FOOD STANDARDS
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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Risk and technical assessment report – Application A1132

Broaden Definition of Steviol Glycosides (Intense Sweetener)

Executive summary

FSANZ received an application from PureCircle seeking to expand the definition of steviol glycosides for use as an intense sweetener to include all minor steviol glycosides present in the *Stevia rebaudiana* Bertoni (for the rest of this report called stevia) leaf. PureCircle claim that the addition of minor steviol glycosides can improve the overall taste profile when added to foods and beverages.

The food technology assessment concludes that broadening the specification for steviol glycosides preparations to include any mixture of individual steviol glycoside compounds is justified. Current analytical methods can be used to identify these other steviol glycoside compounds. A new specification will be written to encompass all the steviol glycosides extracted from the stevia leaf.

In vitro studies consistently showed the biotransformation of steviosides, rebaudiosides and dulcosides to steviol. This is in agreement with earlier studies conducted on stevioside and rebaudioside A which have been evaluated in previous FSANZ assessments. The existing Acceptable Daily Intake (ADI) of 0-4 mg/kg bodyweight, expressed as steviol, is applicable to all steviol glycosides in stevia leaf.

The Applicant intends to market steviol glycoside preparations for use as intense sweeteners under the conditions presently approved for steviol glycoside preparations. No new dietary exposure assessment was considered necessary for this Application.

FSANZ concludes that the expansion of the definition of steviol glycosides to include all steviol glycosides present in the stevia leaf, poses no public health and safety concerns. It is expected that all steviol glycosides will be hydrolysed completely to steviol by gut microflora.

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1 Introduction

PureCircle Limited is seeking to expand the definition of steviol glycosides for use as an intense sweetener to include all steviol glycosides present in the *Stevia rebaudiana* Bertoni (for the rest of this report called 'stevia') leaf. Steviol glycosides are permitted to be added to a variety of food categories in the table to section S15—5. There are specific specifications for steviol glycoside preparations including the specific steviol glycoside rebaudioside M (section S3—32) and for rebaudioside M itself (section S3—31) within Schedule 3 – Identity and purity in the *Australia New Zealand Food Standards Code* (the Code). Currently, there are only 10 permitted steviol glycosides that must make up at least 95% (dried basis) of a steviol glycosides preparation (see S3—32 and subsection 1.3.1—4(7)).

1.1 Objectives of the assessment

There are no permissions for steviol glycosides other than those listed in the Code, to make up 95% of a steviol glycoside intense sweetener preparation. An application to amend the Code to permit the use of other steviol glycosides requires a pre-market assessment.

The objectives of this risk and technical assessment are to:

- determine whether the proposed purpose is clearly stated
- ensure other steviol glycosides achieve their technological function in the quantity and form proposed
- evaluate any potential public health and safety concerns that may arise.

2 Food technology assessment

2.1 Introduction and description of substance

PureCircle is seeking the broadening of the definition of steviol glycosides preparations that are permitted food additives with the technological purpose of intense sweetener and the INS number 960, as the purpose of the Application. Currently, steviol glycosides preparations must contain at least 95% w/w on a dried basis, of any proportion of 10 specifically identified steviol glycosides extracted from the stevia leaf via permissions and specifications for steviol glycosides in the Code. The Application claims there are now around 40 different minor steviol glycosides identified that can be extracted from the Stevia leaf.

The Application is seeking permission for any minor steviol glycoside, including steviol glycoside preparations that can be extracted from the stevia leaf, in addition to the 10 steviol glycosides currently listed.

2.1.1 Identity

There has been a large increase in the number of individual, minor steviol glycosides that have been extracted, isolated and identified from the stevia leaf which the Applicant states is now about 40, and is expected to grow further in time. More detail is provided in the Application, in the steviol glycoside literature, and summarised in section 2.2 (chemical properties) below.

All steviol glycosides share the same stevia backbone structure but have different sugar moieties attached, as conjugated glycosides. These various sugar moieties include glucose, rhamnose, xylose, fructose and deoxyglucose, which can be attached in various combinations, quantity and orientation.

The Applicant differentiates the various steviol glycosides into five groups identified by the various sugar moieties attached to the steviol backbone.

Since the majority of constituents (>95%) in the Applicant's steviol glycoside mixtures are in fact steviol glycosides, the purity specification for steviol glycoside preparations (not less than 95% total steviol glycosides) is consistent with the purity specifications and thus may include some or all of the 10 currently permitted steviol glycosides along with other steviol glycosides present in the stevia leaf. The Applicant demonstrated that steviol glycoside mixtures comply with the existing specifications of impurities concurrent with Joint FAO/WHO Expert Committee on Food Additives (JECFA), Food Chemicals Codex and European Commission specifications for steviol glycosides (JECFA, 2010; Food Chemicals Codex, 2016b; European Commission, 2012).

2.1.2 Technological purpose

Steviol glycosides are permitted in the Code as a food additive with the technological purpose of an intense sweetener. The evaluation of the technological purpose of steviol glycosides has been assessed before by FSANZ in the assessment reports for Applications A540 and A1037 and concluded to be appropriate.

2.1.3 Technological justification

The Applicant's justification for the Application is that other minor, currently not permitted steviol glycosides and glycoside mixtures, added to the already permitted major steviol glycosides provide improvements in the taste and flavour characteristics to various foods. No other changes to the permissions for steviol glycosides are sought in the Code, that is no increase in maximum permitted levels or expansion to other food categories.

2.1.4 Assessment of claimed benefits

The Application contained studies evaluating the impact of the minor steviol glycosides compared to the currently permitted steviol glycosides on the overall taste profile and acceptance when added to three representative food and beverage products. These products were a chocolate flavoured milk beverage, flavoured peanuts and sweetened acidified water. For the first two products the minor steviol glycosides were added in addition to the major steviol glycosides, while for the acidified water beverages they were compared directly to the major steviol glycosides and both compared to sucrose sweetened products as the control.

The findings were:

(a) **chocolate flavoured milk**
Significantly improved chocolate flavour
Slightly improved better overall acceptance, sweetness intensity (noting that extra steviol glycosides were added), less bitter aftertaste and improved dairy note.

(b) **flavoured peanuts**
Significantly improved overall preference
Significantly improved flavour characteristics

(c) **sweetened acidified water**
Slightly improved overall preference, being closer to the sugar control to the major steviol glycoside blend
Significantly improved positive characteristics (sweet aftertaste) and reduced negative characteristics typical of steviol glycosides such as bitterness, astringency and off-note (metallic/liquorice).

The claimed benefit studies provide justification to support the Applicant's claim that the addition of minor steviol glycosides can improve the overall taste profile when steviol glycosides preparations are added to foods and beverages. It needs to be stated that effects would need to be assessed for individual food categories and may vary compared to the above results. It is expected that food companies will conduct their own evaluations to assess whether taste improvements were identified.

