Application for the Approval of Rebaudioside M under Australia and New Zealand Food Standard Code Standard 1.3.1– Food Additives and Standard 1.3.4 – Identity and Purity

PureCircle Limited
915 Harger Road, Suite 250
Oak Brook, Illinois, 60523
USA

December 16, 2014

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ADMINISTRATIVE DATA

Contact Details of Applicant and Organisation

[redacted]
[redacted] & Regulatory Affairs

PureCircle Limited
915 Harger Road, Suite 250
Oak Brook, Illinois, 60523
USA
Telephone (Office): [redacted]
Telephone (Mobile): [redacted]
Fax: [redacted]
Email: [redacted]

Nature of Business

PureCircle Limited (hereafter PureCircle) is the world's leading producer of high purity stevia ingredients for the global food and beverage industry. As part of our business, we are a supplier to the food industry of steviol glycosides.

Details of Other Parties Involved with the Application

The following Scientific and Regulatory Consultant is involved in the preparation submission and stewardship of this application:

[redacted]
Intertek Scientific & Regulatory Consultancy
2233 Argentia Road, Suite 308
Mississauga, Ontario, Canada L5N 2X7
Telephone (Office): [redacted]
Fax: [redacted]
Email: [redacted]

GENERAL REQUIREMENTS

Assessment Procedure

PureCircle considers the most appropriate assessment procedure for the application herein relating to an amendment to *Standard 1.3.1 – Food Additives* of the Australia New Zealand Food Standards Code in order to include rebaudioside M and in *Standard 1.3.4 – Identity and Purity* of the Australia New Zealand Food Standards Code to permit the recognition of an identity and purity specification of steviol glycosides including rebaudioside M. This revision is expected to fall under the General Procedure (Subdivision F), Cost Category Level 2.

Confidential Commercial Information (CCI)

PureCircle requests the information contained within Appendix I be considered confidential commercial information. This section provides a detailed description of the manufacturing process for steviol glycoside preparation including proprietary information and is therefore of significant commercial value to the company. The remainder of the application is considered by PureCircle to be non-confidential.

Exclusive Capturable Commercial Benefit (ECCB)

Steviol glycoside preparations are currently produced by other manufacturing companies, aside from PureCircle. However, rebaudioside M-containing preparations are only produced by PureCircle using a specific manufacturing process. On this basis, PureCircle is seeking permission for the exclusive use of rebaudioside M; therefore, PureCircle anticipates that this application would confer Exclusive Capturable Commercial benefit (ECCB) in accordance with Section 8 of the Food Standards Australia New Zealand (FSANZ) Act with which states:

An **exclusive, capturable commercial benefit** is conferred upon a person who applies for the development of a food regulatory measure or the variation of a food regulatory measure under Section 22 if:

> (a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard or draft variation of the standard that would be prepared in relation to the application; and
>
> (b) any other unrelated persons or bodies, including unrelated commercial entities, would require the agreement of the applicant in order to benefit financially from the approval of the application.

As such, PureCircle is expected to pay the full costs of processing this application.

International and Other National Standards

The safety of steviol glycosides was reviewed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at 4 separate meetings (51st, 63rd, 68 h, and 69th) in 1998, 2004, 2007, and 2008 (JECFA, 1999, 2006b, 2007c, 2009). In 2004, the Committee established a temporary acceptable daily intake (ADI) of 2 mg/kg body weight/day (expressed as steviol) for steviol glycosides based on a study in which pharmacological effects were observed in patients. In 2008, the Committee received additional data on steviol glycosides and upon review of the new data, the Committee concluded that the results from the new human studies were sufficient to remove the additional safety factor of 2 and to establish a full ADI of 4 mg/kg body weight (expressed as steviol) for steviol glycosides. The specifications for steviol glycosides were revised further, requiring not less than 95% of the 7 named steviol glycosides. In 2010, JECFA revised the specifications for steviol glycosides to include 2 additional steviol glycosides, rebaudioside D and rebaudioside F, within the purity criteria (JECFA, 2010). For further details regarding the conclusions of the JECFA meetings, see Section C.2.1.

In March 2011, at the 43rd Session of the Codex Committee on Food Additives (CCFA) recommendations for steviol glycosides provisions in the General Standards of Food Additives (GSFA) were considered (CCFA, 2011).

Other National Standards or Regulations

United States

A notification to the United States (U.S.) Food and Drug Administration (FDA) for purified steviol glycosides with rebaudioside M as the principal component (GRN No. 000473) was recently submitted and the FDA has raised no objections regarding the petitioners' determinations of Generally Recognized as Safe (GRAS) status of the steviol glycoside products for use as general-purpose sweeteners in foods (U.S. FDA, 2013a). Since October of 2014, 31 GRAS notices (GRN Nos. 000252, 000253, 000275, 000278, 000282, 000287, 000303, 000304, 000318, 000323, 000329, 000337, 000348, 000349, 000354, 000365, 000367, 000369, 000375, 000380, 000388, 000389, 000393, 000395, 000418, 000448, 000452, 000456, 000461, 000467, and 000493) for highly-purified steviol glycosides or glucosylated steviol glycosides have been submitted to the FDA for review. The FDA also has raised no objections regarding the petitioners' determinations of GRAS status of the steviol glycoside products for use as general-purpose sweeteners in foods (U.S. FDA, 2008a,b, 2009a-d, 2010a-e, 2011a-i, 2012a-e, 2013b-g). In addition, steviol glycosides with rebaudioside A and stevioside as the principal components proposed for use as a table top sweetener and general purpose non-nutritive sweetener for incorporation into foods, other than infant formulas and meat and poultry products has been submitted to the FDA in May 2014 (GRN No. 000516), the GRAS notification of which is currently "pending" (U.S. FDA, 2014). A high-purity rebaudioside M (GRN No. 000512) and a

high-purity rebaudioside C (GRN No. 000536) also were recently submitted to the FDA for review; however, the GRAS notification of which is currently “pending” (U.S. FDA, 2014).

European Union

The safety of steviol glycosides has been evaluated by the European Food Safety Agency (EFSA) (EFSA, 2010) at the request of the European Commission for purposes of considering permission of steviol glycoside use in food in the European Union (EU). Consistent with JECFA’s evaluation of steviol glycosides, EFSA also allocated an ADI of 4 mg/kg body weight, expressed as steviol equivalents, for steviol glycosides. Subsequently, the European Commission has permitted the use of steviol glycosides as a sweetening agent under Commission Regulation (EU) No 1131/2011 (European Commission, 2011).

Canada

Consistent with other international scientific reviews on the safety of steviol glycosides, Health Canada established an ADI of 4 mg steviol equivalents/kg body weight in July 2012 and recommended that steviol glycosides be approved for use as a sweetening agent (Health Canada, 2012a). Following a brief consultation period, Health Canada approved the use of steviol glycosides as a sweetening agent in a variety of food and beverage categories at levels of up to 0.35%, calculated as steviol equivalents (Health Canada, 2012b). Recently, PureCircle has submitted an application to Health Canada for the approval of the use of rebaudioside M as a high-intensity sweetener. For further details regarding the international status of the use of steviol glycosides, see Section C.2.

Asia

Steviol glycosides are approved for use as a food additive (sweetening agent) in Japan, South Korea, China, Malaysia, Indonesia, Singapore, and Taiwan. In Japan, the Ministry of Health and Welfare approved 3 types of stevia extracts: α-glucosyltransferase-treated stevia, powdered stevia, and stevia extract (Japan Food Chemical Research Foundation, 2014). In addition, purified stevioside (as a crude extract, 50% pure extract, and ≥90% pure extract) and *Stevia rebaudiana* leaf extracts are accepted for general use as sweeteners in a variety of foods and beverages including pickling gum, pickles, dried seafood, meat, fish, soy sauce, bean pastes, sugarless chewing gums, juices, cola, table-top sweeteners, and ice cream in Japan (Marie, 1991; Das *et al.*, 1992; Ferlow, 2005). In India, the Food Safety and Standards Authority of India (FSSAI) has approved the use of steviol glycosides in a number of food and beverage categories as per the official gazette notification (FSSAI, 2012).

Central/South America

Stevioside, *S. rebaudiana* leaves, and highly refined extracts are permitted for use as low-calorie sweeteners in Brazil, Argentina, Paraguay, Uruguay, Mexico, Peru, and Colombia. Rebaudioside M is also permitted as a sweetener of foods and beverages in Colombia.

Nigeria

Rebaudioside M is permitted for use in foods and beverages by the National Agency for Food and Drug Administration and Control. The use-levels of rebaudioside M must be in accordance with maximum levels established for steviol glycosides under Codex Alimentarius Commission's General Standards for Food Additives (GSFA).

Other Jurisdictions

Other countries permitting the use of steviol glycosides include Israel, Russia, Switzerland, Turkey, and Ukraine.

Statutory Declaration

A signed Statutory Declaration for Australia is provided as Appendix G.

Checklist

A completed checklist relating to the information required for submission with this application is provided in Appendix H.

