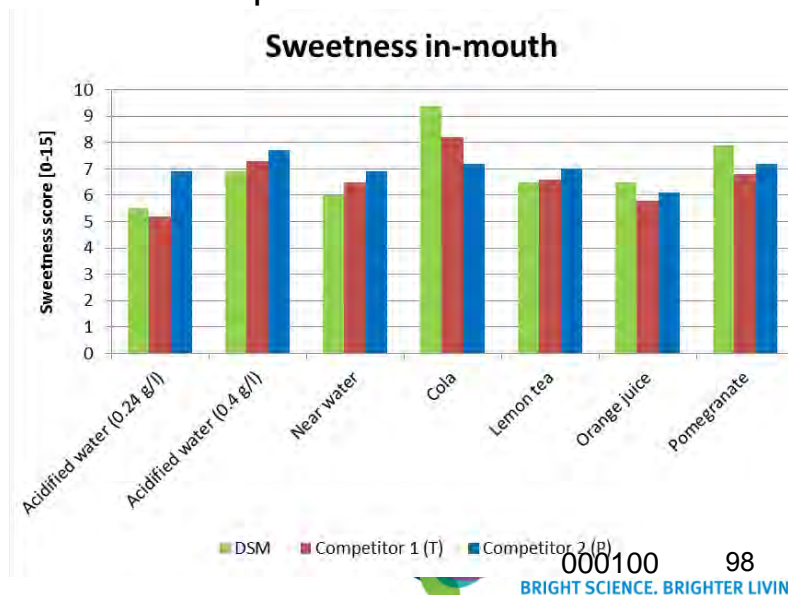
* Degree to which sweetness of sample matches sweetness impression of sugar (sucrose or HFCS)

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Annex 8

Toxicology studies

manuscript

Safety evaluation of Rebaudioside A produced by fermentation

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Abstract – 199 words

Abstract

The safety of rebaudioside A, produced fermentatively by *Yarrowia lipolytica* encoding the *Stevia rebaudiana* metabolic pathway (fermentative Reb A), is based on several elements: first, the safety of steviol glycosides has been extensively evaluated and an acceptable daily intake has been defined; second, the use of *Yarrowia lipolytica*, an avirulent yeast naturally found in foods and used for multiple applications; and third the high purity of fermentative Reb A and its compliance with internationally defined specifications. A bacterial reverse mutation assay and an *in vitro* micronucleus test conducted with fermentative Reb A provide evidence for its absence of mutagenicity, clastogenicity and aneugenicity. The oral administration of fermentative Reb A to Sprague-Dawley rats for at least 91 days did not lead to any adverse effects at consumption levels up to 2057 mg/kg bw/day for males and 2023 mg/kg bw/day for females, which were concluded to be the No Observed Adverse Effect Levels. The results were consistent with outcomes of previous studies conducted with plant-derived rebaudioside A, suggesting similar safety profiles for fermentative and plant-derived rebaudioside A. The results of the toxicity studies reported here support the safety of rebaudioside A produced fermentatively from *Yarrowia lipolytica*, as a general purpose sweetener.

Highlights:

• The safety of fermentative rebaudioside A (Reb A) was assessed. • Genotoxicity tests have shown the absence of mutagenicity of fermentative Reb A. • No adverse effects

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were observed in a 90 day oral toxicity study with fermentative Reb A. • Similar safety profiles were obtained for fermentative and plant-derived rebaudioside A. • The results support the safety of fermentative Reb A, as a general purpose sweetener.

Abbreviations:

ADI: Acceptable Daily Intake

EFSA: European Food Safety Authority

FCC: Food Chemical Codex

FDA: Food and Drug Administration

JECFA: Joint FAO/WHO Expert Committee on Food Additives

GRAS: Generally Recognized As Safe

NOAEL: No Observed Adverse Effect Level

NOEL: No Observed Effect Level

Reb A: Rebaudioside A

SCF: European Scientific Committee for Food

Y. lipolytica: *Yarrowia lipolytica*

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1. Introduction

Rebaudioside A extracted from the leaves of the plant *Stevia rebaudiana* Bertoni is one of the principal steviol glycosides widely used as a non-caloric sweetener in food and beverages. Rebaudioside A can also be produced fermentatively by a strain of *Yarrowia lipolytica (Y. lipolytica)*, genetically engineered to express the steviol glycoside metabolic pathway of the plant *Stevia rebaudiana*, and can be subsequently purified by crystallization to more than 95%.

The safety of rebaudioside A and other steviol glycosides extracted from plants has been extensively evaluated since the 1970's (Carakostas et al. 2008). The use of *Stevia rebaudiana* extracts containing mostly steviol glycosides has been authorized as food additives for many years in a number of South American and Asian countries such as Paraguay, Argentina, Brazil, South Korea, and Japan. The U.S. FDA (United States Food and Drug Administration) has reviewed 35 Generally Recognized As Safe (GRAS) Notices for steviol glycosides since 2008. However, as reported in 1999 and 2000, both the European Scientific Committee for Food (SCF) and the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) were not convinced of the safety of steviol glycosides, notably due to mutagenic effects of steviol glycosides reported *in vitro* in the presence of metabolic activation, and due to the paucity of *in vivo* data available at that time (SCF, 1999; JECFA, 2000). Later on, as purification of steviol glycosides improved, their safety was re-tested in several toxicology studies. Subchronic rat studies conducted with purified rebaudioside A or stevioside did not reveal toxic effects on any organs up to doses of 2500 mg/kg bw/day (Curry and Roberts, 2008; Nikiforov and Eapen, 2008; Aze et al., 1991; Geuns et al.,

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2003). Reproduction studies in rats also showed no effects of purified rebaudioside A on fertility and fetal development (Curry et al., 2008). Overall, the additional mutagenicity studies requested by the SCF did not show evidence of genotoxicity *in vitro* and *in vivo* (Brusick, 2008). In addition, results of a recent study in humans showed the absence of adverse effects of steviol glycosides when consumed at approximately 4 mg/kg bw/day, expressed as steviol equivalent, for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal and low-normal blood pressure for 4 weeks (Maki et al., 2008a Maki et al., 2008b). In 2008, JECFA established an Acceptable Daily Intake (ADI) for steviol glycosides of 0-4 mg/kg bw/day expressed as steviol using a 100-fold uncertainty factor (JECFA, 2008; 2009) based on a chronic (104 weeks) study in rats. This study, performed with stevioside, led to a No Observed Effect Level (NOEL) of 970 mg steviosides/kg bw/day, equivalent to 388 mg steviol equivalents/kg bw/day (Toyoda et al., 1997). JECFA has also established specifications to ensure that the material on the market is equivalent to the material tested in the safety studies.

For the production of rebaudioside A via fermentation, a strain of *Y. lipolytica* was used. *Y. lipolytica* is an avirulent oleaginous yeast species that is used for multiple industrial applications such as the production of citric acid, γ-decalactone, long-chain poly-unsaturated fatty acids and biodiesel fuel (Gonçalves et al., 2014; Zhu and Jackson, 2015). *Y. lipolytica* is naturally found in foods, primarily in foods with high proportions of fat and/or protein, such as in (fermented) dairy products and meat. This yeast is also used to produce food additives such as aroma compounds, organic acids, polyalcohols, emulsifiers, surfactants and carotenoids (Zinjarde, 2014; Zhu and Jackson, 2015). *Candida (=Yarrowia) lipolytica* was approved by the U.S. FDA as a secondary direct

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food additive for human consumption used for citric acid production and recognized as a nonpathogenic organism (21 CFR 173.165). More recently, the U.S. FDA also listed erythritol and eicosapentaenoic acid-rich triglyceride oil produced with *Y. lipolytica* on its inventory of Generally Recognized As Safe (GRAS) notifications for which it has no questions (GRN000382 and GRN000355). The safety of products obtained by fermentation of *Y. lipolytica* was extensively tested: acute (3-6 weeks), subchronic (90 days), chronic (1.5-2 years) and reproduction toxicity was assessed using rats and mice, guppies, chickens and quail. The safety of products obtained by fermentation of genetically engineered strains of *Y. lipolytica* was also assessed. The absence of genotoxic potential of eicosapentaenoic acid-rich triglyceride oil and beta-carotene produced by genetically engineered *Y. lipolytica* was shown in several genotoxicity tests, and no test substance-related adverse effects were observed in a 28-day oral toxicity study performed with eicosapentaenoic acid-rich triglyceride oil and in a 90-day oral toxicity study performed with beta-carotene (Belcher et al., 2011; Grenfell-Lee et al., 2014). A recent review of the safety of *Y. lipolytica* concluded that the yeast did not exert a harmful effect in rats at dietary levels up to 30% of dried biomass for 2 years and over 3 generations, and 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 (Groenewald et al., 2014). In a recent publication, microbiological data from clinical specimens collected over ten years were reviewed for growth of *Y. lipolytica*. It appeared that none of the patients who harbored *Y. lipolytica* had developed an infection despite the low immunity of most patients (Irby et al., 2014), which supports

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previous reports describing *Y. lipolytica* as a nonpathogenic yeast species. The high prevalence of *Y. lipolytica* reported in distal lung tissues even suggests that the yeast should be considered as normal human flora, especially of the adult respiratory tract (Irby et al., 2014). No reports exist on the production of substances by *Y. lipolytica* that are toxic in humans or animals, apart from its potential contribution to the formation of biogenic amines in cheese and meat, at concentrations not toxicologically relevant. *Y. lipolytica* was concluded to be ''safe-to-use'' (Groenewald et al., 2014).

Rebaudioside A is produced by a strain of *Y. lipolytica* genetically engineered to contain and express the steviol glycoside metabolic pathway, by using the technology described in the patent application WO2013/110673 filed in the name of DSM IP Assets B.V. To further support the use of rebaudioside A from *Y. lipolytica* (subsequently referred to as fermentative Reb A) as a food ingredient, the safety of this product was assessed by conducting *in vitro* genotoxicity studies and a 90-day subchronic oral toxicity study in rats. These studies are described in the present paper.

2. Materials and Methods

2.1 Test article preparation

Rebaudioside A (CAS No. 58543-16-1; molar mass: 967.01 g/mol; chemical formula: $C_{44}H_{70}O_{23}$) produced by fermentation using a genetically engineered yeast, *Y. lipolytica*, was obtained from DSM Food Specialties. After separation of the biomass from the supernatant by centrifugation and heat treatment, the process involved clarification, capture of rebaudioside A by chromatography, followed by elution with alcohol, purification and drying. Purified rebaudioside A was then isolated by

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crystallization, dried and packaged. The production process was performed following Good Manufacturing Practices, with appropriate controls of raw materials. The final rebaudioside A from *Y. lipolytica* met the specifications as outlined in the JECFA and Food Chemical Codex. The characteristics of the fermentative Reb A batches tested in the toxicity studies are shown in Table 1.

Table 1

Characteristics of fermentative Reb A batches 1 and 2 that were used respectively in the *in vitro* and *in vivo* toxicity studies.