2.2 Chemical properties

Table 1: Chemical names, identification and structure of steviol glycosides

Chemical name:	Stevioside: 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, β-D-glucopyranosyl ester Rebaudioside A: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D- glucopyranosyl ester
Common Name:	Steviol glycosides (as the group of different steviol glycosides)
INS No.:	960
CAS Registry Number:	Stevioside: 57817-89-7 Rebaudioside A: 58543-16-1
Chemical formula:	Steviol: $C_{20}H_{30}O_3$ Stevioside: $C_{38}H_{60}O_{18}$ Rebaudioside A: $C_{44}H_{70}O_{23}$
Molecular weight:	Steviol: 318.46 Stevioside: 804.88 Rebaudioside A: 967.01

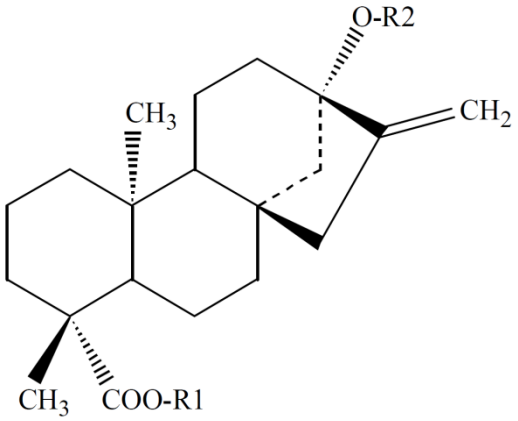
Structural formula:	 Steviol glycoside general structure, R_1 & R_2 are the various sugar moieties Steviol: R_1 & R_2 = H Stevioside: R_1= β -glucose, R_2=β-glucose- β -glucose (2→1) Rebaudioside A: R_1= β -glucose, R_2= β-glucose- β -glucose (2→1) | β -glucose (3→1) Structure taken from the JECFA Chemical and Technical Assessment for steviol glycosides (FAO 2007)

Table B.2.1-1 in the Application contains a detailed list of the different steviol glycosides, in their five groups, their common name, the identity of R1 and R2 and a literature reference (which is not copied here).

2.3 Analytical method for detection

There have been analytical methods available for the detection and quantification of steviol glycosides in food since preparations of steviol glycosides have been commercialised and permitted as intense sweetener food additives. These have been based on High Performance Liquid Chromatography (HPLC). Such analytical methods were mentioned in FSANZ's assessment of Application A1037 (FSANZ 2011). That report referred to the European Food Safety Authority (EFSA) Scientific Opinion on steviol glycosides in 2010 (EFSA 2010). Two HPLC analytical methods have been published (Geuns et al 2008, Gardana et al 2010). Such methods should be readily adaptable for the analysis of all types of steviol glycosides from steviol glycosides preparations added to foods and beverages. The Applicant has optimised the JECFA HPLC analytical method to detect the presence of steviol glycosides in food matrices. The full details of the Applicant's analytical method are available in Appendix E of the Application.

2.4 Manufacturing process

The manufacturing process is based on the process in the JECFA 2010 specification for steviol glycosides and the JECFA 2007 Chemical and Technical Assessment report. The process involves the hot water extraction of stevia leaves and then isolation and purification using ion-exchange resins and solvent extraction (methanol and/or ethanol).

The Applicant's steviol glycoside products are produced in a facility certified under Food Safety System Certification (FSSC) 22000:2010.

2.4.1 Product specification

Specifications for permitted food additives are covered by Schedule 3. They are either covered by primary sources (section S3—2), secondary sources (section S3—3) or individual specifications, section S3—5 to section S3-33 (as noted in subsection S3—2(2)).

The JECFA specifications (Combined Compendium of Food Additive Specifications) are a primary source of specifications being paragraph S3—2(1)(b). The current JECFA steviol glycosides specification (2010) lists only nine individual steviol glycosides. Individual specifications for rebaudioside M (section S3—31) and steviol glycoside mixtures containing rebaudioside M (section S3—32) were added into Schedule 3 as an outcome of the earlier Application A1108 from the same Applicant. This was because the JECFA steviol glycoside specification did not include rebaudioside M.

Both the Food Chemicals Codex (subsection S3—2(10(c)) and the European Commission Regulation (EU) No 231/2012 (subsection S3—2(d)) also contain specifications for steviol glycosides preparations similar to the current JECFA specification in that they relate to specific named individual steviol glycosides.

The 82nd JECFA meeting in June 2016 considered the specifications of steviol glycosides (JECFA 2016). This meeting established new tentative specifications for steviol glycosides, titled “Steviol glycosides from *Stevia rebaudiana* Bertoni”. This distinguishes it from different source materials, such as the newly established specification for rebaudioside A derived via yeast fermentation, also developed at this JECFA meeting. This new tentative steviol glycosides specification includes a new definition and assay analytical method to expand from the current nine named steviol glycosides to include any mixture of steviol glycoside compounds extracted from the stevia leaf, provided the total percentage of steviol glycosides is not less than 95% (w/w). This new tentative steviol glycoside specification is the same approach requested by this Application. This new JECFA specification is only a tentative specification and further work on the new JECFA specification and information is needed by 31 December 2017 before being made final. The new information JECFA has requested relates to the analytical method and results from commercial samples. Because of this delay FSANZ needs to draft a new specification to cover the requests of this Application into Schedule 3, which can be removed when the JECFA tentative specification is made final.

2.4.2 Product stability

JECFA (2007) concluded that the steviol glycosides it assessed for stability are stable when added to foods and beverage under normal processing and storage conditions. They are stable to both temperature (heating) and hydrolysis (reactions with water). Studies conducted by the Applicant for their specific new steviol glycoside preparations were also tested for stability to see if they have similar stability, regardless of the glycoside moiety attached to the steviol structure. These studies concluded that these steviol glycoside preparations had similar stabilities as reported by JECFA, and are pH, temperature and time dependent.

2.5 Conclusion

The food technology assessment concludes that broadening the definition and specification for steviol glycosides preparations to include any mixture of individual steviol glycoside compounds extracted from the stevia leaf is justified. These other individual steviol glycoside compounds are extracted along with the other currently identified steviol glycosides in current specifications by the same manufacturing processes. The same analytical methods currently used for steviol glycosides can be used to identify these minor steviol glycoside compounds. All the extracted steviol glycosides have been concluded to have similar stability, both as the steviol glycosides preparations and when added to foods or beverages.

A new specification needs to be written to encompass all the steviol glycosides extracted from the stevia leaf, since the tentative new specification established by JECFA has not been finalised. Once this JECFA specification is finalised the specification added into the Code can be removed.

3 Hazard assessment

3.1 Background

The safety of steviol glycosides has been assessed extensively since the 1970s by a number of regulatory agencies and scientific bodies including JECFA, EFSA, the US Food and Drug Administration and Health Canada. Interest in the use of steviol glycosides as sweeteners has encouraged significant testing of the compounds and thus the establishment of a large toxicological database, including studies in both animals and humans.

3.1.1 Chemistry

Details of the physiochemical properties of steviol glycosides, including references to product specifications, are included in the food technology assessment (Section 2).