Format of the Application

1. Information related to changes to Standard 1.3.1 – Food Additives

This application for an amendment to clause 2 of Standard 1.3.1 is prepared pursuant to *Section 3.3.1 – Food Additives* of the Application Handbook (FSANZ, 2013) which requires the following structured format to assess an application for a new food additive:

A. General Information on the application

B. Technical Information on the food additive

C. Information on the safety of the food additive

D. Information on dietary exposure to the food additive.

The application is presented in this format. At the start of each section (A-D) the information that must be addressed therein is specified in more detail.

2. Information related to changes to other Standards

It is recognised that in approving the use of steviol glycoside as a food additive, there may also need to be consequential changes to other Standards. These include, but may not be restricted to, the following:

i. Standard 1.3.4 – Identity and Purity

The information on the specifications for steviol glycoside is provided in Section B.6.

ii. Standard 1.3.1 – Food Additives

The information requirements for changes to the commodity standards to allow the addition of steviol glycoside to these foods are provided in all Sections of this application.

A. GENERAL INFORMATION ON THE APPLICATION

In accordance with Section 3.3.1 – Food Additives of the Food Standards Australia New Zealand *Application Handbook* FSANZ (2013) the following general information must be provided:

1. Purpose of the application
2. Justification for the application, including:
    a. technological function of the food additive
    b. the safety of the food additive
    c. the costs and benefits for industry, consumers, and governments associated with the use of the food additive
3. Support for the application.

Each point is addressed in turn in the Section that follows.

A.1 Purpose of the Application

PureCircle is submitting this application concerning steviol glycosides to FSANZ seeking the approval for the use of rebaudioside M at a range of concentrations from ≥50 to ≥95% (see Section B.6.2) for use as an intense sweetener. Currently, steviol glycosides are a mixture comprising of not less than 95% of the 9 named steviol glycosides, which include rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, stevioside, dulcoside A, rubusoside, and steviolbioside as per the steviol glycosides monograph established by JECFA (2010).

A.2 Justification for the Application

A.2.1 Technological Function for the Food Additive

While a wide array of high-intensity sweeteners is already available for use in Australia and New Zealand, including notably other steviol glycoside preparations, rebaudioside M will provide certain additional functional and technological advantages that are presently not attainable with the currently permitted high-intensity sweeteners and in particular existing steviol glycoside preparations. Based on sensory panel studies (see Section B.1.2), rebaudioside M preparations were shown to provide a superior flavour profile compared to that of rebaudioside A. Compared to rebaudioside A-containing products, the test results showed that rebaudioside M-containing products provide a less bitter taste, sweetness linger liquorice taste

and aftertaste in various tests systems at concentrations of 8 or 10%, which was closer to the flavour profile of sucrose. With the continued strive to limit unwanted taste characteristics that are often associated with the use of high-intensity sweeteners in foods and beverages, the specific flavour profiles imparted by high-intensity sweeteners are critical determinants for their practical use as sugar replacement in food. Furthermore, the increased sweetness potency of rebaudioside M-rich preparations will allow for the replacement of greater amounts of sugar in food and beverage at the presently permitted use-levels for steviol glycosides.

A.2.2 Safety of the Food Additive

In order to identify any new data related to the safety of steviol glycosides published since the previous submission to FSANZ regarding steviol glycosides, an updated search of the scientific literature was conducted on November 19, 2014 (*i.e.*, studies published between 2010 and the date of the search). The database searching tool ProQuest was used to search the following scientific databases: MedLine, ToxFile, Agricola, AGRIS, Allied and Complementary Medicine, Biosis Toxicology, Biosis Previews, Foodline: Science, CAB Abstracts, FSTA, NTIS, EMBASE, and Adis Clinical Trials. Keywords related to steviol glycosides (*i.e.*, rebaudioside M or rebaudioside A or rebaudioside B or rebaudioside C or rebaudioside D or rebaudioside F or dulcoside or steviolbioside or rubusoside or steviol or stevioside) were mapped to the relevant date criteria. No other search limitations were incorporated into the search. Reference lists of review articles were searched to ensure all relevant studies published since 2010 were identified. Any new data retrieved are summarised in Section 5 (including metabolism, toxicological, and clinical data).

A.2.3 Costs and Benefits for Industry, Consumers and Government Associated with Use of the Food Additive

Since steviol glycosides are already approved for many food uses at specified use levels within Australia and New Zealand, and PureCircle intends to market rebaudioside M-containing preparations in the same approved food-uses and use-levels, there is no perceived benefit or added cost to government.

As a high-intensity sweetener, rebaudioside M will be used to replace sugar in foods and the group of consumers to whom this would be beneficial would be any individuals that are seeking foods and beverages with reduced calories from sugar for the purposes of maintaining a reduced-calorie diet. This would also include individuals with specific medical conditions that require reduced sugar intakes, such as diabetics, as steviol glycosides do not interfere with glucose homeostasis (EFSA, 2010).

A.3 Support for the Application

Approval of rebaudioside M as a new food additive is of clear interest to the food industry in Australia and New Zealand for a number of reasons:

- The increased sweetness potency of rebaudioside M-rich preparations will allow for the replacement of greater amounts of sugar in food and beverage at the presently permitted use-levels for steviol glycosides.

- Rebaudioside M provides a superior flavour profile compared to that of rebaudioside A.

For these reasons it is expected that rebaudioside M will present an attractive alternative as a sweetener for food manufacturers in Australia and New Zealand. It is anticipated that rebaudioside M may be imported into Australia and New Zealand and that manufacturers would then incorporate it into their products and that in addition, global companies may import the finished product. As mentioned previously, rebaudioside M would benefit individuals who want to maintain a reduced-calorie diet or individuals who have specific medical conditions that require reduced sugar intakes. The application for approval of rebaudioside M as a new food additive in Australia and New Zealand is part of a global regulatory strategy for PureCircle with the equivalent submissions under European food law, and most recently Canada.

B. TECHNICAL INFORMATION ON THE FOOD ADDITIVE

In accordance with Section 3.3.1 – Food additives of the Food Standards Australia New Zealand *Application Handbook* (FSANZ, 2013) the following technical information must be provided:

1. Nature and function of the food additive.
    a. The technological function the additive fulfils
    b. The reason the additive is needed to fulfil this function
2. Information to enable identification of the additive
3. Information on the chemical and physical properties of the additive
4. Information on the impurity profile
5. Manufacturing Process
6. Specifications for identity and purity
7. Information for food labelling
8. Analytical method for detection.

Each point is addressed in turn in the Section that follows.

B.1 Nature and Technological Function of Steviol Glycosides

In Australia and New Zealand, food additives must comply with a monograph published from a specified list of sources, such as JECFA (FSANZ, 2014a). The specifications for steviol glycosides as established by JECFA (2010) have stipulated final steviol glycoside products as containing not less than 95% combined of the 9 named steviol glycosides, which include stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside. However, while steviol glycoside preparations are already available for use in food as sweeteners throughout Australia and New Zealand and many other parts of the world, the steviol glycoside products presently available have technological limitations regarding sweetness quality.

In addition to the already established group of steviol glycosides, more recently, rebaudioside M (also referred to as rebaudioside X) also was identified to occur in the *S. rebaudiana* plant. Rebaudioside M-rich steviol glycoside preparations, similar to other already permitted steviol glycoside preparations for use in food and beverages in Australia and New Zealand, would be used as high-intensity sweeteners for the replacement of sucrose in reduced-calorie or no-sugar-added products. Since sweetness of a high-intensity sweetener is typically dependent on concentration and the matrix, a determination of the sweetness potency of rebaudioside M relative to that of sugar at a range of concentrations was undertaken. It is also well-recognised that the usefulness of high-intensity sweeteners extends beyond its ability to provide sweetness, but also needs to take into account other taste attributes (*i.e*., the taste quality).