Characteristic	Specification	Batch 1	Batch 2
Moisture by loss on drying (%)	≤ 6	Conform	Conform
Ash (%)	≤ 1	Conform	Conform
Solubility in water at RT (%)	> 0.3	Conform	Conform
Rebaudioside A (on dry basis) (%)	≥ 95	Conform	Conform
Total steviol glycosides (on dry basis) (%)	> 95	Conform	Conform
Residual solvents (Ethanol) (ppm)	< 5000	Conform	Conform
pH	4.5 – 7.0	6.2	6.4
Lead (ppm)	< 1	Conform	Conform
Mercury (ppm)	< 1	Conform	Conform
Cadmium (ppm)	< 1	Conform	Conform
Arsenic (ppm)	< 1	Conform	Conform
Total plate count (CFU/g)	≤ 1000	Conform	Conform

For the genotoxicity and 90-day subchronic toxicity studies, fermentative Reb A was provided as a white powder. The rebaudioside A content of the test article was > 95%. The test article was stored at room temperature, protected from light, and was considered stable under these conditions. The genotoxicity studies and 90-day subchronic

toxicity study were performed by WIL Research Laboratories, LLC ('s-Hertogenbosch, The Netherlands, Ashland, Ohio, U.S.A. and Hillsborough, North Carolina, U.S.A, respectively). These studies were performed in accordance with Good Laboratory Practice (GLP).

2.2. Bacterial reverse mutation assay (Ames test)

The mutagenic activity of fermentative Reb A was evaluated using the *Escherichia coli* strain WP$_2$ *uvrA* and *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, in the presence and absence of metabolic activation (S9-mixture). The tester strains were exposed to the test article (batch 1 of fermentative Reb A) according to the direct plate incorporation method. The experimental design followed the ''OECD Guideline for Testing of Chemicals – 471, Bacterial Reverse Mutation Test'' (OECD, 1997).

Bottom agar plates were made of minimal glucose agar that was based on a standard formula: 2% glucose (Fresenius Kabi, Bad Homburg, Germany) in Vogel-Bonner medium E and 1.8% purified agar (Merck, Darmstadt, Germany). The bottom agar plates for the tests with *Salmonella typhimurium* also contained 12.5 µg/plate biotin (Merck) and 15 µg/plate histidine (Sigma Aldrich, Steinheim, Germany) and the agar plates for the tests with the *Escherichia coli* strain contained 15 µg/plate tryptophan (Sigma Aldrich). Top agar was 0.6% bacteriological agar (Oxoid LTD), containing 0.5% NaCl (Merck). Liver microsomal 9000g fraction from liver homogenate (S9 fraction) of male Sprague–Dawley rats treated with Aroclor 1254 was used (Trinova Biochem GmbH, Giessen, Germany).

S9-mix was prepared immediately before use and kept on ice. S9-mix contained per 10 mL: 30 mg NADP (Randox Laboratories Ltd., Crumlin, United Kingdom) and 15.2 mg glucose-6-phosphate (Roche Diagnostics, Mannheim, Germany) in 5.5 mL or 5.0 mL Milli-Q water (first or second experiment, respectively) (Millipore Corp., Bedford, MA., USA); 2 mL 0.5 M sodium phosphate buffer pH 7.4; 1 mL 0.08 M MgCl2 solution (Merck); 1 mL 0.33 M KCl solution (Merck). The above solution was filter (0.22 µm)-sterilized. 0.5 ml S9-fraction was added to 9.5 mL of this solution to achieve the 5% (v/v) S9-mix used in the first experiment and 1.0 ml S9-fraction was added to 9.0 ml of this solution to achieve the 10% (v/v) S9-mix used in the second experiment.

The solvent used to dissolve the test article was DMSO (CAS No. 67-68-5) (SeccoSolv, Merck, Darmstadt, Germany); DMSO alone was therefore used as the negative control for this assay. Positive controls for experiments without S9 were aqueous solutions of sodium azide (CAS No. 26628-22-8) for TA1535; and DMSO solutions of 2-nitrofluorene (CAS No. 607-57-8) for TA98, methylmethanesulfonate (CAS No. 66-27-3) for TA100, ICR-191 (CAS No. 17070-45-0) for TA1537 and 4-nitroquinoline N-oxide (CAS No. 56-57-5) for WP$_2$ *uvrA*. For experiments with S9, 2-aminoanthracene (CAS No. 613-13-8) was dissolved in DMSO. All positive controls were purchased from Sigma Aldrich. Experiments were performed as described by Maron and Ames (1983).

Assays were performed in two independent experiments, using identical procedures, both with and without S9 metabolic activation. Fermentative Reb A was dissolved in DMSO and tested at concentrations of 0, 52, 164, 512, 1600 and 5000 µg per

plate in the first mutation assay and at concentrations of 492, 878, 1568, 2800 and 5000 µg per plate in the second mutation assay. Each concentration, including the controls, was tested in triplicate. The colonies were counted automatically with the Sorcerer Colony Counter (Perceptive Instruments, Bury St Edmunds, United Kingdom). For a test substance to be considered positive it had to generate at least a two-fold increase in the number of reversions (TA100) or at least a three-fold increase in the number of reversions (TA98, TA1535, TA1537 and WP$_2$ *uvrA*) and present a dose-dependent reproducible increase in the number of revertants. To determine the toxicity of fermentative Reb A, the reduction of the bacterial background lawn, the increase in the size of the microcolonies and the reduction of the revertant colonies were examined.

2.3. *In vitro* micronucleus assay in in cultured peripheral human lymphocytes

The potential for fermentative Reb A to induce micronuclei formation in cultured peripheral human lymphocytes was evaluated *in vitro*. The experimental design followed the ''OECD Guideline for the Testing of Chemicals – 487, *In Vitro* Mammalian Cell Micronucleus Test'' (OECD, 2014).

Material from batch 1 of fermentative Reb A was dissolved in DMSO (final concentration 1.0%, unless indicated otherwise), and cultured human lymphocytes were exposed to fermentative Reb A, in the presence and absence of metabolic activation. The liver microsomal fractions were obtained from male Sprague–Dawley rats orally dosed with phenobarbital and β-napthoflavone (Trinova Biochem GmbH, Giessen, Germany).

Cultures were treated with fermentative Reb A for 3 h with metabolic activation (S9-mix) and for 3 or 24 h without metabolic activation. The concentrations of

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fermentative Reb A investigated were 0, 512, 1600 and 5000 µg per mL. A concentration of 5000 µg/mL showed no precipitation in the culture medium and was therefore used as the highest concentration of fermentative Reb A in accordance with the OECD guidelines. Duplicate flasks were used for each dose level.

S9-mix was prepared immediately before use and kept on ice. S9-mix components contained per mL physiological saline: 1.63 mg $MgCl_2.6H_2O$ (Merck); 2.46 mg KCl (Merck); 1.7 mg glucose-6-phosphate (Roche, Mannheim, Germany); 3.4 mg NADP (Randox); 4 µmol HEPES (Life Technologies). The above solution was filter (0.22 µm)-sterilized. 0.5 mL S9-mix was added to 0.5 mL of this solution to yield a 50% (v/v) S9-mix. Metabolic activation was achieved by adding 0.2 mL S9-mix to 5.3 mL of a lymphocyte culture (containing 4.8 mL culture medium, 0.4 mL blood and 0.1 mL (9 mg/ml) phytohaemagglutinin). The concentration of the S9-fraction in the exposure medium was 1.8% (v/v).

All incubations were carried out in a controlled environment, in which optimal conditions were a humid atmosphere of 80 - 100%, containing $5.0 \pm 0.5\%$ CO2 in air in the dark at $37.0 \pm 1.0°C$. Lymphocyte culture (0.4 mL blood from a healthy male donor added, for experiments with and without metabolic activation, respectively to 5.0 or 4.8 mL culture medium [RPMI 1640 medium (Life Technologies), supplemented with 20% (v/v) heat-inactivated (56°C; 30 min) foetal calf serum (Life Technologies), L-glutamine (2 mM) (Life Technologies), penicillin/streptomycin (50 U/mL and 50 µg/mL, respectively) (Life Technologies) and 30 U/mL heparin (Sigma, Zwijndrecht, The Netherlands)], and 0.1 mL (9 mg/mL) phytohaemagglutinin (Remel Europe Ltd., Dartford, United Kingdom) were cultured for 46 ± 2 hours in the presence and absence of

S9 mix, and thereafter exposed to selected doses of fermentative Reb A. DMSO was used as negative control. Metabolic activation was achieved by adding 0.2 mL S9-mix to 5.3 mL of the appropriate (see above) lymphocyte culture (final S9 concentration (v/v): 1.8%).

Mitomycin C (CAS No. 50-07-7) (Sigma, Zwijndrecht, The Netherlands) was used as a clastogen, for cultures not treated with S9, at final concentrations of 0.25 µg/mL and 0.15 µg/mL, respectively, for the studies with 3-hour and 24-hour exposure periods. Colchicine (CAS No. 64-86-8) (Acros Organics, Geel, Belgium) was used as a aneugen for cultures not treated with S9 at final concentrations of 0.1 µg/mL and 0.05 µg/mL, respectively, for the studies with 3-hour and 24-hour exposure periods. Cyclophosphamide monohydrate (CAS no. 50-18-0) (Baxter B.V., Utrecht, The Netherlands) was used in all studies treated with S-9, at a final concentration of 15 µg/mL.

After 3 hours exposure to fermentative Reb A in the absence or presence of S9-mix, the cells were separated from the exposure medium by centrifugation. The supernatant was removed and cells were rinsed with 5 mL HBSS. After a second centrifugation step, HBSS was removed and cells were re-suspended in 5 mL culture medium with Cytochalasine B (5 µg/mL) and incubated for another 24 hours (1.5 times normal cell cycle). The cells that were exposed for 24 hours in the absence of S9-mix were not rinsed after exposure but were fixed immediately.

To harvest the cells, cell cultures were centrifuged and the supernatant was removed. Cells in the remaining cell pellet were resuspended in 1% Pluronic F68 (Applichem, Darmstadt, Germany). After centrifugation, the cells in the remaining pellet

were swollen by hypotonic 0.56% (w/v) potassium chloride (Merck) solution. Immediately after, ethanol (Merck): acetic acid (Merck) fixative (3:1 v/v) was added. Cells were collected by centrifugation and cells in the pellet were fixated carefully with 3 changes of ethanol: acetic acid fixative (3:1 v/v). Fixed cells were dropped onto cleaned slides, which were immersed in a 1:1 mixture of 96% (v/v) ethanol (Merck)/ether (Merck) and cleaned with a tissue. Slides were allowed to dry and thereafter stained for 10 - 30 min with 5% (v/v) Giemsa (Merck) solution in Sörensen's buffer pH 6.8. Thereafter slides were rinsed in water and allowed to dry. The dry slides were automatically embedded in a 1:10 mixture of xylene (Klinipath, Duiven, The Netherlands)/pertex (Histolab, Gothenburg, Sweden) and mounted with a coverslip in an automated Coverslipper (Leica Microsystems B.V., Rijswijk, The Netherlands).

At least 1000 binucleated cells per culture were examined by light microscopy for micronuclei. In addition, at least 1000 mononucleated cells per culture were scored for micronuclei separately. For the test substance to be considered mutagenic a significant dose-related increase in the number of structural chromosomal aberrations is required, and both biological and statistical significance is considered. Test substance significance was established at $p < 0.05$.

2.4. The 90-day subchronic toxicity study in rats

The 90-day toxicity study design was based on the principles of the current test guidelines for repeated-dose toxicity studies as issued by the U.S. FDA Redbook 2000 (US FDA, 2000) and OECD 408 (OECD, 1998).