3.1.2 Previous FSANZ assessments

FSANZ conducted a hazard assessment for steviol glycosides as part of the assessment of Application A540 – Steviol Glycosides as Intense Sweeteners (FSANZ 2008). FSANZ concluded that the toxicological database for steviol glycosides provided an adequate basis for establishing a full ADI. In particular, the evidence surrounding the pharmacological effects of steviol glycosides on blood pressure and blood glucose was considered to be adequate. This means that the additional 2-fold factor used by JECFA in 2005 for uncertainty surrounding its effect in normotensive or diabetic individuals was not required. An ADI of 0–4 mg/kg bodyweight (bw) per day derived by applying a 100-fold uncertainty factor to the no-observed-adverse-effect level (NOAEL) of 970 mg/kg bw/day of stevioside (equivalent to 383 mg/kg bw/day steviol) in a 2-year rat carcinogenicity study was established by FSANZ. This ADI covers steviol glycoside mixtures with different ratios of stevioside/rebaudioside.

FSANZ updated the hazard assessment of steviol glycosides in ice creams and selected beverages as part of the assessment of Application A1037 – Steviol Glycosides, in which the Applicant sought to increase the maximum permitted levels of steviol glycosides in ice-cream and selected beverages (FSANZ 2011). The Applicant submitted several published reviews and studies which have been considered by JECFA but which were not published at the time of FSANZ's previous assessment. The additional published toxicokinetics, metabolism, toxicity, and human data on steviol glycosides adds to the extensive database available for the hazard assessment of steviol glycosides. There were no findings in these publications that indicated a need to change the ADI of 0–4 mg/kg bw/day, expressed as steviol equivalents, which was established by FSANZ in 2008.

For Application A1108 (FSANZ 2015) – Rebaudioside M as a New Steviol Glycoside Intense Sweetener, toxicological and other relevant data published since the FSANZ (2011) assessment were considered. These included an *in vitro* study investigating the hydrolysis of rebaudioside M and other steviol glycosides to steviol by human gut microflora as well as repeat-dose toxicity studies relevant to the hazard assessment of steviol glycosides. No concerns were raised regarding the safety of rebaudioside M. As for other steviol glycosides, rebaudioside M is hydrolysed completely to steviol by gut microflora and the existing ADI, which is expressed on the basis of steviol, is therefore applicable to rebaudioside M.

3.1.3 Assessments by other agencies

Application A540 (FSANZ 2008) summarised the assessments of JECFA and EFSA up until 2008. This information is briefly recapped below.

3.1.3.1 JECFA

JECFA assessed steviol glycosides at its 51st, 63rd, 68th and 69th meetings.

At its 51st meeting, JECFA evaluated toxicological data on stevioside and steviol and concluded that further information was needed (World Health Organization (WHO) 1999).

At its 63rd meeting (WHO 2005, 2006), JECFA determined that the commercial material should be known as "steviol glycosides" and established tentative specifications for material containing not less than 95% of the total of four specified glycosylated derivatives of steviol (i.e. stevioside, rebaudioside A, rebaudioside C and dulcoside A). Additionally, the sum of stevioside and rebaudioside A content was specified at not less than 70% of the four steviol glycosides. JECFA reviewed additional biochemical and toxicological data on the major steviol glycosides and on the aglycone steviol. It was noted that steviol glycosides are poorly absorbed and are metabolised by the intestinal microflora by successive hydrolytic removal of glucose units to steviol, which is well absorbed. Therefore, the toxicity of the glycosides was related to steviol. A temporary ADI of 0–2 mg/kg bw/day for steviol glycosides expressed as steviol was established on the basis of the NOAEL of 2.5% stevioside in the diet, equal to 970 mg/kg bw per day, or 383 mg/kg bw per day when expressed as steviol, in a 2-year carcinogenicity study in rats and applying an uncertainty factor of 200. The factor of 200 incorporated an additional 2-fold factor because of uncertainty surrounding the pharmacological effects of steviol glycosides in humans.

JECFA requested additional information on the effects of steviol glycosides on normotensive and hypotensive individuals and in subjects with type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes. This was because there was limited evidence available at the time to assess whether the reported pharmacological effects of steviol glycosides would also occur at dietary exposure levels.

At its 68th meeting (WHO 2007), JECFA considered the information that had become available since the 63rd meeting. This comprised two submissions, which included a summary of published toxicological studies and some unpublished data, additional information identified from the scientific literature and responses intended to resolve the outstanding issues relevant to the specifications. JECFA was also informed that results of an ongoing toxicity testing program, including clinical studies, would be available by August 2007. JECFA considered that the newly available data did not raise additional concerns regarding the safety of steviol glycosides, but that the ongoing clinical studies would be essential for the evaluation. JECFA therefore extended the temporary ADI of 0–2 mg/kg bw, expressed as steviol, pending submission of the results of the then ongoing clinical studies. The existing tentative specifications were revised by requiring purity of not less than 95% based on the total of seven named steviol glycosides, and deleting the requirement for the sum of stevioside and rebaudioside A content to be not less than 70%. The tentative designation for the specifications was removed at this time.

At its 69th meeting (WHO 2009), JECFA considered a submission that comprised a review of all the available information, including studies completed after the 68th meeting and some older studies not highlighted in the previous JECFA evaluations. The new studies included four toxicological studies with rebaudioside A in experimental animals and clinical trials on the effects of steviol glycosides on blood pressure in healthy volunteers with normal or low-normal blood pressure and on glucose homeostasis in men and women with type 2 diabetes mellitus.

JECFA concluded that the new studies showed no adverse effects at levels up to 4 mg/kg bw per day, expressed as steviol, in individuals with type 2 diabetes mellitus and individuals with normal or low-normal blood pressure. The new data were considered sufficient evidence to remove the temporary ADI designation the Committee had set in 2005. A full ADI for steviol glycosides of 0-4 mg/kg bw/day, expressed as steviol, was established.

JECFA also considered steviol glycosides at the 73rd (2011), 76th (2012) and 82nd (2016) meetings.

At the 73rd meeting (WHO 2011) the Committee was requested to add two new steviol glycosides, rebaudiosides D and F, to the seven named steviol glycosides in the existing specifications. The specifications were revised to include the new steviol glycosides as requested and the method of assay was revised accordingly.

At the 76th meeting (WHO 2012) rebaudioside A and rebaudioside C were scheduled to be evaluated as flavouring agents but were removed from the agenda as the Committee did not consider it appropriate to evaluate these substances as flavouring agents. Specifications for these substances were not prepared as these additives are covered under the existing specifications for steviol glycosides.

The monograph for the 82nd meeting (held June 2016) is not yet available but the summary report confirmed the ADI of 0–4 mg/kg bw, expressed as steviol. With respect to specifications the summary report states, “The Definition and Assay specification was expanded from nine named leaf-derived steviol glycosides to include any mixture of steviol glycoside compounds derived from *Stevia rebaudiana* Bertoni, provided that the total percentage of steviol glycosides is not less than 95%.”

Application A540 (FSANZ 2008) also presented the opinion of EFSA. This information is briefly recapped below.