B.1.1 Sweetness Potency of Rebaudioside M Preparations

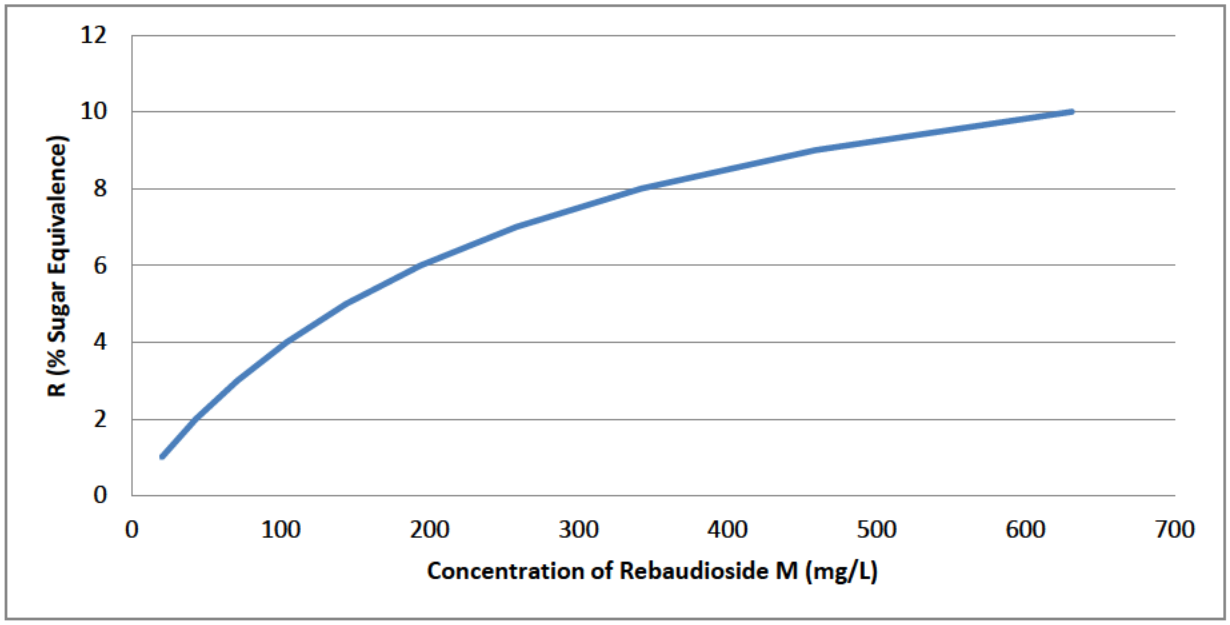
The sweetness potency of rebaudioside M (Lot No. PT010212; 97.4% rebaudioside M) was compared over a range of sucrose equivalencies (see Table B.1.1-1 and Figure B.1.1-1) (Prakash *et al*., 2013). Examined in water (at 4 to 6°C), rebaudioside M was shown to possess a sweetness potency that is approximately 230 to 360 times that of sucrose over a range of sucrose concentrations (4 to 8%). At a sucrose equivalency of 6%, which is customarily used for comparison of the sweetness potency of sweeteners, a preparation rich in rebaudioside M was found to be approximately 300 times as sweet as sucrose (*i.e*., 194 mg/L of the rebaudioside M preparation required to attain the same level of sweetness as a 6% sugar solution). At higher sucrose equivalencies (8 or 10%), representative of sweeter food and/or beverage applications, the potency of rebaudioside M-rich preparations is approximately 160 to 230 times that of sugar. As with most other high-intensity sweeteners the data presented in Table B.1.1-1 and Figure B.1.1-1 show that the sweetness potency of rebaudioside M is concentration-dependent.

Table B.1.1-1 Sweetness Potency of Rebaudioside M Compared to Sugar and Rebaudioside A

R (% Sugar equivalence)	Sweetener Concentration (ppm)		Potency (w/w sugar *vs.* sweetener)	
	Rebaudioside M[a]	Rebaudioside A[b]	Rebaudioside M	Rebaudioside A
1	20	22	500	454
2	43	50	465	400
3	71	86	423	349
4	104	133	385	301
5	144	200	347	250
6	194	300	309	200
7	258	467	271	150
8	342	800	234	100
9	459	1,800	196	50
10	631	-	159	-

[a] Concentration of rebaudioside M = (265 x R)/(14.2 - R) (Prakash *et al.*, 2013).
[b] Concentration rebaudioside A = (200 x R)/(10 - R) (DuBois *et al.*, 1991).

Figure B.1.1-1 Concentration of Sugar (% Sugar Equivalence) *versus* Concentration of Rebaudioside M Required to Achieve the Same Sweetness Intensity



In addition to the presentation of concentrations and sweetness potencies of rebaudioside M at various sucrose equivalencies, for comparison, Table B.1.1-1 also presents the concentrations and sweetness potencies of rebaudioside A over the same range of sucrose equivalencies (calculated based on information provided in DuBois *et al.*, 1991). The data presented in Table B.1.1-1 for both rebaudioside M and rebaudioside A indicate that at every sucrose concentration rebaudioside M has a greater sweetness potency than rebaudioside A, with rebaudioside M being up to 2 times as sweet at rebaudioside A at the higher sucrose concentrations.

Furthermore, it should also be noted that in comparison to rebaudioside A and stevioside, rebaudioside M contains 2 additional glucose units (see Table B.2.2-1). Since it is recognised that all steviol glycosides are hydrolysed to steviol and since there are differences in the molecular weights of different steviol glycosides, the current ADI and the currently permitted use-levels for steviol glycosides are expressed as steviol equivalents. Accordingly, since on a molecular weight basis, steviol comprises less of the overall weight of a rebaudioside M molecule, it will be possible to use greater amounts of rebaudioside M at each permitted use-levels while not exceeding the use-level in terms of steviol content. Combined [the greater sweetness potency and the greater glucose:steviol ratio of rebaudioside M (6:1) *versus* rebaudioside A or stevioside (4 or 3:1)], use of steviol glycoside preparations rich in rebaudioside M will allow, at the currently permitted use-levels, attainment of greater functionality (*i.e*., when used at the permitted use-levels, it will be possible to replace more sugar with rebaudioside M-rich preparations than with other currently available steviol glycoside preparations).

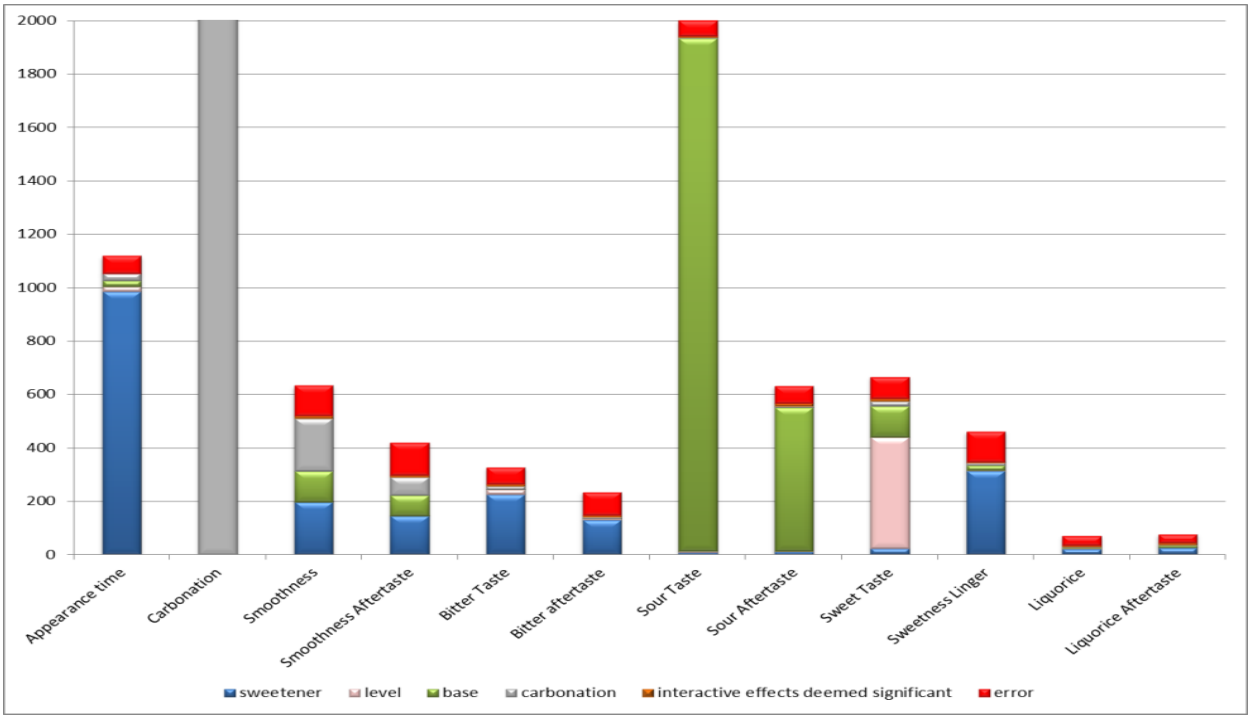
B.1.2 Taste

The sensory profiles of 2 rebaudioside M-containing products (*i.e.,* referred to as RX80 and RX95, with 80 and 95% rebaudioside M purity, respectively) were evaluated by a sensory panel and compared to those of rebaudioside A, aspartame, sucrose, and various blends of the sweeteners in 3 solution systems (phosphoric acid, citric acid, and water) and 2 matrices (carbonated and non-carbonated) at concentrations equivalent to 8 and 10% sucrose [Sensory Research Ltd. (SRL), 2013; see Appendix F for full report]. The experienced panellists reviewed the various beverage samples according to 12 attributes related to appearance of sweetness, taste/flavour, mouthfeel, and aftertaste. There were limited statistical significant interactions between the various factors that distinguish the samples [*i.e*., sweetener type, sweetener concentration, base solution (water or phosphoric or citric acid) and carbonation], indicating that each parameter had a largely independent effect and as such any effects on the sensory experience as a result of changing the sweetener were on “average about the same”, irrespective of the state of the other ‘sample-distinguishing’ parameters. A graphical representation of the weight of each system factor on the various sensory attributes is provided in Figure B.1.2-1, which indicated that:

- appearance time, bitter taste and aftertaste, sweetness linger and liquorice taste, and aftertaste were dictated primarily by the sweetener used,
- sour taste and aftertaste were defined primarily by the solution,
- sweet taste was influenced mostly by the concentration of sweetener, with the solution also having a noticeable effect,
- smoothness and smoothness aftertaste were influenced by the sweetener, the solution, and whether the sample was carbonated, and

- the carbonation attribute was not affected by any factor other than whether the sample was carbonated.