2.4.1. Preparation of test diets

For the control group, an appropriate amount of basal diet was weighed into a properly labeled storage bag on a weekly basis. The test diets were prepared approximately weekly and stored at room temperature. For the test diet groups, an appropriate amount of test article (batch 2 of fermentative Reb A) was broken up using mortar and pestle and added to a portion of the PMI Nutrition International LLC Certified Rodent LabDiet 5002 (meal), and mixed with a medium Hobart mixer for 3 minutes. The resulting premix was then mixed thoroughly with the remaining amount of feed in a large Hobart mixer for 10 minutes. Concentrations were prepared from the lowest to highest concentration. The initial diet concentrations were based on average food consumption and body weights during the pretest period. Test article concentration in the diet was adjusted as necessary based on the mean body weight and food consumption for each group (by sex) to maintain the appropriate target dosage. The test diets were not adjusted for purity of the test article. Stability of the test article in diet at concentrations ranging from 1000 to 55,000 ppm at controlled room temperature for up to 10 days of storage was confirmed previously. Homogeneity of the test article as well as precision and accuracy of the concentrations prepared in the diet were also confirmed during the in-life phase of the 90-day toxicity study, by using a validated HPLC method using UV absorbance detection.

2.4.2. Animals and treatments

A total of 160 rats were used. The test article and control diets were offered ad libitum for 91, 92, or 93 consecutive days, until the day prior to the scheduled necropsy

to four groups of 40 rats (20 males, 20 females). The targeted dosages were 0, 500, 1000 and 2000 mg/kg body weight per day (groups 1, 2, 3, and 4, respectively). Animals in Group 1 received basal diet, which was used to formulate test article diets in Groups 2 through 4.

The weekly dietary inclusion rates at these dosage levels were expected to provide adequate exposure to the test article in all treated groups. These doses were supported by the outcome of a 13-week study (Nikiforov and Eapen, 2008) in which the no-observed-adverse-affect level (NOAEL) was identified as greater than 2000 mg rebaudioside A/kg/day. In this study, lower mean body weight gains were noted in males in the 2000 mg/kg/day group. There were no other rebaudioside A-related changes. Animals used in this study were acclimatized for 2 weeks and treated and cared for in accordance with the guidelines recommended by in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011). The animal facilities at WIL Research are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The experimental protocol for treating the animals was approved by the Institutional Animal Care Committee of WIL Research. Rats used in this study were Sprague-Dawley (*Rattus norvegicus*), aged 5-6 weeks and were obtained from Charles River Laboratories, Inc., Raleigh, NC. Females were nulliparous and non-pregnant. Each animal was uniquely identified with a subcutaneous microchip (BMDS system) implanted in the dorsoscapular area. The rats were housed 4 per cage by sex in solid bottom cages containing ground corncob bedding material (Bed O'Cobs®, The Andersons, Cob Products Division, Maumee, OH) under controlled conditions: temperature of 21.2-21.6°C, relative humidity of 44.1–63.5%, a minimum of 10 fresh air

changes per hour, and a 12-hour light and 12-h dark cycle. Reverse osmosis-treated (on-site) drinking water, delivered by an automatic watering system, and the basal or test diets were provided *ad libitum* throughout the study, except during the period of fasting prior to clinical pathology blood collection when food, but not water, was withheld, or during the conduct of functional observational battery (FOB) and motor activity (MA) assessments, when animals did not have access to diet. All available rats were weighed and examined in detail for physical abnormalities during acclimation. The animals judged suitable for assignment to the study were selected for use in a computerized randomization procedure based on body weight stratification in a block design. These animals were then randomized into 4 study replicates to allow for the reasonable conduct of the FOB and MA assessments. Each dose group and sex were approximately equally represented within each study replicate. The animals were approximately 7 weeks old at the initiation of dose administration. Individual body weights ranged from 160 g to 196 g for males and from 134 g to 168 g for females at randomization.

2.4.3. In-life data

All animals were observed twice daily for mortality and moribundity throughout the study. Clinical examinations were performed once daily during the study period, and detailed physical examinations were conducted on all animals 1 week prior to randomization, on the day of randomization, weekly during the study period, and on the days of the scheduled necropsy. Individual body weights were recorded 1 week prior to randomization, on the day of randomization, on study day 0 (prior to basal or test diet administration), weekly during the study period, and on the day prior to the first day of

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the scheduled necropsy (nonfasted). Final body weights (fasted) were recorded prior to the scheduled necropsy. Cage food weights were recorded weekly, beginning following randomization throughout the study period. The mean amounts of fermentative Reb A consumed (mg/kg bw/day) by each sex per dose group were calculated from the mean food consumed (g/kg bw/day) and the appropriate target concentration of test article in the food (mg/kg). Functional observation battery (FOB) and motor activity (MA) data were recorded for all animals during the final week of test diet administration (study week 12). FOB was performed in a sound-attenuated room equipped with a white-noise generator set to operate at 70 ± 10 dB. Motor activity, recorded after the completion of the FOB assessment was conducted using a personal computer-controlled system that utilizes a series of infrared photobeams surrounding an amber, plastic rectangular cage to quantify each animal's motor activity. Four-sided black plastic enclosures were used to surround the transparent plastic boxes and decrease the potential for distraction from extraneous environmental stimuli or activity by biologists or adjacent animals. The black enclosures rested on top of the photobeam frame and did not interfere with the path of the beams. The motor activity assessment was performed in a sound-attenuated room equipped with a white-noise generator set to operate at 70 ± 10 dB. Each animal was tested separately. Data for ambulatory and total motor activity were collected. Total motor activity was defined as a combination of fine motor skills (*i.e.*, grooming, interruption of 1 photobeam) and ambulatory motor activity (interruption of 2 or more consecutive photobeams). Ophthalmic examinations using an indirect ophthalmoscope and slit lamp biomicroscope preceded by pupillary dilation with an appropriate mydriatic agent were

performed during the acclimation period and near the end of the dosing period (study week 12).

2.4.4. Clinical pathology

Blood samples for hematology and serum chemistry evaluations were collected from 10 animals/sex/group at the end of the second week (study week 1) and on study day 45 (study week 6) from the jugular vein, and blood and urine samples for hematology, coagulation, serum chemistry and urinalysis evaluations were collected from 10 animals/sex/group on the days of the scheduled necropsy (study week 13). The animals were fasted overnight prior to blood collection while in metabolism cages for urine collection, when applicable. On the day of the scheduled necropsy (study week 13), animals were sent to necropsy and placed in a holding room for at least 1 hour to acclimate the animals and minimize stress. Blood was collected within a 3-hour time frame from the vena cava of animals anesthetized by inhalation of isoflurane. Blood was collected into tubes containing potassium (K_2) EDTA (hematology), sodium citrate (coagulation), or no anticoagulant (serum chemistry). Routine clinical pathology investigations were performed as summarized in Tables 4-7. The urine samples were analyzed for specific gravity, pH, urobilinogen, total volume, color, clarity, protein, glucose, ketones, bilirubin, occult blood, leukocytes, nitrites, and sediment microscopy. Hematology parameters were analyzed using an Advia 120 instrument (Siemens Healthcare Diagnostics, Inc), serum chemistry parameters were analyzed using Advia 1800 (Siemens Healthcare Diagnostics, Inc), coagulation parameters were analyzed using

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Stago STA compact (Diagnostica Stago, Inc) and urinalysis using Clinitek Atlas (Siemens Healthcare Diagnostics, Inc).

2.4.5. Pathology

At the time of the scheduled necropsy on study days 91-93, a complete necropsy was conducted on all animals following blood and urine collections for clinical pathology. Animals were anesthetized by isoflurane inhalation and euthanized by exsanguination. The necropsies included, but were not limited to, examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera. Organs that were collected and weighed included the adrenals, brain, epididymides, heart, kidneys, liver, ovaries with oviducts, pituitary, prostate, spleen, testes, thymus, thyroid with parathyroids (weighed after fixation), and uterus. Paired organs were weighed together, and absolute and relative weights (relative to final body weight and brain weight) were calculated. The following organs and tissues were also collected: aorta, bone with marrow (femur with joint, and sternum), brain, cervix, eyes with optic nerves, gastrointestinal tract (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum), harderian gland, larynx, lungs, lymph nodes (axillary, mandibular, and mesenteric), nasal cavity, pancreas, peripheral nerve (sciatic), Peyer's patches, pharynx, salivary glands (mandibular), seminal vesicles, skeletal muscle (rectus femoris), skin with mammary gland, spinal cord (cervical, thoracic, and lumbar), tongue, trachea, urinary bladder, vagina, and any gross lesions. Organs and tissues were fixed in 10% neutral buffered formalin, except for the eyes with optic nerves that were fixed in Davidson's solution, and epididymides and testes that were fixed in modified Davidson's solution.

Trimmed tissues were processed into paraffin blocks, sectioned, mounted on glass microscope slides, and stained with hematoxylin and eosin. Full histopathological examination was performed on tissues from control and high-dose animals, as well as on tissues with abnormal macroscopic findings from all dose groups.

2.4.6. Statistical analysis

Numerical data collected during the course of the study were subjected to calculation of group means, standard deviations and standard errors. Analyses were conducted using two-tailed tests for minimum significance levels of 1% and 5%, comparing each test article-treated group to the control group by sex. Body weight, body weight change, food consumption, continuous FOB, motor activity, clinical pathology, and organ weight data were subjected to a parametric one-way ANOVA (Snedecor and Cochran, 1980) to determine intergroup differences. If the ANOVA revealed statistically significant ($p < 0.05$) intergroup variance, Dunnett's test (Dunnett, 1964) was used to compare the test article-treated groups to the control group. Functional observational battery parameters that yielded scalar or descriptive data were analyzed using Fisher's Exact Test (Steel and Torrie, 1980). All repeated measures analysis of variance (RMANOVA) statistical analyses for total and ambulatory motor activity counts recorded prior to the initiation of dose administration and after dosing were conducted.

3. Results

3.1 Bacterial reverse mutation assay (Ames test)

A standard bacterial reverse mutation assay was used to determine the mutagenic potential of fermentative Reb A. The test substance was in the first mutation experiment tested at a concentration range from 52 to 5000 μg/plate in the absence and presence of 5% S9 mix. The numbers of revertant colonies on fermentative Reb A-treated plates were at levels similar to the corresponding negative controls for all test conditions, i.e. no biologically relevant increase in the number of revertant colonies was observed upon treatment with the test substance. In contrast, the included positive controls induced a significant increase in the number of revertant colonies. In the second mutation assay, fermentative Reb A was tested at a concentration range of 492 to 5000 μg/plate in the absence and presence of 10% (v/v) S9-mix. Like in the first experiment fermentative Reb A showed no biologically relevant increase in the number of revertant colonies. In addition, no cytotoxic effects were observed at any concentration when exposed to fermentative Reb A, both with and without incubation with S9, in any of the two experiments. Based on the two experiments it was concluded that the test conditions were valid and that fermentative Reb A was not mutagenic in the bacterial reverse mutation assay. See Table 2 for details.

Table 2

Summary of bacterial reverse mutation assay (Ames test) results

F RebA dose (µg/plate)	TA98 -S9	TA98 +S9	TA100 -S9	TA100 +S9	TA1535 -S9	TA1535 +S9	TA1537 -S9	TA1537 +S9	WP2uvrA -S9	WP2uvrA +S9
First mutation assay										
0	19	26	87	101	22	14	23	18	34	34
52	28	35	99	88	26	22	16	26	29	29
164	19	31	86	107	29	17	18	17	27	27
512	28	32	120	99	22	13	21	26	34	34
1600	19	31	112	105	25	15	21	26	32	32
5000	23	32	102	110	22	14	21	24	40	40
Positive control	1136	1499	867	1530	719	255	560	485	1523	217
Second mutation assay										
0	11	22	11	78	11	15	9	12	34	51
492	18	25	18	88	17	7	13	3	35	46
878	15	27	15	92	14	10	16	6	39	40
1568	15	28	15	91	16	13	8	9	34	40
2800	17	34	17	86	13	22	14	7	29	48
5000	22	22	22	88	12	14	7	7	39	36
Positive control	858	508	657	531	691	126	586	289	1388	277

Note: Data represent the mean of the revertant colonies of 3 plates per experiment. F RebA: fermentative Reb A.