3.1.3.2 EFSA

In 2010, EFSA assessed the available data on steviol glycosides and concluded that steviol glycosides complying with JECFA specifications are not carcinogenic, genotoxic or associated with any reproductive/developmental toxicity. EFSA established an ADI for steviol glycosides, expressed as steviol equivalents, of 0–4 mg/kg bw/day based on application of a 100-fold uncertainty factor to the NOAEL in the 2-year carcinogenicity study in rats (EFSA 2010).

In 2015 EFSA provided a Scientific Opinion regarding the safety of an amendment of the specifications of steviol glycosides. Available data was assessed to decide whether it was appropriate to add rebaudioside M to the list of steviol glycosides that may comprise the assay value of 95% total steviol glycosides content, and to delete the requirement in the EC regulation definition specifying that steviol glycosides contain at least 75% stevioside and/or rebaudioside A. EFSA concluded that extending the current specifications to include rebaudiosides D and M as alternatives to rebaudioside A in the predominant components of steviol glycosides would not be of safety concern. In addition, the Panel considered that the specific steviol glycoside composition would not be a safety concern, provided that the total amount of steviol glycosides were greater than 95%. The Panel also concluded that the ADI of 4 mg/kg bw/day can also be applied where total steviol glycosides comprise more than 95% of the material.

3.1.3.3 United States of America

In 2013 the current Applicant submitted to the United States Food and Drug Administration (USFDA) a Generally Recognised as Safe (GRAS) notice for steviol glycosides with Reb M as the principal component (> 50% of its total steviol glycosides content (FSANZ 2015). In February 2016 Cargill submitted a GRAS notice to the USFDA for steviol glycosides from *Saccharomyces cerevisiae* for use as a general purpose sweetener in foods, excluding infant formula and products under USFDA's jurisdiction at levels determined by good manufacturing practices, as well as use as a table top sweetener. The USFDA had no questions regarding this GRAS notice.

3.1.3.4 Canada

Health Canada established an ADI of 4 mg steviol equivalents/kg bw/day and recommended that steviol glycosides be approved for use as a sweetening agent (Health Canada 2012). Health Canada also recently expanded the definition of steviol glycosides to include rebaudioside M (Health Canada 2016).

3.1.4 Scope of the hazard assessment

The aims of the current assessment were to:

- evaluate any newly submitted data relevant to the hazard assessment of steviol glycosides
- search the published literature for any relevant studies on steviol glycosides which have not been previously considered by FSANZ
- determine whether there are any public health and safety concerns associated with all the steviol glycosides extracted from the Stevia leaf, compared to the currently permitted 10 steviol glycosides

3.2 Evaluation of submitted data

The Applicant submitted a dossier that included summary information related to the safety of steviol glycosides including information on toxicokinetics and metabolism, degradation products and major metabolites. FSANZ also conducted a literature search using EBSCO Discovery Service (includes PubMed, EconLit, SIFT, Food Science Source and Food Science and Technology Abstracts) in August 2016.

Overall, three studies were identified that had been published since the previous FSANZ evaluation of steviol glycosides, and were considered relevant to the current application. These were Purkayastha et al (2015, 2016) and Roberts et al (2016). The Applicant also included an unpublished study (Kwok (2015)) of relevance to the current application.

3.2.2 Absorption, metabolism and excretion

Purkayastha et al 2016 reported a number of *in vitro* studies comparing the metabolism of steviol glycosides, including rebaudioside B, C, D, E, F, M, steviolbioside and dulcoside A. The study includes previously unpublished material but also the results of earlier studies conducted by the same research group Purkayastha et al (2014, 2015). A comparative pharmacokinetics study by Roberts et al 2016 in rats and humans administered stevioside has also been evaluated.

Purkayastha et al 2014

FSANZ has previously evaluated this study as a part of Application A1108 (FSANZ 2015).

The hydrolysis of the steviol glycosides rebaudioside A, B, D, M and steviolbioside to steviol was evaluated *in vitro,* using human faecal homogenates from healthy donors without known gastrointestinal disease or exposure to laxatives of antimicrobial drugs for at least 7 days prior to faecal sample collection, and without previous ingestion of stevia-based natural sweeteners.

Incubations were carried out in triplicate at 37 °C under anaerobic conditions. Separate incubations were conducted with pooled faecal homogenates from male and female donors (n = 3/sex). Each set of incubation experiments was conducted twice. Rebaudioside A, B and D were evaluated at concentrations of 0.2 and 2.0 mg/mL. Rebaudioside M and steviolbioside were evaluated only at a concentration of 0.2 mg/mL because these compounds precipitated out of solution at higher concentrations. Incubation time-courses were 0, 4, 8, 24, and 48 h (rebaudioside B and D) or 0, 8, 16, and 24 h (rebaudioside A and M and steviolbioside). The extent of hydrolysis of each compound was based on the amount of steviol generated over the course of the incubation periods. A liquid chromatography/mass spectrometry (LC-MS) method was used for the quantification of steviol in incubation mixtures.

Results for rebaudioside A and M incubated at a concentration of 0.2 mg/mL are shown in Table 2. After 8 h of incubation, the extent of hydrolysis of rebaudioside A and M was 52-101% and 46-91%, respectively. Complete hydrolysis of both rebaudioside A and rebaudioside M was evident after 16 h incubation with faecal homogenate samples from both sexes.

Table 2: Formation of steviol from incubation of rebaudioside A and M in pooled male and female faecal homogenate samples (0.2 mg/mL)

Steviol glycoside	Incubation time (h)	Males		Females	
		Per cent hydrolysed to steviol[a]		Per cent hydrolysed to steviol	
		M1	M2	F1	F2
Reb A	8	52	77	94	101
	16	99	98	100	107
	24	97	98	98	104
Reb M	8	46	83	91	82
	16	116	107	108	109
	24	115	108	107	108

Source: (Purkayastha et al 2014)

Abbreviations: F1, female faecal homogenate samples #1; F2, female faecal homogenate samples #2; M1, male faecal homogenate samples #1; M2, male faecal homogenate samples #2.
[a] Percent hydrolysed to steviol was calculated based on the theoretical maximum concentration of steviol that could be formed from nominal complete hydrolysis. Each value is the mean of three replicates. Results are for incubations conducted at a rebaudioside concentration of 0.2 mg/mL.

At a concentration of 0.2 mg/mL, hydrolysis of rebaudioside B and D to steviol was essentially complete after 24 h and 8 h incubation (Table 3), respectively, however, the extent of hydrolysis was lower at a 10-fold higher substrate concentration of 2 mg/mL. The yield of steviol from steviolbioside (tested at 0.2 mg/mL only) was consistently lower than 100% (77–82% hydrolysis to steviol after 24 h incubation) (Purkayastha et al 2014).

Table 3 Formation of steviol from incubation of rebaudioside B and D in pooled male and female faecal homogenate samples (0.2 mg/mL)

Steviol glycoside	Incubation time (h)	Males		Females	
		Per cent metabolised[a]		Per cent metabolised	
		M1	M2	F1	F2
Reb B	4	22	20	21	16
	8	87	72	90	58
	24	90	100	106	89
Reb D	4	39	30	35	26
	8	108	111	112	85
	24	112	108	101	100

Source: (Purkayastha et al 2014)

Abbreviations: F1, female faecal homogenate samples #1; F2, female faecal homogenate samples #2; M1, male faecal homogenate samples #1; M2, male faecal homogenate samples #2. [a]The percent rebaudioside remaining was calculated based on the theoretical maximum concentration of steviol that could be formed from nominal complete metabolism. Each value is the mean of 3 replicates. Results are for incubations conducted at a rebaudioside concentration of 0.2 mg/mL.