Figure B.1.2-1 Sums of Squares of the Analysis of Variance of the Data for Samples with Single Sweeteners



Note: the carbonation bar is truncated.

Overall, the results indicate that RX80 and RX90 provided similar sensory profiles that were categorised between that of aspartame (closest sensory experience to sucrose) and rebaudioside A (furthest from sucrose). Aspartame was associated with a more bitter taste and aftertaste, sweetness linger, and liquorice taste and aftertaste than sucrose; rebaudioside A was reported to have the highest of these tastes/aftertastes, while RX80 and RX90 fell between aspartame and rebaudioside A on all attributes reviewed. Mixing of sweeteners generally followed predictable gradual sensory changes based on the percentage of each sweetener; however, some synergistic effects were reported for blends of RX80 or RX90 that differed from individual sweetener experiences (*i.e.*, lower appearance time, lower sweetness linger and liquorice taste). An additional sensory study also evaluating the individual sweeteners (RX80 and RX90), as well as blends of the sweeteners corroborates the previous conclusions for the taste profile of the individual sweeteners and their mixtures (Coca-Cola Co., 2013; see Appendix F for full report).

B.2 Information to Enable Identification of Steviol Glycosides

Information to enable the identification of steviol glycosides, including the chemical structure, the chemical name, the molecular weight and formula, and the common name, are presented below.

B.2.1 Identity of Substance

As mentioned previously, steviol glycoside preparations comprising rebaudioside M may be prepared to contain rebaudioside M at a range of concentrations, from ≥50 to ≥95% (see Section B.6.2). In the case of the high rebaudioside M content products (≥95%), the remaining <5% would largely comprise small amounts of non-glycosidic components including saccharides. Likewise, the remainder of the composition of preparations consisting of lower quantities of rebaudioside M would be accounted by higher levels of related steviol glycosides (mainly rebaudioside D, A, and B). In all cases, however, the total steviol glycoside content of the rebaudioside M-containing preparations would be at least 95% as per the requirement for total steviol glycoside content specified in the existing specifications for steviol glycosides.

B.2.1.1 Chemical Name and Chemical Abstract Service (CAS) Number

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

Chemical Abstract Number (CAS) Number: 1220616-44-3

B.2.1.2 Synonyms, and Common Names

Synonyms: Reb X, Reb M, Stevia Leaf Extract

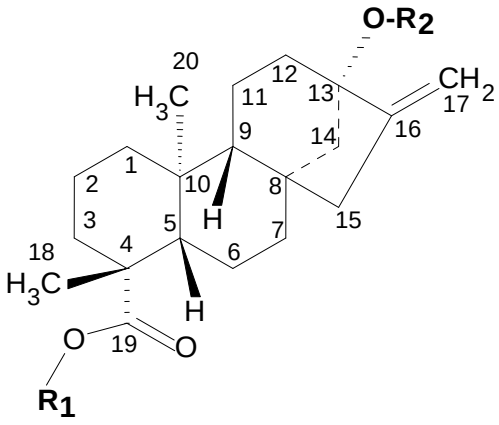
Common Name: Rebaudioside M

B.2.2 Molecular and Structural Formulae, and Molecular Weights of the Components of the Mixture

Rebaudioside M alone or in combination with other steviol glycosides will consist of not less than 95% total steviol glycosides, with rebaudioside M representing at least 50% of the finished product and the remainder comprising the following 9 related steviol glycosides, in any combination and ratio: stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside, steviolbioside, and rubusoside.

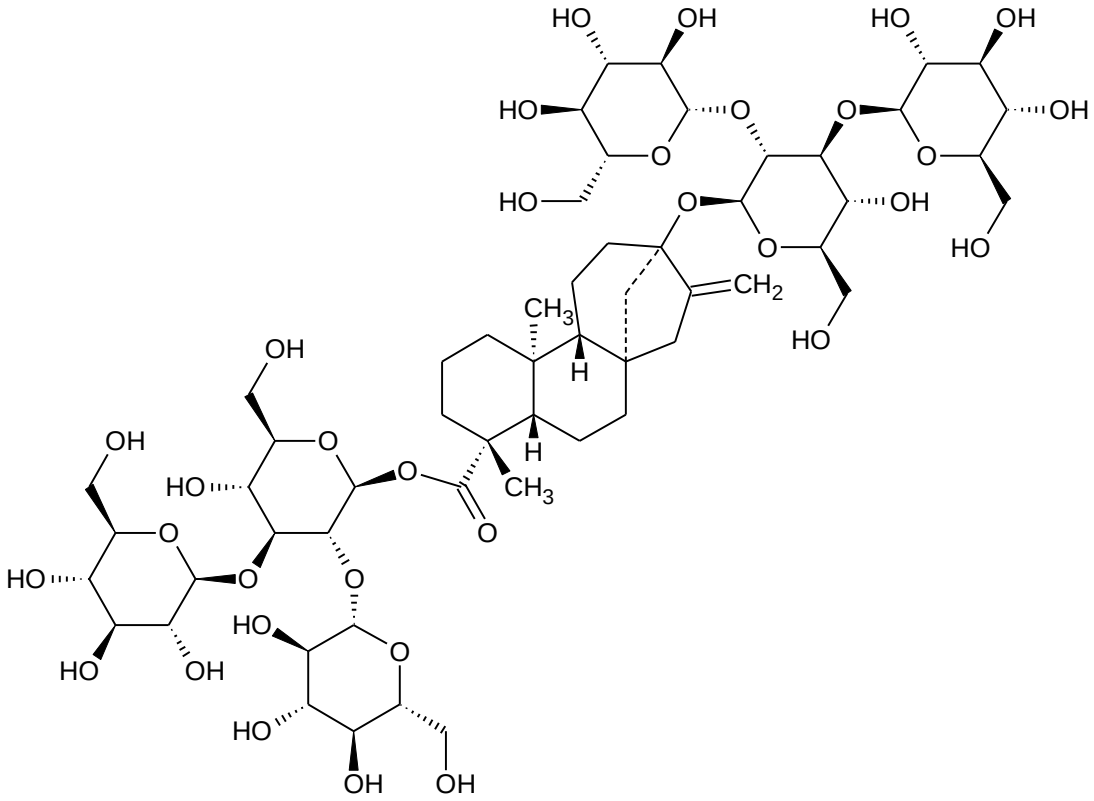
All steviol glycoside constituents are glycosylated derivates of the aglycone steviol and as such, all share the same backbone structure (see Figure B.2.2-1), differing only with respect to the type and number of glycoside units (glucose, xylose, or rhamnose) at positions R_1 and R_2.

Figure B.2.2-1 Backbone Structure for Steviol Glycosides



The structural formula for rebaudioside M is presented in Figure B.2.2-2.

Figure B.2.2-2 Rebaudioside M Structure



The molecular weight and formula, as well as the chemical structure (R_1 and R_2 groups) of rebaudioside M are presented in Table B.2.2-1. For comparison, the molecular weights and formulae, and the R_1 and R_2 groups for each of the 9 steviol glycosides that are already recognised under the purity specifications for steviol glycosides (JECFA, 2010), as well as for the aglycone steviol, also are summarised in Table B.2.2-1. The chemical structure of rebaudioside M is similar to that of rebaudioside A, except that rebaudioside M contains 2 additional β-D-glucosyl moieties at the C-2' and C-3' positions.

Table B.2.2-1 Molecular Weight and Formula, and R-Groups in Backbone Structure (see Figure B.2.2-1)				
Steviol Glycoside	**Molecular Weight**	**Molecular Formula**	**R-Groups in Backbone Structure**	
			R_1	**R_2**
Steviol	318.46	$C_{20}H_{30}O_3$	H	H
Rebaudioside M	**1,291.3**	**$C_{56}H_{90}O_{33}$**	**β-Glc-β-Glc(2→1) | β-Glc(3→1)**	**β-Glc-β-Glc(2→1) | β-Glc(3→1)**
Stevioside	804.88	$C_{38}H_{60}O_{18}$	β-Glc	β-Glc-β-Glc(2→1)
Rebaudioside A	967.01	$C_{44}H_{70}O_{23}$	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside B	804.88	$C_{38}H_{60}O_{18}$	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside C	951.02	$C_{44}H_{70}O_{22}$	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
Rebaudioside D	1,129.15	$C_{50}H_{80}O_{28}$	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside F	936.99	$C_{43}H_{68}O_{22}$	β-Glc	β-Glc-β-Xly(2→1) | β-Glc(3→1)
Dulcoside A	788.88	$C_{38}H_{60}O_{17}$	β-Glc	β-Glc-α-Rha(2→1)
Rubusoside	642.73	$C_{32}H_{50}O_{13}$	β-Glc	β-Glc
Steviolbioside	642.73	$C_{32}H_{50}O_{13}$	H	β-Glc-β-Glc(2→1)