3.2. *In vitro* micronucleus assay in cultured peripheral human lymphocytes

The potential for fermentative Reb A to induce micronuclei formation in cultured peripheral human lymphocytes was assessed. No cytotoxic effects were observed at any concentration when exposed to fermentative Reb A for 3 h, both with and without incubation with S9. When exposed to fermentative Reb A for 24 h in the absence of S9, there was a slight decrease (of 17%) in relative cell growth at 5000 µg/mL fermentative Reb A. The lower concentrations did not show any cytotoxicity. For all three experimental conditions, the number of mononucleated and binucleated cells with

micronuclei of human lymphocyte cultures treated with fermentative Reb A were similar to the negative control cultures. As demonstrated in Table 3, no relevant increases in the number of cells with micronuclei after treatment with any concentration of fermentative Reb A, either with or without metabolic activation, were observed. In contrast, positive control samples incubated with Mitomycin C, Colchicine or Cyclophosphamide monohydrate showed a significant increase in mononucleated or binucleated cells with micronuclei. Therefore it was concluded that the test conditions were valid and that fermentative Reb A was not clastogenic or aneugenic in the *in vitro* micronucleus assay.

Table 3

Summary of *in vitro* micronucleus assay results

Pooled data from two cultures of each 1000 cells. [1] 1.7% Dimethyl sulfoxide. *** Significantly different from control group (Chi-square test), $p < 0.001$. F RebA: fermentative Reb A, MMC-C: Mytomycin C, CP: Cyclophosphamide, Colch: Colchicine

3.3. The 90-day subchronic toxicity study in rats

3.3.1. In-life data

A 90-day subchronic toxicity study was performed in Sprague-Dawley rats for fermentative Reb A. All animals survived until the scheduled necropsy, and daily clinical

observations and weekly detailed physical examinations showed no test article-related systemic or local toxicity in groups dosed from 500 to 2000 mg/kg bw/day for a minimum of 91 consecutive days. Changes in body weight (Fig. 1), body weight gain and cumulative body weight gain were generally statistically significantly lower for males in the 2000 mg/kg bw/day group, compared to control At the end of the dosing period, the mean body weight in the 2000 mg/kg bw/day group males was 5.9% lower than the control group. Some statistically significant decreases in body weight were also observed in females in the 2000 mg/kg bw/day, throughout the course of the study, however, there was no significant difference observed in final body weight compared to female control body weight. There were no other test article-related effects on body weight. There were slight fluctuations in body weight gains beginning during study week 9 to 10 in the 500 and 1000 mg/kg bw/day group males; however, these were not considered test article-related due to a lack of a time-related trend. There were no test article-related effects on food consumption. However, some statistically significant differences were observed when the control and test article-treated groups were compared. Statistically significantly higher mean food consumption was noted in the 500 and 200 mg/kg bw/day group females from study week 4 to 5. Due to the lack of a dose-responsive trend, these differences were not considered test article-related. Average compound consumptions (mg/kg bw/day) were based on theoretical dietary concentrations of the test article and were 516, 1026, and 2057 mg/kg bw/day for males and 509, 1016, and 2021 mg/kg bw/day for females for 500, 1000, and 2000 mg/kg bw/day groups. Neurological evaluations conducted during the last week of dosing did not show test article-related differences between the control animals and animals in the test article-treated groups. A

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statistically significantly lower incidence of animals sitting or standing normally was noted in the 1000 mg/kg bw/day female group compared to the control group at the study week 12 evaluation primarily because of a higher number of animals that were asleep, lying on their side or curled up. However, this did not occur in a dose-related manner and there was no indication of lethargy,hypoactivity or impaired mobility in these same females during the open field observations. Therefore, the slightly lower number of females noted to be sitting or standing normally was not considered test article-related. There was a statistically significantly higher number of females in the 2000 mg/kg bw/day group that were very easy to remove from the cage at the study week 12 evaluation. The difference was not considered treatment-related. Frequently-handled animals are commonly easily to remove from the cage as evidenced by 100% of control males being found very easy to remove from the cage, and the lower number of control females found to be very easy to remove from the cage is lower than the historical control data. There were no ophthalmological findings that were considered test article-related.

3.3.2. Clinical pathology and pathology

Summaries of the hematology and coagulation parameters at the end of treatment are presented in Table 4 (males) and Table 5 (females). There were some statistically significant differences between the control and test article-exposed groups; however, none were considered test article-related. These differences included higher mean prothrombin (PT) times in the 500, 1000, and 2000 mg/kg bw/day group males and mean absolute lymphocyte count in the 2000 mg/kg bw/day group males at study week 13, lower mean absolute lymphocyte count in the 2000 mg/kg bw/day group females at study

week 1, and lower mean hematocrit in the 2000 mg/kg bw/day group females at study week 1, lower mean absolute reticulocyte count in the 2000 mg/kg bw/day group females at study week 13, and lower red cell distribution width (RDW) (%) in the 500 and 1000 mg/kg bw/day group females at study week 13. These were not considered test article-related because of lack of clear dose-response, lack of histologic correlates, changes not observed in the other sex or consistent among sexes, changes not observed in other intervals, and/or values were within the WIL Research historical control data ranges. The statistically significant higher PT in males (only) was also of small magnitude, and there were no statistically significant differences observed for other measures that could indicate a potential effect on blood clotting, e.g., no differences between treatment groups in measures of APTT, RBC and platelet count. Summaries of the serum chemistry parameters at the end of treatment are presented in Table 6 (males) and Table 7 (females). There were some statistically significant differences in serum chemistry parameters between the control and test article-exposed groups; however, none of the differences were considered test article-related. These differences included lower mean albumin and higher mean gamma glutamyl transferase (GGT) values in the 2000 mg/kg bw/day group males at study week 6 and lower mean urea nitrogen values in the 500 and 2000 mg/kg bw/day group females at study week 1. Both the albumin and GGT differences were considered spurious findings, as there was no temporal consistency in the findings and there was no effect in females at any time point. Lower urea nitrogen values were not noted in males and not noted with correlating urea nitrogen values, lacked clear dose-response, and had no histologic correlates. Urinalysis parameter results were unremarkable. The review of gross pathological findings (Table 8) and organ weights

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(Table 9) indicated that the treatments with fermentative Reb A did not induce any changes, and histopathological evaluation did not indicate any test-article related changes. All histologic changes were considered to be incidental findings or related to some aspect of experimental manipulations. There were no test article-related alterations in the prevalence, severity, or histologic character of those incidental tissue alterations.

Table 4

Hematology profile of male rats at the end of treatment

Data are means ± S.D. *Statistically significant difference from control group (p<0.05). **Statistically significant difference from control group (p<0.01). N = 10. WBC, white blood cells; RBC, red blood cells; Hbg, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PT, prothrombin time; APTT, activated partial thromboplastin time; LUC, large unstained cell; RDW, red cell distribution width; HDW, hemoglobin distribution width

Table 5

Hematology profile of female rats at the end of treatment

Data are means ± S.D. *Statistically significant difference from control group (p<0.05). **Statistically significant difference from control group (p<0.01). n = 10. WBC, white blood cells; RBC, red blood cells; Hbg, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PT, prothrombin time; APTT, activated partial thromboplastin time; LUC, large unstained cell; RDW, red cell distribution width; HDW, hemoglobin distribution width

Table 6

Serum chemistry profile for male rats at the end of treatment

	Group 1 Basal (0 mg/kg)	Group 2 Low dose (500 mg/kg)	Group 3 Mid dose (1000 mg/kg)	Group 4 High dose (2000 mg/kg)
Albumin (g/dL)	3.7 ± 0.2	3.8 ± 0.2	3.9 ± 0.3	3.7 ± 0.2
Total protein (g/dL)	6.4 ± 0.2	6.5 ± 0.4	6.7 ± 0.4	6.5 ± 0.3
Globulin (g/dL)	2.7 ± 0.1	2.7 ± 0.2	2.8 ± 0.2	2.7 ± 0.2
A/G ratio	1.41 ± 0.06	1.37 ± 0.05	1.41 ± 0.06	1.38 ± 0.11
Total bilirubin (mg/dL)	0.01 ± 0.03	0.01 ± 0.03	0.03 ± 0.05	0.00 ± 0.00
Urea nitrogen (mg/dL)	13.4 ± 1.0	12.6 ± 1.2	13.8 ± 1.8	13.3 ± 2.0
Creatinine (mg/dL)	0.30 ± 0.06	0.31 ± 0.06	0.36 ± 0.08	0.33 ± 0.07
ALP (U/L)	92 ± 17	89 ± 14	103 ± 26	95 ± 19
ALT (U/L)	46 ± 22	44 ± 9	37 ± 10	38 ± 9
AST (U/L)	93 ± 19	93 ± 14	83 ± 14	85 ± 13
GGT (U/L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Glucose (mg/dL)	154 ± 20	143 ± 26	151 ± 37	138 ± 22
Cholesterol (mg/dL)	55 ± 15	48 ± 8	56 ± 9	59 ± 25
Calcium (mg/dL)	10.9 ± 0.4	10.6 ± 0.4	10.8 ± 0.6	10.6 ± 0.4
Chloride (mEq/L)	107 ± 1	106 ± 1	106 ± 1	106 ± 1
Phosphorus (mg/dL)	8.2 ± 0.9	7.7 ± 0.7	8.2 ± 1.3	8.1 ± 0.9
Potassium (mEq/L)	5.22 ± 0.74	4.86 ± 0.53	5.54 ± 1.39	4.90 ± 0.51
Sodium (mEq/L)	147 ± 2	147 ± 2	147 ± 2	147 ± 1
SDH (U/L)	16 ± 13	21 ± 7	20 ± 6	19 ± 7
Triglyceride (mg/dL)	48 ± 20	40 ± 12	45 ± 11	41 ± 14

Data are means ± S.D. *Statistically significant difference from control group ($p<0.05$). **Statistically significant difference from control group ($p<0.01$). N = 10. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyltransferase; SDH, sorbitol dehydrogenase