Purkayastha et al 2015

In 2015, the same research group carried out a study comparing the *in vitro* metabolism of another steviol glycoside, rebaudioside E, to that of rebaudioside A. Rebaudioside A was used as a positive control. The stability of steviol was also monitored. Triplicate samples at a concentration of 0.2 mg/mL were evaluated using anaerobic incubation conditions at 37°C over the time course of 0, 4, 8 16 and 24 hours. Faecal homogenates from Caucasian and Asian donors were used. The faecal samples were collected from healthy male and female donors with no history of gastrointestinal disease and without ingestion of stevia-based natural sweeteners, laxatives and antimicrobial drugs for at least 7 days prior to sample collection. The extent of hydrolysis of each compound was based on the amount of steviol generated over the course of the incubation periods. The results for rebaudioside A and E at a concentration of 0.2 mg/mL are shown in Table 4 below.

Table 4: Formation of steviol from incubation of rebaudioside A and rebaudioside E in pooled male and female Asian and Caucasian faecal homogenate samples (0.2 mg/mL)

Steviol glycoside[a]	Incubation time (h)	Females		Males	
		Per cent metabolised to steviol[b]		Per cent metabolised to steviol	
		F1[c]	F2[d]	M1[e]	M2[f]
Reb A	4	27.2	16.2	10.4	10.3
	8	85.8	72.4	52.9	45.9
	16	86.8	89.6	82.9	84.0
	24	81.1	87.6	83.3	85.6
Reb E	4	30	23.2	15.9	19.5
	8	85.2	86.9	57.6	65.1
	16	93.5	89.3	88.9	91.5
	24	92.9	94.6	93.7	85.6

Source: Purkayastha et al 2015

[a] Results are for incubations carried out at a rebaudioside concentration of 0.2 mg/mL
[b] The percent rebaudioside was calculated based on the theoretical maximum concentration of steviol that could be formed from nominal complete metabolism

[c] Asian, female, n=3 replicates
[d] Caucasian, female, n=3 replicates
[e] Asian, male, n=3 replicates
[f] Caucasian, male, n=3 replicates

Purkayastha et al 2016

In 2016, the same group presented previously published data (Purkayastha et al 2014, 2015 above) together with new data on additional steviol glycosides with differing glycosidic moieties, with the objective of demonstrating that all steviol glycosides found in stevia leaf extract undergo the same metabolic fate. Methods were as previously described.

Overall, the paper reports five separate experiments:

- Experiment 1 compared the metabolism of rebaudioside A with rebaudiosides B and D (all at 0.2 and 2.0 mg/mL) over incubation times of 4, 8, 24 and 48 h. These results were previously reported in Purkayastha et al 2014.

- Experiment 2 compared the metabolism of rebaudioside A with rebaudioside C (all at 2.0 mg/mL) over incubation times of 4, 8, 24 and 48 h. Hydrolysis at 48 h for rebaudioside C was generally more complete at 48 h than for rebaudioside A.

- Experiment 3 compared the metabolism of rebaudioside A with rebaudioside M (each at 0.2 mg/mL) over incubation times of 8, 16 and 24 h. These results were previously presented in Purkayastha et al 2014.

- Experiment 4 compared the metabolism of rebaudioside A with rebaudioside E and steviolbioside (each at 0.2 and 2.0 mg/mL) over incubation times of 4, 8, 16 and 24 h. Data were presented for the incubation at 2.0 mg/mL and are shown below in Table 5.

- Experiment 5 compared the metabolism of rebaudioside A (at 0.2 and 2.0 mg/mL) with rebaudioside F, rebaudioside M and dulcoside A, (each at 0.2 and 2.0 mg/mL) over incubation times of 8, 16 and 24 h. At a concentration of 0.2 mg/mL hydrolysis was essentially complete after 48 hours for rebaudioside A, M and dulcoside A (Table 6).

  Rebaudioside F showed a lower rate of hydrolysis at 0.2 mg/mL, ranging from 64 to 82 per cent. Results from the concentration of 2.0 mg/mL were also provided and also show lower rates of hydrolysis of rebaudioside F, reaching between 15 and 31 per cent. While rebaudioside M at 0.2 mg/mL showed complete hydrolysis after 24 hours incubation, only limited hydrolysis was seen at 2.0 mg/mL due to low solubility at this concentration. The authors suggested that given the common metabolic pathway, safety data available for individual steviol glycosides can be used to support the safety of other purified steviol glycosides.

Table 5 Formation of steviol from rebaudioside A, E and steviolbioside in pooled human faecal homogenates (at 2.0 mg/mL)

Steviol glycoside (2.0 mg/mL)	Incubation time (hours)	Females		Males	
Reb A		F1[a]	F2[b]	M1[c]	M2[d]
	4	2.1	1.6	1.3	1.1
	8	10.7	5.9	4.5	3.1
	16	61.9	32.7	26.5	16.0
	24	78.9	64.7	60.1	34.8
Reb E	4	2.0	2.3	1.8	2.2
	8	11.2	7.9	4.6	5.9
	16	56.2	37.5	28.0	26.1
	24	67.8	59.4	58.4	54.2
Steviolbioside	4	7.9	7.2	5.4	6.2
	8	25.3	20.0	13.8	14.6
	16	60.5	62.4	35.3	31.4
	24	77.9	66.8	54.0	50.4

Source: Purkayastha et al 2016

[a] Female, Asian n=3 replicates
[b] Female, Caucasian n=3 replicates
[c] Male, Asian n=3 replicates
[d] Male, Caucasian n=3 replicates

Table 6: Formation of steviol from rebaudiosides A, F and M, and of dulcoside A in pooled human faecal homogenates (at 0.2 mg/mL)