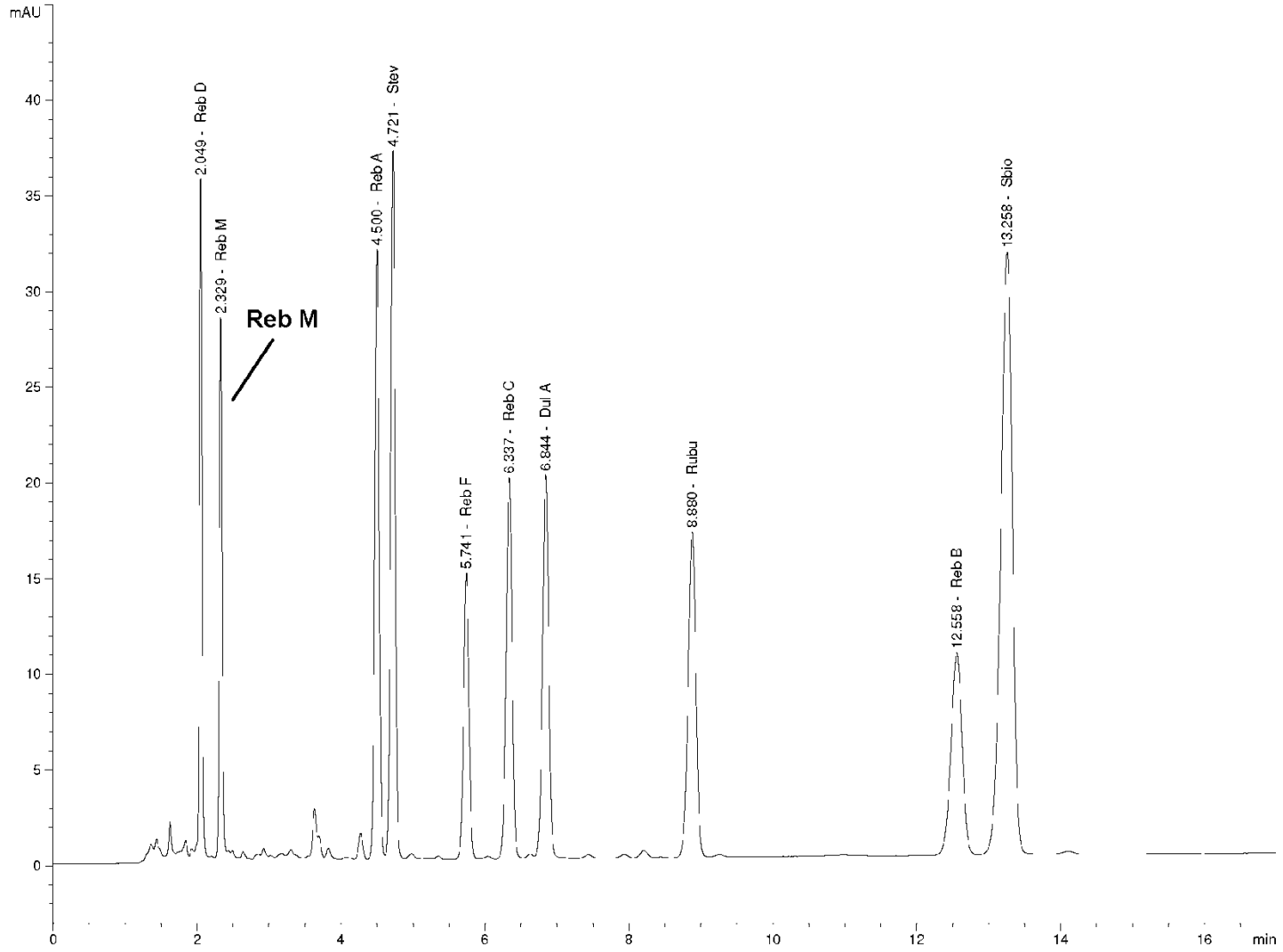
Glc = Glucose; Rha = Rhamnose; Xyl = Xylose.

B.2.3 Spectroscopic and Chromatographic Data

The presence and specific chemical identity of rebaudioside M, isolated from the leaves of *S. rebaudiana*, have been confirmed by 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC), Nuclear Magnetic Resonance (NMR) spectroscopy, as well as high-resolution mass spectroscopic data as outlined by Prakash *et al.* (2013; rebaudioside M is referred to as rebaudioside X in the publication).

A sample chromatogram of steviol glycosides reference standards obtained using high-performance liquid chromatography (HPLC) showing the separation of the different steviol glycosides, including a distinct peak for rebaudioside M, is presented in Figure B.2.3-1.

Figure B.2.3-1 High-Performance Liquid Chromatography Chromatogram for Steviol Glycosides Reference Standards (Prakash *et al*., 2013)



B.3 Information on the Chemical and Physical Properties of Steviol Glycoside Preparation

B.3.1 Physical and Chemical Properties

The pH (pH of approximately 5 to 7; 1% solution) and solubility (slightly soluble) of rebaudioside M are provided as part of the batch analysis of PureCircle's high rebaudioside M content product (rebaudioside M purity ≥96%) (see Table B.6.2.2-1) conducted to demonstrate composition of the product, as well as general conformance with the existing specifications for steviol glycosides established by JECFA.

B.3.2 Solubility

Rebaudioside M is slightly soluble in water, which is similar to the existing solubility requirement for steviol glycosides of "freely soluble in water" as per the established JECFA specifications for steviol glycosides (JECFA, 2010). The solubility of rebaudioside M was assayed as part of the batch analysis conducted for the high rebaudioside M product (see Section B.6.2.2 and Appendix A for Certificates of Analysis) using the JECFA method for determination of solubility.

B.4 Information on the Impurity Profile

B.4.1 Analysis and Limits for Environmental Contaminants - Microbiological Characteristics

Five (5) sample, non-consecutive lots of a steviol glycoside preparation rich in rebaudioside M (96.14 to 96.60% rebaudioside M; see Section B.6.2.2-1 for full analysis) (Lot Nos. PT090212, PT100412, PT110212, PT200212, and PT230212) were analysed for a series of possible microbiological contaminants common to the food industry using standard microbial tests (for certificates of analysis see Appendix A). The results of the analysis are presented in Table B.4.1-1. Microorganisms (total levels) in the preparations were consistently either not detectable or identified at very low levels (10 to 120 colony forming units/gram). Individual types of microorganisms, including *Escherichia coli*, *Salmonella*, and *Listeria*, were not present in any of the tested samples.

Table B.4.1-1 Summary of the Microbiological Product Analysis for Five (5) Lots of a Steviol Glycoside Preparation Rich in Rebaudioside M

Microbiological Parameter	Manufacturing Lot Nos.				
	PT090212	PT100412	PT110212	PT200212	PT230212
Total Plate Count (CFU/g)[a]	ND	20	ND	10	120
Yeast (CFU/g)[b]	ND	ND	ND	ND	ND
Mould (CFU/g)[b]	ND	ND	ND	ND	ND
Total Coliforms (MPN/g)[c]	ND	ND	ND	ND	ND
Escherichia coli count (MPN/g)[d]	ND	ND	ND	ND	ND
Thermophilic Acidophilus bacteria (CFU/g)[e]	ND	ND	ND	ND	ND
Guaiacol producing bacteria (in 1 gram)[e]	Negative	Negative	Negative	Negative	Negative
Staphylococcus aureus (CFU/g)[f]	ND	ND	ND	ND	ND
Salmonella sp. (in 25 grams)[g]	Absent	Absent	Absent	Absent	Absent
Listeria (in 25 grams)[h]	Absent	Absent	Absent	Absent	Absent

CFU = colony forming units; ND = not detected.

[a] AOAC (2005). Method 966.23. In: Official Methods of Analysis of the Association of Official Analytical Chemists: Vols. 1&2, 18th edition, Current through Revision 1, 2006). Arlington (VA): Association of Official Analytical Chemists (AOAC).

[b] Standards Australia (1997). Food microbiology. Method 2.2: Examination for specific organisms—Colony count of yeasts and moulds. (Australian/New Zealand Standard no Standard AS 1766.2.2). Sydney, Australia: Standards Association of Australia/SAI Global.

[c] BSi (1991). Methods for Microbiological examination of food and animal feeding stuffs — Part 3: Enumeration of coliforms — Most probable number technique. (BS 5763-3:1991 ISO 4831:1991). British Standard (BS) / International Organization for Standardization (ISO).

[d] BSi (1993). Methods for Microbiological examination of food and animal feeding stuffs — Part 8: Enumeration of presumptive Escherichia coli. Most probable number technique. (BS 5763-8:1994 ISO 7251:1993). British Standard (BS) / International Organization for Standardization (ISO).

[e] Japan Fruit Juice Association (2007). The United Test Method for Thermo-Acidophilic Bacilli. Tokyo, Japan, Japan Fruit Juice Association, Working Group for Unification of Test Method for TAB, Research Committee for Fruit Juice Technologies.

[f] U.S. FDA (2001). Staphylococcus aureus (Chapter 12). In: Bacteriological Analytical Manual (BAM). College Park (MD): U.S. Food and Drug Administration (U.S. FDA). Available at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm [Page Last Updated: 02/21/2014].