Table 7

Serum chemistry profile for female rats at the end of treatment

	Group 1 Basal (0 mg/kg)	Group 2 Low dose (500 mg/kg)	Group 3 Mid dose (1000 mg/kg)	Group 4 High dose (2000 mg/kg)
Albumin (g/dL)	4.6 ± 0.3	4.5 ± 0.4	4.8 ± 0.4	4.7 ± 0.3
Total protein (g/dL)	7.7 ± 0.5	7.5 ± 0.5	7.8 ± 0.7	7.8 ± 0.6
Globulin (g/dL)	3.1 ± 0.3	3.0 ± 0.1	3.1 ± 0.3	3.1 ± 0.3
A/G ratio	1.50 ± 0.11	1.53 ± 0.08	1.54 ± 0.08	1.54 ± 0.07
Total bilirubin (mg/dL)	0.07 ± 0.05	0.06 ± 0.05	0.07 ± 0.05	0.10 ± 0.12
Urea nitrogen (mg/dL)	14.8 ± 2.1	14.4 ± 1.8	15.5 ± 2.8	14.5 ± 2.4
Creatinine (mg/dL)	0.42 ± 0.06	0.44 ± 0.07	0.46 ± 0.06	0.45 ± 0.07
ALP (U/L)	53 ± 21	51 ± 14	54 ± 18	63 ± 26
ALT (U/L)	45 ± 19	35 ± 12	27 ± 5	34 ± 20
AST (U/L)	104 ± 28	91 ± 34	82 ± 25	98 ± 41
GGT (U/L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Glucose (mg/dL)	148 ± 15	149 ± 33	153 ± 15	143 ± 25
Cholesterol (mg/dL)	81 ± 34	64 ± 13	59 ± 16	67 ± 14
Calcium (mg/dL)	11.7 ± 0.6	11.6 ± 0.6	11.9 ± 0.7	11.8 ± 0.7
Chloride (mEq/L)	105 ± 1	107 ± 1	105 ± 2	105 ± 2
Phosphorus (mg/dL)	8.3 ± 0.8	7.9 ± 0.7	9.0 ± 1.3	8.4 ± 1.3
Potassium (mEq/L)	6.27 ± 1.23	5.55 ± 0.60	6.91 ± 1.63	6.21 ± 0.96
Sodium (mEq/L)	145 ± 2	146 ± 1	146 ± 1	145 ± 2
SDH (U/L)	16 ± 6	14 ± 7	12 ± 3	18 ± 14
Triglyceride (mg/dL)	31 ± 6	30 ± 8	33 ± 9	29 ± 10

Data are means ± S.D. *Statistically significant difference from control group ($p < 0.05$). **Statistically significant difference from control group ($p < 0.01$). N = 10. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyltransferase; SDH, sorbitol dehydrogenase

Table 8

Incidence of gross necropsy findings

	Males		Females	
	Basal 0 mg/kg/day N = 20	High dose 2000 mg/kg/day N=20	Basal 0 mg/kg/day N = 20	High dose 2000 mg/kg/day N=20
Kidneys				
Examined	20	20	20	20
Within Normal Limits	20	20	20	18
Dilated Pelvis	0	0	0	2
Liver				
Examined	20	20	20	20
Within Normal Limits	20	19	20	20
Area(s), White	0	1	0	0
Lungs				
Examined	20	20	20	20
Within Normal Limits	19	19	20	20
Area(s), Dark Red	1	1	0	0
Thymus				
Examined	20	20	20	20
Within Normal Limits	17	19	18	20
Area(s), Dark Red	3	1	2	0
Ureters				
Examined	20	20	20	20
Within Normal Limits	20	20	20	19
Distended	0	0	0	1
Uterus				
Examined	NA	NA	20	20
Within Normal Limits	NA	NA	15	16
Contents, Clear Fluid	NA	NA	5	4

Only findings that were noted in the high dose group are mentioned in the table.

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Table 9

Organ/body weight ratios (mean ± SD, relative to final body weights)

	Group 1 Basal (0 mg/kg)	Group 2 Low dose (500 mg/kg)	Group 3 Mid dose (1000 mg/kg)	Group 4 High dose (2000 mg/kg)
Males				
Final body weight (g)	535 ± 35	526 ± 36	524 ± 39	505 ± 40
Adrenal glands	0.011 ± 0.001	0.011 ± 0.002	0.011 ± 0.001	0.012 ± 0.002
Brain	0.414 ± 0.025	0.416 ± 0.030	0.419 ± 0.037	0.431 ± 0.032
Epididymides	0.261 ± 0.016	0.269 ± 0.025	0.257 ± 0.025	0.269 ± 0.021
Heart	0.311 ± 0.025	0.338 ± 0.049	0.302 ± 0.035	0.332 ± 0.039
Kidneys	0.583 ± 0.043	0.598 ± 0.048	0.559 ± 0.034	0.589 ± 0.045
Liver	2.380 ± 0.130	2.341 ± 0.149	2.335 ± 0.215	2.454 ± 0.218
Pituitary	0.003 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.003 ± 0.000**
Prostate	0.215 ± 0.031	0.225 ± 0.041	0.204 ± 0.038	0.224 ± 0.055
Spleen	0.175 ± 0.020	0.169 ± 0.023	0.159 ± 0.020	0.179 ± 0.026
Testes	0.679 ± 0.067	0.698 ± 0.063	0.678 ± 0.066	0.703 ± 0.070
Thymus	0.043 ± 0.012	0.040 ± 0.013	0.048 ± 0.011	0.050 ± 0.011
Thyroid with parathyroids	0.003 ± 0.001	0.004 ± 0.001	0.003 ± 0.001	0.003 ± 0.001
Females				
Final body weight (g)	276 ± 18	275 ± 20	268 ± 27	268 ± 20
Adrenal glands	0.025 ± 0.004	0.027 ± 0.005	0.025 ± 0.003	0.025 ± 0.003
Brain	0.715 ± 0.060	0.726 ± 0.049	0.753 ± 0.067	0.720 ± 0.061
Heart	0.377 ± 0.037	0.355 ± 0.048	0.370 ± 0.034	0.357 ± 0.031
Kidneys	0.612 ± 0.046	0.621 ± 0.052	0.617 ± 0.049	0.598 ± 0.039
Liver	2.537 ± 0.208	2.571 ± 0.204	2.645 ± 0.244	2.673 ± 0.202
Ovaries with oviducts	0.048 ± 0.012	0.045 ± 0.007	0.050 ± 0.008	0.047 ± 0.006
Pituitary	0.007 ± 0.001	0.007 ± 0.002	0.007 ± 0.001	0.007 ± 0.001
Spleen	0.206 ± 0.038	0.202 ± 0.028	0.209 ± 0.033	0.205 ± 0.029
Thymus	0.094 ± 0.023	0.081 ± 0.013	0.081 ± 0.026	0.086 ± 0.014
Thyroid with parathyroids	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001
Uterus	0.236 ± 0.090	0.215 ± 0.057	0.262 ± 0.095	0.250 ± 0.088

N = 20. Results are presented as means ± S.D.

4. Discussion

The fermentative production of Rebaudioside A by a strain of *Yarrowia lipolytica*

is an alternative production method of rebaudioside A. It meets specifications for steviol glycosides defined by the FCC and JECFA and is therefore alike to commercially available products. For confirmation of the safety-in-use of fermentative Reb A, a standard battery of toxicity studies was performed with this product in order to assess if there is any potential to induce gene mutations and structural or numerical chromosome aberrations, and to test its subchronic toxicity in rodents. The results of the two *in vitro* genotoxicity studies (bacterial reverse mutation test and *in vitro* micronucleus test) confirmed that fermentative Reb A is neither mutagenic to *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2 uvrA, nor clastogenic or aneugenic in cultured peripheral human lymphocytes. The 90-day subchronic toxicity study conducted in rats did not reveal any adverse effects of fermentative Reb A at consumption levels up to 2057 mg/kg bw/day for males and 2023 mg/kg bw/day for females. No deaths were reported during the study. Clinical observations did not reveal any adverse effects and there were no between-group differences in either food consumption or clinical pathology parameters that were considered test-article-related. Similarly, no test-article-related ophthalmic, macroscopic or microscopic findings or changes in organ weights were found. Functional observational battery and motor activity assessments were unaffected by test article administration. The only observation that was considered possibly treatment-related was a slightly (up to ~6% by study end) decreased body weight in males in the high-dose (2000 mg/kg bw/day) group, compared to the body weight of males in the control group, which was not associated with a decrease in food intake. The lower mean body weights were not considered to be adverse. Because of the proportion of basal diet that was

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replaced with the test article containing little caloric value, the lower mean body weight gains may have been the result of the animals not consuming an equivalent number of calories as the control group. Additionally, the changes were not considered to be adverse due to the small magnitude of difference from the control group value. This finding and further absence of toxicity of the tested material is consistent with results of previous subchronic toxicity studies performed in rodents with high purity rebaudioside A and stevioside extracted from *Stevia rebaudiana* (Curry and Roberts, 2008; Nikiforov and Eapen, 2008; Aze et al., 1991). The oral administration of rebaudioside A (purity 99.5%) to Sprague-Dawley rats for 90 days up to doses of 2000 mg/kg bw/day did not lead to any adverse effects on clinical and functional observations, hematology, serum chemistry or urinalysis. No organ weight changes, macroscopic or microscopic tissue changes were attributed to the treatment (Nikiforov and Eapen, 2008). However, a slight decrease in food conversion efficiency was observed in the high-dose group for males only, associated with a decreased body weight gains (9.1% lower compared to the control group). Similar findings were also reported after administration of rebaudioside A (purity > 97%) to Wistar rats at concentrations up to 50,000 mg/kg diet (corresponding with 4161 and 4645 mg/kg bw/day for males and females) during 90 days, after which statistically significant decreases in body weight were observed in both sexes at 25,000 and 50,000 mg rebaudioside A/kg diet, particularly during the first days of the studies, while no clear differences in food consumption and limited effects on food conversion efficiency were reported (Curry and Roberts, 2008). The No Observed Adverse Effect Level (NOAEL) was concluded to be at the highest dose tested, i.e. 50,000 mg rebaudioside A/kg diet (Curry and Roberts, 2008). Although the administration of

stevioside (95.6% purity) to Fischer 344 rats up to 5% of their diet for 90 days did not lead to statistically significant difference in body weight gain or food intake between the control and treated groups, the terminal body weights were statistically significantly decreased in the female 2.5%-dose group and male and female 5%-dose group in comparison to the controls (Aze et al., 1991). These effects are also consistent with results of other studies conducted with intense sweeteners dosed at high level, with reported 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 a recent re-evaluation of steviol glycosides, JECFA did not consider these changes in body weight gain of toxicological significance and concluded that the decrease in body weight gain observed in rats given rebaudioside A for 90 days can be attributed to decreased caloric density of the diet (JECFA, 2009). Based on all the previous studies reported as discussed above, the decrease in body weight gain observed in the 90 day study conducted with fermentative Reb A is not considered an adverse effect, but likely an indirect effect related to the lower caloric density of the diet. Based on the overall results of the investigations reported here, it is concluded that the NOAEL for fermented Reb A is the highest dose tested, 2057 mg/kg bw/day for males and 2023 mg/kg bw/day for females. This NOAEL is equivalent to 679 mg steviol equivalent/kg bw/day for males and 668 mg steviol equivalent/kg bw/day for females[1]. During its sixty-third meeting, based on a review of biochemical data of the major steviol glycosides used as sweeteners, JECFA's Committee observed that steviol glycosides are poorly absorbed and are metabolized by the intestinal microflora by successive hydrolytic removals of glucose units, leading to

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[1] The conversion from rebaudioside A to steviol equivalent is made by using a factor 0.33 that is calculated based on the ratio of molecular weights of rebaudioside A (967.01 g/mol) and steviol (318.45 g/mol).

the formation of steviol, which is well absorbed. Since then, the toxicity of the steviol glycosides is expressed as steviol equivalent, based on the molecular weight of steviol and of the different steviol glycosides evaluated (JECFA, 2009).

The results of the toxicity studies described in this paper demonstrate that fermentative Reb A has a safety profile comparable to plant derived rebaudioside A, which can be explained by the high homology of these products and the absence of safety concerns for *Y. lipolytica*. The production of rebaudioside A by a genetically engineered *Y. lipolytica* can therefore be seen as a safe alternative source of rebaudioside A. In addition, fermentative Reb A will be metabolized in the same manner as plant-derived rebaudioside A, which is converted either to stevioside or to rebaudioside B before being metabolized to steviol (Koyama et al., 2003; Gardana et al., 2003). An Acceptable Daily Intake (ADI) of 4 mg/kg bw/day expressed as steviol equivalent was derived by JECFA for all steviol glycosides from the No Observed Effect Level (NOEL) of 388 mg steviol equivalents/kg bw/day obtained in a chronic (104 weeks) rat study performed with stevioside, and using a 100-fold uncertainty factor. This ADI is therefore applicable to fermentative Reb A as well.