Steviol glycoside	Incubation time	Female n=3 replicates		Male n=3 replicates	
		Mean % metabolised	SD	Mean % metabolised	SD
Reb A	4	6.5	1.6	4.0	1.3
(0.2 mg/mL)	8	22.9	10.6	7.6	3.1
	16	80.3	14.9	34.4	13.0
	24	101.5	4.6	74.8	6.0
	48	114.4	1.9	114.0	3.9
Reb A	4	0.6	0.2	0.5	0.1
(2.0 mg/mL)	8	2.4	0.9	1.2	0.4
	16	10.3	4.9	4.5	1.4
	24	32.5	7.0	12.2	4.7
	48	87.5	10.9	66.2	11.4
Reb F	4	0.9	0.2	0.7	0.1
(0.2 mg/mL)	8	3.1	0.4	1.6	0.7
	16	12.2	4.1	4.7	1.3
	24	41.2	6.0	15.9	9.4
	48	82.0	7.8	63.6	22.3
Reb F	4	0.1	0.0	0.4	0.0
(2.0 mg/mL)	8	0.4	0.0	0.3	0.1
	16	1.9	0.2	2.0	1.6
	24	6.6	1.0	2.9	0.9
	48	31.1	6.5	15.0	6.6
Dulcoside A	4	2.3	1.2	2.2	0.7
(0.2 mg/mL)	8	6.4	4.0	5.5	2.0
	16	19.7	4.1	15.0	7.4
	24	60.3	11.4	42.8	19.8
	48	89.3	16.2	96.7	3.7
Dulcoside A	4	0.4	0.1	0.3	0.1
(2.0 mg/mL)	8	1.3	0.8	1.1	0.5
	16	5.4	1.6	5.1	1.6
	24	29.5	16.7	43.2	9.5
	48	75.4	37.7	104.5	7.9
Reb M	4	5.6	1.8	3.2	1.3
(0.2 mg/mL)	8	24.9	14.1	7.2	2.6
	16	67.6	11.6	32.3	10.2
	24	96.6	5.6	75.5	8.5
	48	105.0	2.0	102.2	3.2
Reb M[e]	4	0.2	0.1	0.1	0.1
(2.0 mg/mL)	8	0.2	0.1	0.1	0.1
	16	0.3	0.1	0.2	0.1
	24	0.4	0.1	0.3	0.1
	48	0.6	0.3	0.6	0.2

Source: Purkayastha et al 2016.

[e] The Purkayastha et al 2016 paper reports this as 'rebaudioside F' rather than 'rebaudioside M'. This appears to be a typographical error in the text.

The results of the unpublished study provided by the Applicant are consistent with the findings of the studies summarised above. Complete metabolism of rebaudioside A and a test article consisting of a mixture of steviol glycosides was found within 24 hours when incubated in male and female pooled human faecal homogenates under anaerobic conditions.

Roberts et al 2016

Roberts et al (2016) conducted a comparative pharmacokinetic study in rats and humans.

Male and female Sprague-Dawley rats (6/sex/group; aged between 7 to 9 weeks; fasted overnight) were administered a single dose of stevioside (in deionised water or polyethylene glycol) by oral gavage at dose levels of 40 and 1000 mg/kg bw, respectively. These doses were equivalent to 16 and 40 mg/kg bw expressed as steviol. Blood samples were collected prior to dosing and at 0.5,1, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h following administration. Plasma samples were analysed for steviol and steviol glucuronide concentration using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Toxicokinetic parameters were calculated from the mean plasma concentration data and included the maximum achieved concentration of the analyte (C_{max}), time to maximum achieved concentration (T_{max}), area under the plasma concentration time curve (AUC) and the terminal half-life ($T_{1/2}$).

Ten healthy male subjects between the ages of 20 and 45 with a Body Mass Index (BMI) in the normal range (18.50 to 29.99 kg/m^2) were employed in the human study. A dose of 40 mg/kg bw stevioside (reported to be ≥95% pure), equivalent to 16 mg/kg bw steviol, mixed with 50 mL of deionised water was consumed by subjects over a 5 minute period. Subjects had been fasted overnight and then received breakfast 0.5 h after dosing. Lunch, snacks and dinners were provided up to 11 h after dosing. Blood samples were collected at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48 and 72 h. Analysis for plasma steviol and steviol glucuronide was performed using LC-MS/MS.

Plasma steviol concentrations were below the limit of quantification (20 ng/mL) for both male and female rats in the 40 mg/kg bw dose group at 0, 0.5 and 1.0 hours post dosing. Steviol was detected in the plasma of both male and female rats at 2 h post dosing, and increased through 4 and 6 hours post dosing. At 36 h in males, and 24 h in females, plasma steviol concentrations were below the limit of quantification. In rats administered 1000 mg/kg bw stevioside, measureable plasma steviol concentrations were detected one hour after dosing and increased through 6 and 12 hours in both males and females, with a greater increase seen in females. Toxicokinetic parameters in rats are summarised in Table 7.

Sex-related differences were noted in C_{max}, AUC and T_{max} at the high dose for steviol and steviol glucuronide.

Table 7: Summary of toxicokinetic parameters for steviol and steviol glucuronides in male and female rats following administration of stevioside at 40 or 1000 mg/kg bodyweight

Parameter	Females	Males	Females	Males
	40 mg/kg bw		1000 mg/kg bw	
Steviol				
C_{max}(ng/mL)	87.1 (17.2)	76.0(14.9)	1960 (1580)	539 (163)
T_{max} (h)	6	4	12	6
AUC_{last}(ng*h/mL)	605 (66.7)	581 (81.5)	25,700 (12,800)	9290 (918)
$T_{1/2}$ (h)	NC	7.8	10	5.0
Steviol glucuronide				
C_{max}(ng/mL)	200 (45.0)	160 (32.2)	6550 (5480)	1410 (314)
T_{max} (h)	4	6	12	8
AUC_{last}(ng*h/mL)	2500 (204)	2310 (316)	79,700 (44,100)	29,2000 (4470)
$T_{1/2}$ (h)	6.5	NR	8.9	7.5

Source: Roberts et al (2016)

NR not reported; NC not calculated

The authors noted that all human volunteers were included in the Safety, Efficacy Evaluable (EE) and first Per Protocol (PP1) populations. One subject had to be excluded from the second Per Protocol Population (PP2) due to outlying baseline values of steviol glucuronide. Plasma steviol was detected in nine of the 10 members of the EE and PP1 populations, and in eight of the nine members of the PP2 population. The peak plasma concentration of steviol was seen at 19 – 20 hours post consumption of 40 mg/kg stevioside, while that of steviol glucuronide was seen at 21– 22 hours post dosing. Toxicokinetic parameters are described in Table 8. C_{max} for steviol was similar in rats and humans however, T_{max} occurred later in humans than in rats.

Table 8: Summary of the mean (SEM) pharmacokinetic data for steviol and steviol glucuronide in the plasma of healthy males following consumption of stevioside

Parameter	Plasma steviol			Plasma steviol glucuronide		
	EE (n=10)	PP1 (n=10)	PP2 (n=9)	EE	PP1 (n=10)	PP2 (n=9)
Total $AUC_{-0.75-72h}$(ng*h/mL)	1630 (394)	1630 (394)	1680 (438)	136,000 (25,100)	136,000 (25,100)	137,000 (28,000)
C_{max} (ng/mL)	77.2 (17.4)	77.2 (17.4)	72.45 (18.7)	4470 (702)	4470 (702)	4400 (781)
T_{max} (hour)	19.2 (4.08)	19.2 (4.08)	20.0 (4.47)	21.6 (3.49)	21.6 (3.49)	22.7 (3.71)
$T_{1/2}$ (hour)	15.4 (NA)	15.4 (NA)	15.4 (NA)	18.6 (4.03)	18.6 (4.03)	18.8 (4.64)

Source: Roberts et al 2016

3.2.3 Safety of Steviol Glycosides

3.2.3.1 Genotoxicity

The weight of evidence (JECFA 2010; A1037 FSANZ 2011 and EFSA 2011 assessments) indicates that steviol glycosides are unlikely to be genotoxic. A recent review also concluded that the database for steviol glycosides is robust and does not indicate stevioside or rebaudioside A are genotoxic (Urban et al 2013).