[g] BSi (2012). Microbiology of Food and Animal Feed. Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella. Enumeration by a miniaturized most probable number technique. (PD CEN ISO/TS 6579-2:2012). British Standard (BS) / International Organization for Standardization (ISO). Information available at: http://shop.bsigroup.com/ProductDetail/?pid=000000000030278960.

[h] U.S. FDA (2011). Detection and Enumeration of Listeria monocytogenes (Chapter 10). In: Bacteriological Analytical Manual (BAM). College Park (MD): U.S. Food and Drug Administration (U.S. FDA). Available at: http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm2006949.htm [Page Last Updated: 02/21/2014].

Additionally, 3 samples of the lower content rebaudioside M extract also were tested for potential environmental contaminants (heavy metals, microorganisms, and pesticides) consistent with the analysis for extracts comprising ≥95% rebaudioside M. Results of the additional microbiological analysis are presented in Table B.4.1-2 (results are included as part of the Certificates of Analysis of Appendix A). No microorganisms were detected in the tested samples at the specified limits.

Table B.4.1-2 Summary of the Microbiological Product Analysis for Three (3) Lots of a Steviol Glycoside Extract Containing Approximately 50% Rebaudioside M

Microbiological Parameter	Manufacturing Lot Nos.		
	PT170513	PT240714	PT300714
Total Plate Count (<1,000 CFU/g)[a]	ND	ND	ND
Yeast (<10 CFU/g)[b]	ND	ND	ND
Mould (<10 CFU/g)[b]	ND	ND	ND
Total Coliforms (<10 MPN/g)	ND	ND	ND
Escherichia coli count (Not detected)	ND	ND	ND
Thermophilic Acidophilus bacteria (Not detected)	ND	ND	ND
Guaiacol producing bacteria (Negative in 1 gram)	Negative	Negative	Negative
Staphylococcus aureus (<10 CFU/g)	ND	ND	ND
Salmonella sp. (Negative in 25 grams)	Negative	Negative	Negative
Listeria (Negative in 1 gram)	Negative	Negative	Negative

CFU = Colony Forming Unit; MPN = Most Probable Number; ND = Not Detected.

B.4.2 Analysis and Limits for Environmental Contaminants - Pesticide Analysis

Pesticide analyses were conducted on the same 5 samples of a rebaudioside M-rich steviol glycoside product as those used for the microbiological analysis (Lot Nos. PT090212, PT100412, PT110212, PT200212, and PT230212). Specifically, the samples were subjected to a multi-residue pesticide screen that covered a range of commonly applied pesticides. No pesticide residues were detected in the finished product (see Appendix B for the analytical reports).

Similarly, additional pesticide analysis also was conducted on the 3 lots of product of lower rebaudioside M content. As for the high-rebaudioside M-content preparations, multi-residue pesticide screens were conducted on the samples. Among others, the samples were screened for organophosphate, organochlorine, and ethylenebisdithiocarbamate pesticides. In all cases, no pesticide residues were detected (detection limits ranged from 0.01 to 0.05 ppm; for Certificates of Analysis, see Appendix B).

B.4.3 Analysis and Limits for Environmental Contaminants - Heavy Metals

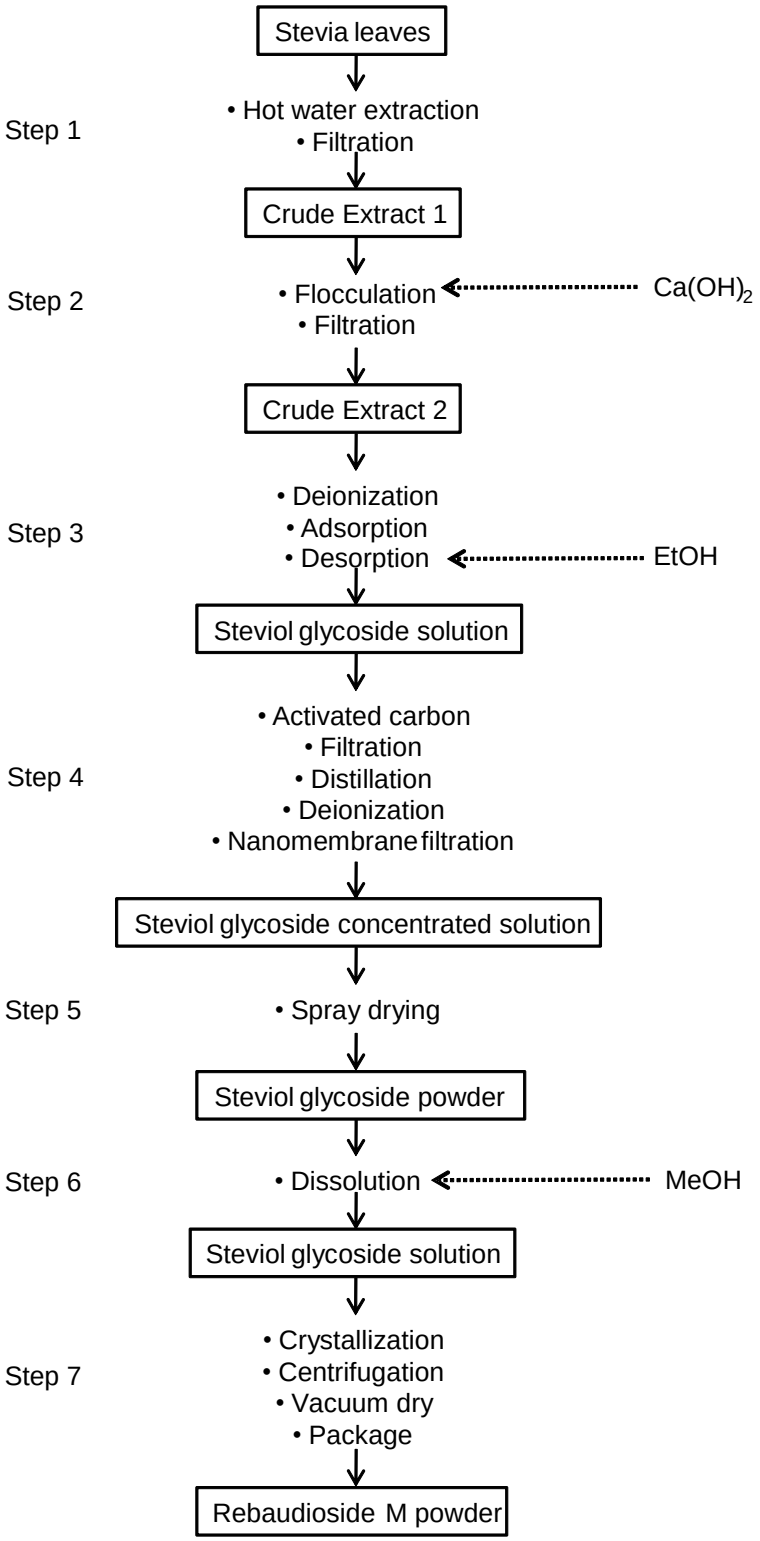
The 5 sample lots (Lot Nos. PT090212, PT100412, PT110212, PT200212, and PT230212) also were analysed for levels of arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) content. Results of the analyses revealed levels below <1 ppm for all 4 parameters (see Appendix A for certificates of analysis). As per the existing specifications for steviol glycosides (see Section 1.3.1), limits of ≤1 mg/kg are specified for As and Pb. The heavy metal analyses, therefore, indicate that steviol glycoside preparations enriched in rebaudioside M would be consistent with the existing specifications for heavy metals for steviol glycosides.

B.5 Manufacturing Process

B.5.1 Overview of the Manufacturing Process

A schematic overview of the production process for a steviol glycoside preparation rich in rebaudioside M is illustrated below in Figure B.5.1-1. The production process for steviol glycoside preparations enriched in rebaudioside M falls within the scope of the processes generally accepted for the production of steviol glycosides that meet the current specifications. Additional details of the manufacturing process are provided within Appendix I, which contains confidential information. Specifically, the overall scheme for the production of a rebaudioside M-rich preparation is consistent with that described in the Chemical and Technical Assessment (CTA) of steviol glycosides as published by JECFA (2007b). The CTA describe a biphasic process, whereby crushed stevia leaves are extracted with hot-water and the resulting extract is subject to isolation and purification (by use of ion-exchange chromatography) at the first stage. This initial stage may be subsequently followed by additional purification steps, including further recrystallisation and separation steps, involving use of either ethanol or methanol as the solvent. The additional purification steps are needed to yield final products with high rebaudioside A or high stevioside and/or rebaudioside A, in which the composition of the purified extract that is recognised as a food additive contains no less than 95% of the 9 known steviol glycosides (listed in Table B.6.1-1) (JECFA, 2010). It is well recognised that the difference in crystallisation rate and solubility of a particular molecule can be effectively utilised to achieve selective crystallisation of the molecule of interest. Thus, much like the processes that have been manipulated to achieve preparations rich is rebaudioside A and stevioside, it is possible to specifically modify certain parameters at the recrystallisation stage (*i.e*., specific number of crystallisation steps, solvent concentration, as well as temperature and duration of the process) resulting in selective crystallisation of rebaudioside M. The compositional analysis of high rebaudioside M content (≥95%) and lower grade (≥50%) rebaudioside M products produced using the above described process is presented in Section B.6.2.1. Therefore, by appropriately modifying the manufacturing process, products that contain 95% of steviol glycosides, with rebaudioside M representing more than 50% of the finished product can be obtained. The remainder of the rebaudioside M-containing products' composition could contain any combination of the following 9 related steviol glycosides: stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside, steviolbioside, and rubusoside for a total steviol glycoside content of not less than 95%.