Fermentative Reb A is intended to be used as general purpose sweetener. It is anticipated that fermentative Reb A will be used in a manner similar to that of other non-nutritive sweeteners, in consumer products such as beverages, dairy products, baked goods and confections. The daily intake of rebaudioside A and other steviol glycosides has been estimated by national and international food safety and regulatory agencies based on crude estimates, on more specific food consumption data, or on per capita estimates. In 2006, JECFA has done a conservative international estimate of human

exposure to steviol glycosides by using the database of the World Health Organization Global Environment Monitoring System - Food Contamination Monitoring and Assessment Programme (GEMS/Food), by assuming that steviol glycosides would replace all sweeteners used in or as food, which is very unlikely due to technical and sensorial barriers, and by using the minimum reported relative sweetness of steviol glycosides and sucrose of 200:1 (JECFA, 2006). JECFA came to an estimated intake of 1.3 to 3.5 mg/kg bw/day expressed as steviol equivalent. Estimates through food consumption in Japan, in the USA and in China were also calculated, based on annual production or annual demand data of steviol glycosides. Only the very conservative assumption that all added sugar in the diet in the USA would be replaced by steviol glycosides, at a ratio of 200:1 based on sweetness, led to estimates higher than the ADI of 4 mg steviol equivalent/kg bw/day (JECFA, 2006). This is very improbable because not every consumer is interested in consuming low or non-caloric sweeteners – Ng et al. 2012 calculated that only 6% of the products purchased from 2005 to 2009 in the USA contained non-caloric sweeteners, and there are several alternatives to Stevia among the sweeteners. 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 children and adults (EFSA, 2010). EFSA's exposure assessments of steviol glycosides were revised in 2011, 2014 and 2015 based on their up-to-date authorized uses and by using European consumption data. Although conservative estimates of exposure suggested in 2010 that the ADI of 4 mg steviol equivalent/kg bw/day would be exceeded both for adults and children at the maximum proposed use level (EFSA, 2010), revised exposure assessments concluded

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that the ADI is not likely to be exceeded for all population groups, with the exception of toddlers, and only at the upper range of the high-level exposure estimates (95th percentile: 4.3 mg/kg bw per day) (EFSA, 2014; 2015). Renwick (2008) used a different approach to estimate the human exposure to steviol glycosides, basing his calculation on the observed exposure data for aspartame and considering relative sweetness potencies in relation to sucrose, of 180 for aspartame and 200 for rebaudioside A. By using this approach, Renwick came to similar conclusions as EFSA since he predicted an average dietary exposure to rebaudioside A for children (aged 1-14 years), including diabetics, ranging from 0.4 to 1.3 mg steviol equivalents/kg bw/day, and from 1.5 to 4.2 mg/kg bw/day at the high percentile (90th/97.5th). For adults, the mean dietary exposure to rebaudioside A, expressed as steviol equivalents, including diabetics, ranged from 0.3 to 0.7 mg/kg bw/day, and from 1.5 to 3.1 mg/kg bw/day at the high percentile (90th/97.5th). The relative sweetness intensity of rebaudioside A generally ranges from 200-400 times that of sucrose. This is also the case for fermentative Reb A, as confirmed by sweetness profile comparisons of fermentative Reb A and plant derived rebaudioside A from available market samples where the relative sweetness of both plant derived rebaudioside A and fermentative Reb A was found to be between 200 and 300 times sweeter than sucrose. By using the highly improbable assumption that all consumer steviol glycosides would be replaced by fermentative Reb A, the approach used by Renwick would result in exposure values all within the ADI of 4 mg steviol equivalent/kg bw/day. Overall, it can be concluded that all approaches used to assess exposure to steviol glycosides lead to estimations within the ADI established by JECFA, or slightly above the ADI at the high level of the exposure estimate (typically 95th percentile of consumers only). Because

fermentative Reb A is equivalent in physical characteristics and sensorial properties to commercial plant-derived rebaudioside A products, there is no reason to believe that the fermentative material would have an exposure higher than the commercially available plant-extracted steviol glycosides.

5. Conclusion

The genotoxicity and subchronic toxicity of rebaudioside A from *Yarrowia lipolytica* was assessed by using accepted toxicity assays. The bacterial reverse mutation assay and the *in vitro* micronucleus test concluded the absence of mutagenicity, clastogenicity and aneugenicity of the tested product. The 90-day toxicity study did not result in any test-article related changes in clinical chemistry, hematology, coagulation, or urinalysis parameters, organ weights, nor macroscopic or microscopic pathology, and was therefore considered safe for rats under the experimental conditions. From the study, a NOAEL could be derived of at least 2000 mg/kg bw/day, or at least 660 mg steviol equivalent/kg bw/day, which was the highest dose tested. These results were consistent with outcomes of previous studies performed with plant-derived rebaudioside A, which suggests very similar safety profiles of fermentative and plant-derived rebaudioside A. The results of the toxicity studies reported here support the safety of fermentative Reb A produced from *Yarrowia lipolytica* in the manner described herein.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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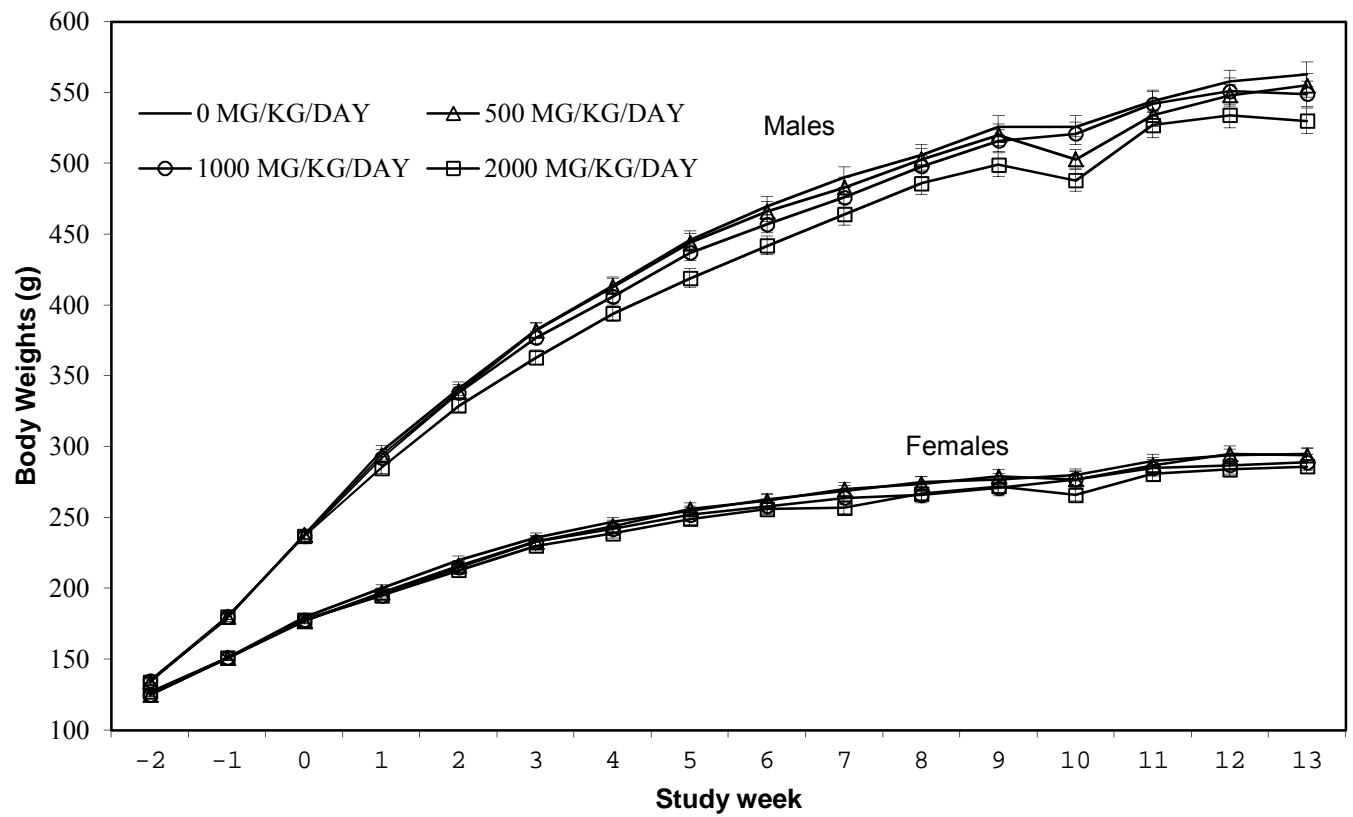
Figure 1

Body weights of male and female rats during the 90-day subchronic toxicity study. Fermentative Reb A was administered daily for at least 91 days at the doses indicated in the figure legend.

Annex 9

Secondary

Metabolite report

TNO innovation for life

TNO report

TNO 2015 R10227 | Final report

Evaluation of the genotoxicity of kaurenoic acid and elaboration of a health limit value for risk assessment

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Contents

1   Introduction

DSM Food Specialties B.V. (hereafter called DSM) requested TNO to perform a literature search and evaluation for the genotoxicity and/or carcinogenicity endpoints of kaurenoic acid. DSM reported that kaurenoic acid is an impurity present in the food additive rebaudioside A (with a purity >95%) for which reportedly an ADI of 12.1 mg/kg body weight/day is set, as deduced from the ADI of 4 mg steviol equivalent/kg body weight/day set for steviol glycosidesby the JECFA . The results of a literature search previously performed by DSM were provided. Taken into account the results of the genotoxicity evaluation of kaurenoic acid, DSM additionally requested TNO to elaborate a health limit value for kaurenoic acid based on the hazard information available in public literature and to determine a maximum limit for the amount of kaurenoic acid in Rebaudioside A.

TNO performed a literature search using the tox search strategy module of the TNO Food Safety Assessment Tool covering amongst others Toxnet, IPCS, ECB, OECD chemportal, ECHA and EFSA databases. Moreover, additional searches were performed using Pubmed, Toxline and Scopus based on the CAS No and chemical name. In case too many hits were obtained as result of the literature search, the qualifiers 'mutagen*, genotox* or carcinogen*' were included in the search. For the elaboration of a health limit value for kaurenoic acid additional qualifiers to the CAS No and chemical name used were 'repeated', 'hazard or risk or health' and 'not hplc'.

Moreover, additional searches were performed to obtain specific information on some aspects found in this toxicological literature on the hazard of kaurenoic acid in general. The results of the literature search were used for the evaluation of the genotoxicity and/or carcinogenicity of kaurenoic acid and elaboration of a health limit value for risk assessment, which is discussed in the following chapters. The results of the literature search and evaluation for the genotoxicity and carcinogenicity endpoints are described in chapter 2 and 3, subsequently. In chapter 4, the overall health limit value for kaurenoic acid is elaborated.

2 Genotoxicity tests evaluated

In public literature and databases a total of 4 publications covering mutagenicity, and genotoxicity data could be retrieved. Three of these publications were also provided by DSM. No carcinogenicity data were found in the public domain. A short description of these publications is provided in the following paragraphs.