3.2.3 Discussion

FSANZ conducted a hazard assessment for steviol glycosides as part of the assessment of Application A540 – Steviol Glycosides as Intense Sweeteners (FSANZ 2008). An ADI of 0–4 mg/kg bodyweight (bw) per day was established by applying a 100-fold uncertainty factor to the no-observed-adverse-effect level (NOAEL) of 970 mg/kg bw/day of stevioside (equivalent to 383 mg/kg bw/day steviol) in a 2-year rat carcinogenicity study. This ADI covers steviol glycoside mixtures with different ratios of stevioside/rebaudioside.

The Applicant submitted a dossier that included summary information related to the safety of steviol glycosides including information on toxicokinetics and metabolism, degradation products and major metabolites. FSANZ also conducted a literature search to update its 2015 review conducted as a part of Application A1108 (FSANZ 2015). There were no findings in these publications that indicated a need to change the ADI of 0–4 mg/kg bw/day, expressed as steviol equivalents, established by FSANZ in 2008.

In vitro studies consistently showed the biotransformation of steviosides, rebaudiosides and dulcosides to steviol. This is in agreement with earlier studies conducted on stevioside and rebaudioside A (e.g. Gardana et al 2003; evaluated in FSANZ 2008). It is expected that all steviol glycosides will be hydrolysed completely to steviol by gut microflora. The existing ADI of 0-4 mg/kg bodyweight, which is expressed on the basis of steviol, is therefore applicable to all steviol glycosides in stevia leaf.

FSANZ's conclusions are consistent with those of the 82nd meeting of JECFA which confirmed the ADI of 0–4 mg/kg bw, expressed as steviol. With respect to specifications the summary JECFA report states, "The Definition and Assay specification was expanded from nine named leaf-derived steviol glycosides to include any mixture of steviol glycoside compounds derived from *Stevia rebaudiana* Bertoni, provided that the total percentage of steviol glycosides is not less than 95%."

4 Dietary exposure assessment

The Applicant intends to market steviol glycoside mixtures for use as intense sweeteners under the same conditions as those presently approved for steviol glycoside preparations and as such no new dietary exposure assessment was considered necessary for this Application. The use levels for steviol glycosides are expressed as 'steviol equivalents' and as such are not specified for any one particular individual steviol glycoside but are instead based on the total content of the aglycone steviol in the final food product.

The Dietary Exposure Assessment for Application A1037 (seeking to increase the maximum permitted levels of steviol glycosides in ice-cream and a range of beverages) based on a 30% market uptake scenario for broad food groups at maximum levels specified, indicated that estimated dietary exposures to steviol glycosides were less than 60% of the ADI for both mean and 90th percentile exposures for all population groups assessed. The major contributor of steviol glycosides for all assessed population groups (except Australian children aged 2 – 6 years) were water based flavoured drinks (soft drinks, cordials and formulated beverages). Flavoured milk products, closely followed by water based flavoured drinks, were the greatest contributors to total steviol glycosides exposure for Australian children aged 2 – 6 years (FSANZ 2011).

The summary report of the 82nd JECFA meeting (held June 2016) notes that the predicted maximum dietary exposure to steviol glycosides of 4.0 – 4.4 mg/kg bw per day for young children who were high consumers exceeded the upper bound of the ADI (up to 110%). The ADI was not exceeded for other groups.

However, the JECFA assessment does not consider that steviol glycosides are likely to present a health concern for any age group, considering the conservative nature of the dietary exposure estimate.

4.1 Conversion factors

As previously described in this document, a series of *in vitro* and *in vivo* metabolism studies suggest that all steviol glycosides are degraded to steviol by bacteria in the human colon. Part of the steviol is absorbed by the colon wall and transported to the liver by portal blood. Steviol glucuronide is formed in the liver and this is then excreted in the urine (Guens et al 2006). Steviol is considered to be the sole absorbed and common metabolite of steviol glycosides in the human (Renwick and Tarka 2008). As such, the ADI of steviol glycosides is expressed as steviol equivalents.

As the current steviol glycoside concentrations are provided in terms of steviol equivalents, conversion factors for all potential steviol glycosides present in the stevia leaf would normally be required to be provided and added to the conversion factor table. Conversion factors are currently provided for each of the permitted steviol glycosides (Table 4.1) as indicated in Standard 1.3.1 – Food additives.

Table 4.1: Conversion Factors of Steviol Glycosides in Order to Determine Steviol Equivalents

Steviol glycoside	Conversion factor
Dulcoside A	0.40
Rebaudioside A	0.33
Rebaudioside B	0.40
Rebaudioside C	0.33
Rebaudioside D	0.28
0Rebaudioside F	0.34
Rebaudioside M	0.25
Rubusoside	0.50
Steviol	1.00
Steviolbioside	0.50
Stevioside	0.40

Adapted from subsection 1.3.1—(7) in Standard 1.3.1

Any additional conversion factors will be required to be added to the conversion factor table in Standard 1.3.1.

5 Risk characterisation

No evidence was found to suggest that the expansion of the definition of steviol glycosides for use as sweetener to include all steviol glycosides present in *S. rebaudiana* poses any public health and safety risks. It is expected that all steviol glycosides will be hydrolysed completely to steviol by gut microflora.

References

Awney HA, Massoud MI, El-Maghrabi S (2011) Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing male rats. J Appl Toxicol, 31(5):431–438.

Awney HA (2012) Letter to the Editor regarding 'Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing rats'. J Appl Toxicol, 32(2):152.

Carakostas M (2012) Letter to the Editor regarding 'Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing rats'. J Appl Toxicol, 32(2):149–151.

EFSA (2010) Scientific Opinion on safety of steviol glycosides for the proposed uses as a food additive. European Food Safety Authority. 10 March 2010.
http://www.efsa.europa.eu/fr/efsajournal/pub/1537.htm

EFSA (2011) Revised exposure assessment for steviol glycosides for the proposed uses as a food additive. European Food Safety Authority. EFSA J 9(1) 1972 [19pp]. doi:10.29-03/j.efsa.2011.1972.
http://www.efsa.europa.eu/en/efsajournal/pub/1972

EFSA (2015) Scientific Opinion on the safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive. (EFSA Panel on Food Additives and Nutrient Sources Added to Food/ANS) (Question no EFSA-Q-2014-00002, adopted on 17 November 2015 by European Food Safety Authority). EFSA J 13(12):4316 [29 pp.]. doi: 10.2903/j.efsa.2015.4316.
http://www.efsa.europa.eu/en/efsajournal/pub/4316.

FSANZ (2008) Application A540 – Steviol Glycosides as Intense Sweeteners. Food Standards Australia New Zealand.
http://www.foodstandards.gov.au/code/applications/Pages/applicationa540stevi3096.aspx

FSANZ (2011) Application A1037 – Steviol Glycosides: Increase in Permitted Use Levels. Food Standards Australia New Zealand.
http://www.foodstandards.gov.au/code/applications/Pages/applicationa1037stev4605.aspx

FSANZ (2015) Approval Report – Application A1108: Rebaudioside M as a Steviol Glycoside Intense Sweetener. Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ).
http://www.foodstandards.gov.au/code/applications/Documents/A1108-AppR.pdf.