Figure B.5.1-1 Schematic Overview of the Production Process for a Steviol Glycoside Preparation with Rebaudioside M as the Characterising Steviol Glycoside



$Ca(OH)_2$, calcium hydroxide; EtOH, ethanol; MeOH, methanol.

B.5.1.1 Summary of Manufacturing Process of Rebaudioside M

The manufacturing process of rebaudioside M is similar to the general method of extracting steviol glycosides from the leaves of *S. rebaudiana*. Briefly, the crushed stevia leaves are extracted with hot-water and the resulting extract is subject to isolation and purification (by use of ion-exchange chromatography). This initial stage is followed by additional purification steps, including further and repeated recrystallisation and separation steps. Through the manipulation of these purification steps (*i.e.*, specific number of crystallisation steps, solvent concentration, as well as temperature and duration of the process) the manufacturer is able to selectively crystallise a preparation high in rebaudioside M. This process results in a preparation that contains 95% of steviol glycosides, with rebaudioside M representing more than 50% of the finished product and the remainder comprising the following 9 related steviol glycosides, in any combination and ratio: stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside, steviolbioside, and rubusoside.

B.5.2 Raw Materials

The production process utilised to obtain steviol glycoside preparations with a high rebaudioside M content involves use of processing aids that are already recognised for use in the manufacture of steviol glycoside preparations. These are briefly outlined below. As with all other already recognised steviol glycoside preparations, the leaves of the *S. rebaudiana* Bertoni plant are used as the starting material for the production of preparations which contain high levels of rebaudioside M.

B.5.2.1 Identity of the Plant Source of the Additive

Like all other recognised steviol glycosides listed in the steviol glycoside specification, rebaudioside M occurs naturally in the leaves of *S. rebaudiana* plant, which comprises the primary source for steviol glycoside preparations rich in rebaudioside M. S*. rebaudiana* is a shrub of the *Asteraceae* plant family that was originally native to north-eastern Paraguay and abutting regions of Brazil and Argentina. Current cultivation of this plant, however, has expanded globally throughout parts of North and South America, Europe, and Asia (Lemus-Mondaca *et al*., 2012). The plant, which is harvested on an annual basis, can grow to 1 meter in height or more, in a range of sub-tropical soils that provide sufficient moisture and drainage. The leaves of the plant are harvested for their steviol glycoside content, which is estimated to be approximately 15% of the dry leaf weight (Lemus-Mondaca *et al*., 2012). These leaves have been used by native tribes in South America for centuries to sweeten herbal teas (Lewis, 1992; Geuns, 2003).

B.5.2.2 Alcohol Solvents

Consistent with the previously described manufacturing process, food-grade ethanol [meeting the specifications of the Food Chemicals Codex (FCC, 2014)] is used as a desorption solvent during the production process of rebaudioside M (see Appendix C for the certificate of analysis). High-purity methanol, also meeting the specifications of the FCC (2014), is used to re-crystallise rebaudioside M from the refined steviol glycoside powder (see Appendix C for the certificate of analysis). Both ethanol and methanol are removed from the product during the crystallisation phase of the production process in accordance with Good Manufacturing Practice (GMP). Residual levels of ethanol and methanol in the final product are discussed in Section 1.3.2.2. As presented in Section 1.3.2.2, analysis of the final product revealed levels of not more than 70 and 850 mg/kg of methanol and ethanol, respectively, which are well below the acceptable limits for residues of either solvent as per the existing purity criteria for steviol glycosides established by JECFA in the steviol glycoside specifications (JECFA, 2010**)**.

B.5.2.3 Resins

The adsorption resin used in the production of rebaudioside M-containing extracts consists of a styrene and divinyl benzene copolymer. Ion-exchange resins are used during the production process to remove various impurities, including minerals, from the steviol glycoside extract. The crude steviol glycoside solution is passed through the ion-exchange column during 2 steps of the production process (Steps 3 and 4 of Figure B.5.1-1). The resin was tested for levels of heavy metals (cadmium, lead, mercury, and chromium), as well as polybrominated biphenyls (PBBs) and polybrominated biphenyl ethers (PBBEs) (for the certificate of analysis, see Appendix C).

An adsorption resin consisting of divinyl benzene is used to purify the steviol glycoside extract (at Step 3 of Figure B.5.1-1) by separating the steviol glycosides from other impurities. The resin was submitted for testing to determine levels of lead and volatile organic compounds including benzene, toluene, m-, p-, and o-xylene (for the certificate of analysis, see Appendix C).

The ion-exchange columns also comprise copolymers of styrene and divinyl benzene, with $-SO_3Na$ as the functional group in the case of the cation exchange column and $–N(CH_3)_3Cl$ as the functional group in the case of the anion exchange column (Data Sheets are provided in Appendix C). Sulphonated copolymer of styrene and divinylbenzene are permitted for use as ion exchange resins during the manufacturing process of any food in accordance with GMP (as per Standard 1.3.3 Processing Aids; FSANZ, 2014b).

Product samples of low- and high-rebaudioside M content were tested for potential resin leachates, including styrene, divinylbenzene (DVB), trimethylamine (TMA), and triethylamine (TEA). In the case of the lower rebaudioside M content samples (50 to 60%), the same product

lots used for the analysis of environmental contaminants/pesticide residues (Lot Nos. PT170513, PT240714, and PT300714 – see Appendix B for the Analytical Report) were subjected to the leaching analysis. Testing of higher rebaudioside M content product (≥95% rebaudioside M) was performed with samples from 3 new production lots (Lot Nos. PT310714, PT050814, and PT130814[1]). In all cases, the results of the analysis demonstrated absence of any resin residues in the final products, with the analytes testing below the respective limits of detection (styrene: 0.1 ppm; divinylbenzene: 0.1 ppm; trimethylamine: 0.05 ppm; and triethylamine: 0.04 ppm).

B.5.2.4 Other Processing Aids

High-purity calcium hydroxide [$Ca(OH)_2$], meeting the current specification listed in the FCC (2014), is used as a flocculant during the production of the intermediate steviol glycoside extract (for the certificate of analysis, see Appendix C). Activated carbon, meeting appropriate food-grade specifications, is used as a decolourising agent in the production process of the intermediate steviol glycoside extract.

B.6 Specification for Identity and Purity

B.6.1 Current Official JECFA Chemical Specifications for Steviol Glycosides

Commercially available steviol glycoside preparations must comply with the current specifications for steviol glycosides established by JECFA (2010), as presented in Figure B.6.1-1. Presently, the specifications stipulate such preparations as containing not less than 95% of 9 named steviol glycosides: stevioside, rebaudiosides A, B, C, D, and F, steviolbioside, rubusoside and dulcoside.

[1] Additional analyses (batch analysis for conformance with specifications and environmental contaminants analyses) also were performed with the 3 new lots of high rebaudioside M content extracts. Results of the analyses are included along with the additional analyses on the low rebaudioside M content extracts in the respective Appendices.

Figure B.6.1-1 Current Specifications for Steviol Glycosides as Established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2010)

STEVIOL GLYCOSIDES

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). An ADI of 0 – 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

SYNONYMS INS no. 960

DEFINITION The product is obtained from the leaves of *Stevia rebaudiana* Bertoni. The leaves are extracted with hot water and the aqueous extract is passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is washed with a solvent alcohol to release the glycosides and product is recrystallised from methanol or aqueous ethanol. Ion exchange resins may be used in the purification process. The final product may be spray-dried.

Stevioside and rebaudioside A are the component glycosides of principal interest for their sweetening property. Associated glycosides include rebaudioside C, dulcoside A, rubusoside, steviolbioside, rebaudioside B, rebaudioside D, and rebaudioside F generally present in preparations of steviol glycosides at levels lower than stevioside or rebaudioside A.