2.1 Pezzuto et al., 1985 and 1986

Kaurenoic acid was tested for its potential to induce gene mutations in *S. typhimurium* strain TM677 in the absence and presence of an Aroclor 1254 induced S9 liver fraction (Pezzuto et al., 1985[1] and 1986[2]). Although test results were not provided in the 1985 publication, according to the authors kaurenoic acid did not induce gene mutations up to 5 mg/ml under the conditions used. In the 1986 publication, the study results for kaurenoic acid were reported and showed no induction of gene mutations under the conditions used and up to 5 mg/ml.

2.2 Cavalcanti et al., 2006

In a study by Cavalcanti (2006)[3] the potential genotoxicity of kaurenoic acid was evaluated in Chinese hamster lung fibroblast (V79) cells *in vitro*, using the Comet and the micronucleus assays. Kaurenoic acid was tested at concentrations of 2.5, 5, 10, 30 and 60 µg/mL for a treatment time of 3 hours. Kaurenoic acid induced DNA damage in both the comet and micronucleus assay at the 30 and 60 µg/ml concentrations. Unfortunately, no explanation was provided for the concentration range chosen and, more important, no information is provided on the cytotoxicity of the test substance at the concentrations used.

2.3 Cavalcanti et al. 2010

Cavalcanti et al. (2010)[4] studied the potential genotoxic effects of kaurenoic acid and some structural related compounds using several standard *in vitro* and *in vivo* protocols (Comet, chromosomal aberration, micronucleus and *Saccharomyces cerevisiae* assays). Based on the outcome of the tests the authors concluded that kaurenoic acid is considered to be genotoxic and mutagenic in human peripheral blood leukocytes (PBLs), yeast (*S. cerevisiae*) and mice (bone marrow, liver and kidney) probably due to the generation of DNA double-strand breaks and/or inhibition of topoisomerase. Some more

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[1] Pezzuto et al. (1985) Metabolically activated steviol, the aglycone of stevioside, is mutagenic. Proc. Nati. Acad. Sci., vol 82, pp. 2478-2482
[2] Pezzuto et al. (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, vol 169, pp. 93-103
[3] Cavalcanti et al. (2006) Food and Chemical Toxicology, vol 44, pp. 388–392
[4] Cavalcanti et al (2010) Mutation Research, vol 701, pp. 153–163

information on the different studies performed are given in the sections 2.3.1 and 2.3.2.

2.3.1   *In vitro data*
Peripheral lymphocyte cultures were treated with 10, 30 and 60 µg/mL kaurenoic acid. The percentage of apoptotic cells, demonstrated by visualization of normal or aberrant chromatin organization using fluorescent DNA-binding dyes, showed a clear concentration related effect ($p < 0.001$) reflected in the percentage of apoptotic cells: 6.55; 25.11; 39.55 and 46.11%, for 0, 10, 30 and 60 µg/mL kaurenoic acid respectively.

In peripheral lymphocytes, positive effects were observed in the neutral and alkaline comet assay at concentrations of 30 and 60 µg/ml. Moreover, in the chromosome aberration test, kaurenoic acid induced mainly chromatid and chromosome breaks at 30 and 60 µg/ml for which in these concentrations also the mitotic index was significantly reduced to 58% and 35%, respectively. The presence of micronuclei was also observed the *in vitro* micronucleus assay in peripheral lymphocytes, also at the concentrations of 30 and 60 µg/ml.

In *S. Cerevisiae*, kaurenoic acid induced increased frequencies of point and frameshift mutations during the stationary and exponential phases. In all cases where induced mutations are observed, at least moderate (ca. 50%) cytotoxicity is observed.

2.3.2   *In vivo data*
Male Swiss mice were treated with a single dose of 25, 50, and 100 mg/kg kaurenoic acid via the intraperitoneal route of exposure. Micronuclei in bone marrow and DNA strand breaks in the Comet assay were evaluated after 24 and 48 hours of exposure. The Comet assay was performed on cell preparations of liver, kidney and spleen. In the micronucleus test, a dose related induction was observed for multinucleated polychromatic erythrocytes together with a dose related induction of cytotoxicity based on the ratio of polychromatic and normochromatic erythrocytes.

In the Comet assay, induction of DNA strand breaks were observed in liver and kidney cells, but not in spleen cells, at the two highest doses levels tested, whereas in liver in the lowest dose level an induction of DNA strand breaks is found as well. No indicators for cytotoxicity were evaluated for liver and kidney under the conditions used.

3   Evaluation of genotoxicity data available

The tests published were performed with kaurenoic acid of unknown purity and from different sources. The relevance thereof for the conclusions drawn by the authors cannot be evaluated and is therefore not further taken into account. For the current evaluation it is assumed that high purity test substances were used in the studies published.

Moreover, in none of the publications the pH at the concentrations used was provided. As kaurenoic acid is an acid, the pH of the exposure medium may have been dropped below a pH of 6.5[5], which may induce false positive effects in genotoxiticity tests *in vitro*[5, 6]. For the current evaluation it is assumed that the pH during exposure was within physiological levels.

Although only one strain is used, no induction of the mutation frequency in *S. typhimurium* TM677 is found by Pezzuto et al.[1, 2].

The genotoxity data as published by Cavalcanti et al.[3, 4], showed positive effects on chromosome aberrations, point and frameshift mutations, micronuclei and DNA strand breaks *in vitro* and/or *in vivo*. However, for some of the results no cytotoxity data are provided whereas in case these are provided, significant cytotoxicity is present at the concentrations in which positive effects are observed. Most relevant in this respect are the *in vivo* tests performed. It should be noted however that for the target cells covered by the Comet assay, no information is present on the amount of cytotoxicity induced. Moreover, in the discussion also no information is provided on the shape of the comets detected which may give an indication of the type of DNA damage covered, which may also include cytotoxicity and/or apoptosis. Taken into account the results of the *in vitro* tests performed it is more than likely that next to necrosis as result of cytotoxicity, also an apoptosis related mechanism is concerned. Moreover, the relevance of effects observed upon intraperitoneal dosing related to oral exposure can be considered questionable.

According to OECD guidelines of *in vitro* tests, tests concentrations up to 2000 µg/ml for non-cytotoxic, or up to a certain amount of cytotoxicity depending on the test protocol, should be tested. Note that excessive cytotoxicity may induce artifacts for which the test results are not taken into account to conclude for a genotoxic or mutagenic effect of the substance.

Information on the cytotoxicity of kaurenoic acid in *in vitro* tests are shown by Cavalcanti et al.[4], and are confirmed for several cell lines *in vitro* published by Batista et al. (2013)[7] and Fernandez et al. (2013)[8] in which cytotoxicity induced by kaurenoic acid was determined based on trypan blue staining and MTT conversion, respectively. For kaurenoic acid, depending on the cell lines concerned, 50% cytotoxicity was reported at >9.92 µM (equivalent to 3 µg/ml) by Batista[7] and at 60 µg/ml by Fernandez[8]. Considering that the reported cytotoxicity data are in the range where DNA effects were reported by

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[5] Morita et al. (1992) Mutation Research, vol 268, pp. 297-305
[6] Kirkland et al. (2007) Mutation Research, vol 628, pp 31–55
[7] Batista et al. (2013) European Journal of Medicinal Chemistry, vol 62, pp. 168-176
[8] Fernandez et al. (2013) Genetics and Molecular Research, vol 12 (2), pp. 1005-1011

Cavalcanti[3] in the micronucleus and Comet assay in V79 cells, i.e. at 30 and 60 µg/ml, it cannot be excluded that the effects observed are the result of cytotoxicity. Moreover, induced DNA damage *in vitro* is observed exclusively in the presence of cytotoxicity in the studies as published by Cavalcanti et al.[4].

In the OECD guideline for the *in vitro* mammalian cell micronucleus test (OECD 487[9]) the following is noted/required regarding the cytotoxicity of the test substance:

- [9] '*Care should be taken to avoid conditions that could lead to artifactual positive results which do not reflect the genotoxicity of the test chemicals. Such conditions include changes in pH (41) (42) (43) or osmolality, interaction with the cell culture medium (44) (45) or excessive levels of cytotoxicity*';
- [13] '*For all protocols, it is important to demonstrate that cell proliferation has occurred in both the control and treated cultures, and the extent of test chemical-induced cytotoxicity or cytostasis should be assessed in all of the cultures that are scored for micronuclei*'
- [29] '*If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve 55 ± 5% cytotoxicity using the recommended cytotoxicity parameters (i.e. reduction in RICC and RPD for cell lines when cytoB is not used, and reduction in CBPI or RI when cytoB is used to 45± 5% of the concurrent negative control) (72). Care should be taken in interpreting positive results only found in the higher end of this 55 ± 5% cytotoxicity range*'.

For the comet assay no adopted *in vitro* OECD guideline is available. However, a guideline for the *in vivo* Comet assay is recently adopted (OECD 489[10]). As the effects of cytotoxicity with respect to the conclusion to be drawn are rather similar, the *in vivo* guideline can be used for reference on the effects of cytotoxicity in the *in vitro* Comet assay as well. It is noted that:

- [54]'*where increases in DNA migration are observed, it is recommended that an examination of one or more indicators of cytotoxicity is performed as this can aid in interpretation of the findings. Increases in DNA migration in the presence of clear evidence of cytotoxicity should be interpreted with caution*'., and,
- [64] '*To assess the biological relevance of a positive or equivocal result, information on cytotoxicity at the target tissue is required (see paragraphs 54-55). Where positive or equivocal findings are observed solely in the presence of clear evidence of cytotoxicity, the study would be concluded as equivocal for genotoxicity unless there is enough information that is supportive of a definitive conclusion.*'

As no information was provided on the cytotoxicity at the concentrations for which an induction of DNA damage was observed *in vitro*[3] or *in vivo*[4] and the concentrations inducing the positive result show statistically significant

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[9] OECD TG 487, In vitro mammalian cell micronucleus test, adopted 26 September 2014
[10] OECD TG 498, In vivo mammalian alkaline comet assay, adopted 26 September 2014

cytotoxicity at the concentrations/dose levels inducing DNA damage[7, 7, 8], conclusions on the genotoxicity of kaurenoic acid should be evaluated carefully and take into account the mechanism of cytotoxicity observed or expected.

Cavalcanti et al.[4] demonstrated that kaurenoic acid is concentration related inducing apoptosis at the levels at which cytotoxity is observed in peripheral leukocytes *in vitro*, whereas necrosis is concentration related induced to a lesser degree (see figure 1).
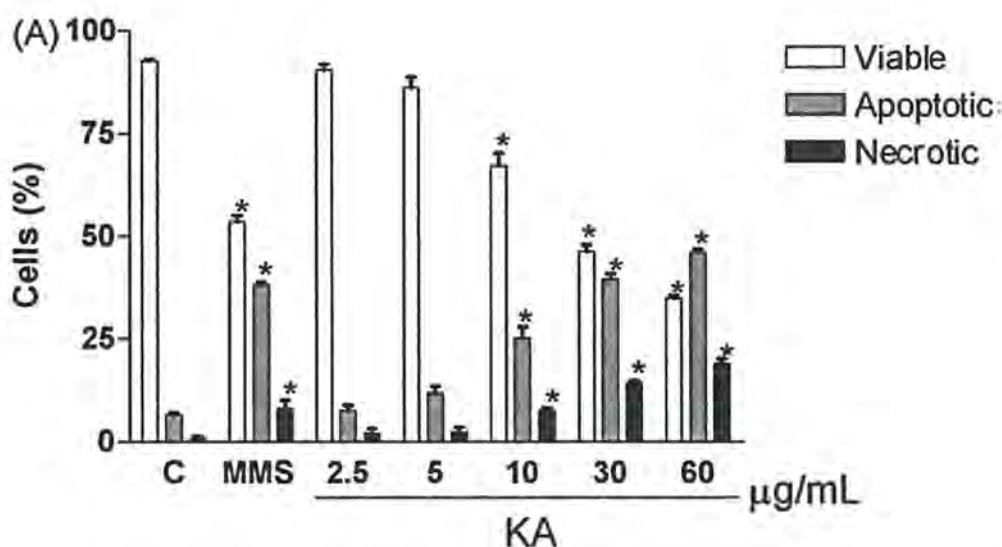

Figure 1: Effect of kaurenoic acid (KA) on human leukocyte viability, apoptosis and necrosis (from Cavalcanti et al. 2010[4])

Furthermore, kaurenoic acid is reported to be cytotoxic through induction of apoptosis in several *in vitro* systems as published by Fernandez et al.[8] and Lizarte Neto et al.[11], reportedly via inhibition of NF-κB and activation of caspase 8, and via suppression of anti-apoptotic signals[8] and as an effect on the regulation of several genes involved in the apoptotic pathway, including c-FLIP, caspase 3, caspase 8, and miR-21[11], respectively.