Gardana C, Simonetti P, Canzi E, Zanchi R, Pietta P (2003) Metabolism of stevioside and rebaudioside A from *Stevia rebaudiana* extracts by human microflora. J Agric Food Chem, 51, 6618–6622.

Gardana C, Scaglianti M, Simonetti P (2010) Evaluation of steviol and its glycosides in *Stevia rebaudiana* leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry. J Chromatogr A, 1217(9):1463–1470.

Geuns JMC, Buyse J, Vankeirsbilck A, Temme EHM, Compernolle F, Toppet S (2006). Identification of steviol glucuronide in human urine. J Agric Food Chem 54(7):2794–2798.

Geuns JMC, Buyse J, Vankeirsbilck A, Temme EHM (2007). Metabolism of stevioside by healthy subjects. Exp Biol Med 232(1):164–173.

Geuns JMC (2008). Analysis of steviol glycosides: validation of the methods. In: Steviol Glycosides: Technical and Pharmacological Aspects: Proceedings of the 2nd Stevia Symposium. Heverlee, Belgium: Euprint ed., pp. 59–78.

Health Canada (2016). *Notice of Modification to the List of Permitted Sweeteners to Enable the Use of Rebaudioside M as a Sweetener in Various Unstandardized Foods*. (Reference Number: NOM/ADM-0065). Ottawa (ON): Health Canada, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch. http://www.hc-sc.gc.ca/fn-an/consult/nom-adm-0065/index-eng.php

Koyama E, Kitazawa K, Ohori Y, Izawa O, Kakegawa K, Fujino A et al (2003). *In vitro* metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. Food Chem Toxicol 41(3):359–374.

Nikiforov AI, Rihner MO, Eapen AK, Thomas JA (2013) Metabolism and toxicity studies supporting the safety of rebaudioside D. Int J Toxicol, 32(4):2612–73.

Prakash I, Bunders C, Devkota KP, Charan RD, Ramirez C, Priedemann C, Markosyan A (2014a) Isolation and characterisation of a novel rebaudioside M isomer from a bioconversion reaction of rebaudioside A and NMR comparison studies of rebaudioside M isolated from *Stevia rebaudiana* Bertoni and *Stevia rebaudiana* Morita. Biomolecules, 4(2):3743–89.

Prakash I, Markosyan A, Bunders C (2014b) Development of next generation Stevia sweetener: Rebaudioside M. Foods, 3:16217–5.

Purkayastha S, Pugh G, Lynch B, Roberts A, Kwok D, Tarka SM (2014) *In vitro* metabolism of rebaudioside B, D, and M under anaerobic conditions: comparison with rebaudioside A. Regul Toxicol Pharmacol, 68(2):259–268.

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

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

Renwick AG (2007) Principles and methods of toxicology (5th ed.) Editor W Hayes. Philadelphia (PA) Taylor and Francis/CRC Press p 188.

Renwick AG, Tarka SM (2008). Microbial hydrolysis of steviol glycosides. Food Chem Toxicol 46(7, Suppl.):S70S–74.

Roberts A, Lynch B, Rogerson R, Renwick A, Kern H, Coffee M, Cuellar-Kingston N, Eapen A, Crincoli C, Pugh G, Bhusari S, Purkayastha S, Carakostas M (2016) Chemical-specific adjustment factors (inter-species toxicokinetics) to establish the ADI for steviol glycosides. Regul Toxicol Pharmacol, 79: 91–102.

Toyoda K, Matsui H, Shoda T, Uneyama C, Takada K, Takahashi M (1997) Assessment of the carcinogenicity of stevioside in F344 rats. Food Chem Toxicol, 35(6):597–603.

USFDA (2013) Agency Response Letter GRAS Notice No. GRN 000473. US Food and Drug Administration. 2 December 2013. http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm382202.htm

United States Pharmacopeial Convention (2014) Food Chemicals Codex. 9th ed, United States Pharmacopeial Convention, Rockville, MD, Steviol glycosides. http://online.foodchemicalscodex.org/online/pub/index?fcc=10&s=0&oYr=2016&oMo=6&oDa=1

Urban JD, Carakostas MC, Brusick DJ (2013) Steviol glycoside safety: Is the genotoxicity database sufficient? Food and Chemical Toxicology 51: 386–390.

Waddell WJ (2011) Letter to the Editor regarding 'Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing rats'. J Appl Toxicol, 31(6):59–5596.

Wheeler A, Boileau AC, Winkler PC, Compton JC, Prakash I, Jiang X et al (2008). Pharmacokinetics of rebaudioside A and stevioside after single oral doses in healthy men. Food Chem Toxicol 46(7, Suppl.):S54–S60.

WHO (2005) Steviol glycosides. In: Evaluation of certain food additives. Sixty-third report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 8-17, 2004, Geneva. Geneva: World Health Organization (WHO); 343–9, 138. (WHO Technical Report Series, no. 928; http://whqlibdoc.who.int/trs/WHO_TRS_928.pdf)

WHO (2006) Steviol glycosides. In: *Safety Evaluation of Certain Food Additives*. Sixty-third Meeting of the Joint FAO/WHO Expert Committee on Food Additives, June 8-17, 2004, Geneva, Switz. (WHO Food Additives Series, no 54). Geneva, Switz.: World Health Organization (WHO) / International Programme on Chemical Safety (IPCS), pp. 1171–44, 638. Available at: http://whqlibdoc.who.int/publications/2006/9241660546_eng.pdf.

WHO (2007) Chemical and Technical Assessment of Steviol Glycosides, prepared for the 69th meeting of the Joint FAO/WHO Expert Committee of Food Additives. http://www.fao.org/fileadmin/templates/agns/pdf/jecfa/cta/69/Steviol_glycosides.pdf

WHO (2009) Steviol glycosides (addendum). In: *Safety Evaluation of Certain Food Additives and Contaminants*. Sixty-ninth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 17-26-29, 2008, Geneva, Switz. (WHO Food Additives Series, vol 60). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 1832–19. Available at: http://whqlibdoc.who.int/publications/2009/9789241660600_eng.pdf.

WHO *(Joint FAO/WHO Expert Committee on Food Additives)*, 2010. *Steviol glycosides*. In: Combined Compendium of Food Additive Prepared at the 73rd JECFA (2010) and published in FAO JECFA Monographs 10 (2010), superseding specifications prepared at the 69th JECFA (2008) and published in FAO JECFA Monographs 5 (2008).

WHO (2011) Seventy-third Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting – Food Additives. Summary and conclusions.

WHO (2012) Seventy-sixth Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting – Food Additives. Summary and conclusions.

WHO (2016) Eighty-second Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting – Food additives. Summary and conclusions, 7-16 June 2016, Geneva, Switzerland. Issued 21 June 2016. http://www.fao.org/3/a-bl839e.pdf

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