Lizarte Neto et al.[11] and Cavalcanti et al.[4] reported the induction of apoptosis in the same order of magnitude than the concentrations used in the study of Cavalcanti et al.[3]. Moreover it should be noted that Lizarte Neto et al.[11] referred to the study of Cavalcanti et al.[3] in which it was stated that the results 'supports a dose dependent cytotoxic effect of kaurenoic acid on non-cancer cells'.

Taken into account that apoptosis can induce DNA-strand breaks, chromosome and chromatid breaks and micronuclei as a result of defragmentation of the DNA, considering that the induction of DNA damage as observed in several tests is related to cytotoxic concentrations at which apoptosis is the main underlying effect, and considering that the apoptosis induced by kaurenoic acid is related to apoptotic pathways covering the regulation of several genes, a

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[11] Lizarte Neto et al. (2013) Brazilian Journal of Medical and Biological Research, vol. 46, pp. 71-78

mutagenic mode of action for the DNA damage observed is not to be considered. Therefore, the effects on DNA damage as observed in the studies of Cavalcanti et al.[3, 4] are considered to be related to a threshold related effect. It should be noted that at the concentrations/ dose levels concerned in which no apoptosis is observed, also no induction of DNA damage related to genotoxicity is observed which further indicates that a genotoxic mechanism is not likely..

Considering the above, and taken into account:
- Lacking explanation on the concentration range used in the study of Cavalcanti et al.[3];
- Lacking information on the cytotoxicity of the test substance at the concentrations inducing positive effects in the study of Cavalcanti et al.[3];
- Information on the induction of cytotoxicity by kaurenoic acid observed in other cell lines[8, 11] at concentrations in the order of magnitude of the concentrations in which effects were observed in the study of Cavalcanti et al.[3];
- Observed apoptosis induced by kaurenoic acid at concentrations inducing cytotoxicity (Cavalcanti et al.[4]);
- Observed DNA damage *in vitro* at concentrations in which cytotoxicity and apoptosis is observed (Cavalcanti et al.[4]);
- Observed induction of micronucleï *in vivo* at dose levels inducing cytotoxicity, which may be related to apoptosis considering the results of the *in vitro* tests (Cavalcanti et al.[4]);
- Observed induction of DNA strand breaks in the comet assay *in vivo* without cytotoxicity parameters included in the test set-up, however at dose levels inducing cytotoxicity in the micronucleus assay (Cavalcanti et al.[4]);
- The *in vivo* tests are dosed by intraperitoneal injection, for which the relevance upon oral exposure is unknown (Cavalcanti et al.[4]);
- The published mechanism of induction of apoptosis by kaurenoic acid in *in vitro* systems at dose levels also inducing cytotoxicity (Cavalcanti et al.[4], Fernandez et al.[8] and Lizarte Neto et al.[11])

no classification with respect to the genotoxicity of kaurenoic acid can be drawn taken into account the results of the studies as reported by Cavalcanti et al.[3, 4].

Considered in coherence, the DNA damage observed is likely the result of artefacts due to DNA fragmentation as a result of apoptosis and should therefore not be considered the result of a genotoxic mode of action. These effects are therefore considered to be a threshold related effect and not considered as a carcinogen via a genotoxic mode of action.

4   Health based limit value for Kaurenoic acid

In public literature, no hazard information could be retrieved which could be used to elaborate a health limit value for kaurenoic acid. Moreover, an additional search for structural related compounds (>90% comparable) of kaurenoic acid was performed in order to determine possible read-across candidates. For the structural related compounds argyrophilic acid (CAS No. 20316-84-1), beyer-15-en-18-oic acid (CAS No. 120852-64-4) and grandiflorenic acid (CAS No. 22338-67-6), additional literature searches were performed, however also for these substances no relevant toxicity data could be retrieved.

With respect to the toxicological relevance of exposure to not specified substances and/or to substances for which toxicological information is lacking, one may refer to the point of view of the World Health Organization (WHO), the US Food and Drug Administration (FDA) and the European Food Safety Authority (2012)[12], concerning the Threshold of Toxicological Concern concept (TTC concept). The TTC concept is based on the assumption that there is a level of exposure to a given substance below which no significant risk is expected to occur. Intake levels of a substance below 1.5 µg/kg bodyweight/day, for substances lacking (structural alerts for) genotoxicity, not containing organophosphate or carbamate groups in their chemical structure, and classified as a Cramer structural class III compound, are generally considered to be without significant risks on adverse health effects, even after long term (life-long) exposure (Kroes et al. 2004[13]).

Kaurenoic acid is not considered to be genotoxic based on the conclusions in chapter 3 and is a Cramer structural class III compound for which a TTC threshold of 1.5 µg/kg bodyweight/day applies. The Cramer classification was obtained using Toxtree-v2.6.0. This TTC threshold is accepted by the EFSA. Recently, a re-evaluation of the Munro dataset underlying the TTC thresholds was published by Leeman et al. (2014)[14]. This re-evaluation revealed that for Cramer structural class III substances not containing halogens and organophosphates in their molecular structure, the toxicologically relevant daily intake can be increased to a level of 4 µg/kg bodyweight/day. It should be noted however, that this threshold is not (yet) accepted by authorities.

Based on the information above, a health based limit value for kaurenoic acid, based on the re-evaluation of the TTC dataset, may be considered of 4 µg/kg bodyweight/day. However, considering that this threshold is not yet accepted by authorities, the TTC threshold of 1.5 µg/kg bodyweight/day is taken into account in the current case.

For the current case kaurenoic acid is an impurity in the food additive rebaudioside A. Considering that reportedly, for rebaudioside A an ADI of 12.1 mg/kg body weight/day is elaborated by the JECFA, and when taken into account that intake of rebaudioside A up to a level of 12.1 mg/kg bw/d is assumed, the maximum level of

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[12] EFSA Journal 2012; 10(7):2750
[13] Food and Chemical Toxicology 2004; 42(1):65-83
[14] Leeman et al. 2014 Reevaluation of the Munro dataset to derive more specific TTC thresholds. Reg. Toxicol. Pharm. 69:273–278

the impurity kaurenoic acid in rebaudioside A can be calculated. For the current case it is assumed that exposure to kaurenoic acid is exclusively via intake of rebaudioside A. As such, 0.0015 mg kaurenoic acid may be present in 12.1 mg rebaudioside A, which is equivalent to 124 mg kaurenoic acid/kg rebaudioside A (124 ppm).

5 Conclusion

Based on the current evaluation, it is concluded that the effects observed in the genotoxicity tests performed with kaurenoic acid can be attributed to apoptosis and should therefore not be considered the result of a genotoxic mode of action. These effects are therefore considered to be a threshold related effect whereas carcinogenicity via a genotoxic mode of action is not to be considered.

A health based limit value of 1.5 µg /kg bodyweight/day for kaurenoic acid is elaborated, considering the TTC threshold for Cramer structural class III substances.

When considering that exposure to kaurenoic acid is exclusively related to the food additive rebaudioside A, a maximum level of 124 ppm of kaurenoid acid in rebaudioside A is considered safe at a maximum intake of 12.1 mg rebaudioside A /kg body weight/day, being the ADI thereof.

6 Signature

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Annex 10

DSM and Commercial Stevia

Products Composition Report

Commercial & DSM Samples - analysis

Company	Product	Batch	steviol ppm	KA ppm	KA1-Glc ppm	reb A LC-UV %	stevioside LC-UV %	reb B LC-UV %	reb C LC-UV %	reb D LC UV %
DSM	Reb A	Reb A NBK-5203-0008-1210BPF1-2	15	5		96.1	0	1.5		
DSM	Reb A	Reb A NBK-5203-009-2207BPF2-2	11	6		96.3	0.2	1.4		
DSM	Reb A	Reb A NBK-5203-010-310BPF3-1	0	<3		96.8	0	1		
DSM	Reb A	Reb A NBK-05203-011-076	100	5		96.9	0.1	2.4		
Company H	Commercial Sample	12101021814	17							
Company A	Commercial Sample	131302-01				95.6	0.2	1.9	0.2	1.9
Company A	Consumer Product	E187-2				86.0	5.2	3.0	1.7	2.7
Company B	Consumer Product	BTUD16R036				77.0	0.4	29.0	0.6	0.6
Company C	Commercial Sample	20131007	3							
Company B	Consumer Product	BT3E09c059	28	<0.3	2.3					
Company D	Commercial Sample	422076/10	2			96.0	0.4	2.0	1.1	
Company D	Commercial Sample	421974/10	2			88.5	1.3	1.6	2.3	0.5
Company D	Commercial Sample	460617/10	2		1					
Company D	Commercial Sample	421974/10	3							
Company F	Commercial Sample	1201141	2							
Company F	Commercial Sample	1201131	1							
Company E	Commercial Sample	3010042	2		3.1					
Company E	Commercial Sample	3020513	4		1.3					
Company C	Commercial Sample	20110502	1		0.8	100	0.4	0.8	0.4	0.7
Company C	Commercial Sample	20120609	3			97.0	0.6	1.2	0.4	0.8
Company C	Commercial Sample	20120611	40	0.3	25	90.0	0.6	1.6	0.3	

Company	Product	Batch	steviol ppm	KA ppm	KA1-Glc ppm	reb A LC-UV %	stevioside LC-UV %	reb B LC-UV %	reb C LC-UV %	reb D LC-UV %
Company E	Commercial Sample	GGT120513	2							
Company K	Commercial Sample	20130526			1.1					
Company J	Commercial Sample	1304111			5.9					
Company J	Commercial Sample	1303051			0.6					
Company B	Consumer Product		19	<0.3						
Company G	Commercial Sample		<0.3	<0.3		52.9/53.7	27.4/30.8	1.8/2.1	11.1/11.8	0.4/0.4
Company G	Commercial Sample	GSG0713484				4.5	2.3	0.8	1.0	
Company G	Commercial Sample	DR0813395	<0.3	<0.3		99.4/101.4	0.2/0.4	0.5/0.9	0.2/0.5	
Company G	Commercial Sample	SGA011204				81.3	0.1	19.3	0.2	
Company A	Consumer Product	130531-01				95.5	0.1	2.3		1.1

"Safety evaluation of rebaudioside A produced by fermentation", Rumelhard M, Hosako H, Eurlings IM, Westerink WM, Staska LM, van de Wiel JA, La Marta *J. Food Chem Toxicol*. 2016 Mar; 89:73-84. doi: 10.1016/j.fct.2016.01.005. Epub 2016 Jan 15.

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