Chemical name

<u>*Stevioside*</u>: 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
<u>*Rebaudioside A*</u>: 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

CAS. number

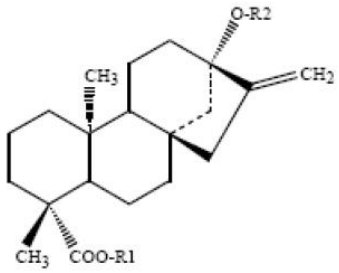
Stevioside: 57817-89-7
Rebaudioside A: 58543-16-1

Chemical formula

Stevioside: $C_{38}H_{60}O_{18}$
Rebaudioside A: $C_{44}H_{70}O_{23}$

Structural formula

The 9 named steviol glycosides:



<u>Compound name</u>	<u>R1</u>	<u>R2</u>
Stevioside	*β*-Glc	*β*-Glc-*β*-Glc(2→1)
Rebaudioside A	*β*-Glc	*β*-Glc-*β*-Glc(2→1) |*β*-Glc(3→1)
Rebaudioside B	H	*β*-Glc-*β*-Glc(2→1) |*β*-Glc(3→1)
Rebaudioside C	*β*-Glc	*β*-Glc-*α*-Rha(2→1) |*β*-Glc(3→1)
Rebaudioside D	*β*-Glc-*β*-Glc(2→1)	*β*-Glc-*β*-Glc(2→1) |*β*-Glc(3→1)
Rebaudioside F	*β*-Glc	*β*-Glc-*β*-Xyl(2→1) |*β*-Glc (3→1)
Dulcoside A	*β*-Glc	*β*-Glc-*α*-Rha(2→1)
Rubusoside	*β*-Glc	*β*-Glc

	Steviolbioside H *β*-Glc-*β*-Glc(2→1) Steviol (R1 = R2 = H) is the aglycone of the steviol glycosides. Glc, Rha and Xyl represent, respectively, glucose, rhamnose and xylose sugar moieties.
Formula weight	Stevioside: 804.88 Rebaudioside A: 967.03
Assay	Not less than 95% of the total of the 9 named steviol glycosides on the dried basis.
DESCRIPTION	White to light yellow powder, odourless or having a slight characteristic odour. About 200 - 300 times sweeter than sucrose
FUNCTIONAL USES	Sweetener
CHARACTERISTICS	
IDENTIFICATION	
Solubility (Vol. 4)	Freely soluble in water
Stevioside and rebaudioside A	The main peak in the chromatogram obtained by following the procedure in Method of Assay corresponds to either stevioside or rebaudioside A.
pH	Between 4.5 and 7.0 (1 in 100 solution)
PURITY	
Total ash (Vol. 4)	Not more than 1%
Loss on drying (Vol. 4)	Not more than 6% (105 °C, 2h)
Residual solvent (Vol. 4)	Not more than 200 mg/kg methanol and not more than 5000 mg/kg ethanol (Method I in Vol. 4, General Methods, Organic Components, Residual Solvents)
Arsenic (Vol. 4)	Not more than 1 mg/kg Determine by the atomic absorption hydride technique (Use Method II to prepare the test (sample) solution)
Lead (Vol. 4)	Not more than 1 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under “General Methods, Metallic Impurities)

B.6.2 Analysis of a Steviol Glycoside Product with Rebaudioside M as the Principal Steviol Glycoside and Conformance with Current Steviol Glycoside Specifications

B.6.2.1 Steviol Glycoside Composition

Several related steviol glycosides have been identified in the leaves of the *S. rebaudiana* plant, which possess intense sweetening properties. In order to utilise the steviol glycosides as sweeteners in food, extraction processes have been developed to isolate and purify these plant constituents. While the steviol glycosides share a characteristic backbone (the aglycone, steviol), they do differ with respect to the number and/or type of sugar (glucose, xylose, or rhamnose) moieties attached to the aglycone. As a result, while related, each steviol glycoside is characterised by a somewhat differing sweetness profile. Consequently, it is recognised that slight modifications can be applied at certain stages of the production processes, while still

adhering to the same general principles, to achieve steviol glycoside preparations of a specifically desired composition and thus specific sweetness profile.

Until now, the primary steviol glycosides of interest with respect to commercial uses of steviol glycoside preparations as sweeteners have been rebaudioside A and, to a somewhat lesser degree, stevioside. As such, rebaudioside A and stevioside were until now typically characterised as the principal constituents of currently available steviol glycoside preparations. As previously mentioned, the existing specifications for steviol glycosides as established by JECFA (2010) stipulate that final steviol glycoside products as containing not less than 95% combined of the 9 named steviol glycosides, which include stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside. In addition to the already established group of steviol glycosides, more recently, rebaudioside M also was identified to occur in the *S. rebaudiana* plant. While the related steviol glycoside was first identified in *S. rebaudiana* Morita (Ohta *et al*., 2010), further analysis also revealed its presences in *S. rebaudiana* Bertoni (Prakash *et al*., 2013). Furthermore, analysis of commercial stevia extracts, including highly purified extracts (*e.g*., SG95, RebA 97), demonstrates the existing presence of rebaudioside M, albeit at very low levels, in such preparations in addition to the other already recognised steviol glycosides (see Table B.6.2.1-1). Specifically, in preparations that comprised not less than 95% total steviol glycosides, rebaudioside M was identified at levels in the range of 0.02 to 0.2%. In a less pure stevia preparation comprising only approximately 92% total steviol glycosides, rebaudioside M occurred at a level of 0.26%.

Table B.6.2.1-1 Overview of Analysis of Commercial Steviol Glycoside Preparations from *Stevia rebaudiana* (Bertoni) for Steviol Glycoside Content (Prakash *et al*., 2013)

Steviol Glycoside	Steviol Glycoside Preparation						
	Stevia Extract	SG95	SG95	RebA 80	RebA 80	RebA 97	RebA 97
Reb M	0.26	0.20	0.20	0.15	0.08	0.02	0.02
Rub	ND	0.22	0.18	0.18	ND	ND	ND
Dul A	ND	0.60	0.48	0.15	ND	ND	ND
Stev	28.12	28.82	27.25	4.70	2.46	0.17	0.20
Reb C	11.02	8.53	8.37	2.25	2.62	0.19	0.35
Reb F	2.21	1.56	1.55	0.98	0.90	0.30	0.35
Reb A	49.59	54.86	57.11	85.79	87.82	99.18	98.22
Reb D	0.74	0.86	0.86	1.29	1.74	0.41	1.03
Sbio	ND	0.17	0.13	ND	ND	ND	ND
Reb B	ND	0.84	0.79	1.30	0.46	0.16	0.75
Total	**91.68**	**96.44**	**96.73**	**96.63**	**95.99**	**100.4**	**100.9**

DulA = dulcoside A, ND = not detected; Reb = rebaudioside; Rub = rubusoside, Sbio = Steviolbioside; SG = steviol glycoside; Stev = stevioside.

As part of the continuing efforts to further explore the sweetening potential of the various steviol glycosides, a production process has been developed (Section B.5.1) which allows for the selective isolation of rebaudioside M and results in the production of steviol glycoside preparations enriched in rebaudioside M specifically. As discussed in Section B.1.2, rebaudioside M and high-purity preparations thereof have been found to be sweeter, as well as to possess an improved sweetness quality compared to other currently available steviol glycoside preparations, including those of high rebaudioside A purity. As discussed in greater detail in Section B.5.1, while the general principles underlying the production process used to obtain steviol glycoside preparations of varying steviol glycoside compositions are largely similar, the precise details of the process, particularly at the crystallisation stages, can be adjusted such that desired compositions are achieved. Depending on the number of crystallisation steps employed, it is possible to obtain preparations that are characterised by a rebaudioside M content of at least 50%, including preparations that contain more than 95% rebaudioside M. Table B.6.2.1-2 presents analysis of sample lots of steviol glycoside preparations produced specifically to contain higher levels of rebaudioside M, including high rebaudioside M content preparations (as per the manufacturing process outlined in Section B.5.1; see Appendix A for analysis results/certificates of analysis).

Table B.6.2.1-2 Levels of Steviol Glycosides (% wt/wt) in Several Sample Production Lots of a Steviol Glycoside Preparation Rich in Rebaudioside M

Steviol Glycoside[a]	Lot Numbers				
	PT040213	PT110213	PT010313	PT070213	PT090212, PT100412, PT110212, PT200212, and PT230212[b]
Rebaudioside M (%)	71.17	68.13	83.17	83.35	96.14-96.60
Rebaudioside D (%)	19.74	23.12	15.00	14.09	1.23-1.25
Rebaudioside A (%)	7.57	7.37	0.49	1.00	0.02-0.06
Rebaudioside B (%)	0.73	0.72	0.75	0.91	1.87-1.88
Rebaudioside F (%)	ND	ND	ND	ND	0.02-0.03
Total Steviol Glycosides (%)	99.20	99.34	99.41	99.35	99.35-99.75

ND = Not detected.
[a] Stevioside, rebaudioside F, dulcoside A, rubusoside, and steviolbioside also were analysed, but were not detected in these manufacturing lots. Further analyses are detailed below in Table B.6.2.1-3.
[b] These lots of high rebaudioside M content preparations also were analysed for conformance with current chemical and physical specifications for steviol glycosides (see Section1.3.1.2).

As presented in Table B.6.2.1-2, in the case of less pure rebaudioside M preparations (produced using processes with fewer crystallisation steps), the remainder of the preparations is accounted for by other already recognised steviol glycosides (*i.e.,* rebaudioside A, D, and B) that are presently allowed among the 9 in Australia and New Zealand that may comprise the total steviol glycoside content of not less than 95%, such that in all cases the finished ingredient product meets the purity specification for steviol glycosides (not less than 95% steviol

glycosides). Furthermore, analyses illustrating the steviol glycoside composition of extracts containing ≥95% rebaudioside M are presented below in Table B.6.2.1-3.

Table B.6.2.1-3 Steviol Glycoside Composition of Extracts Containing ≥95% Rebaudioside M

Steviol Glycosides (% w/w on anhydrous basis)	Manufacturing Lot Nos. (%)				
	PT100412	**PT110212**	**PT200212**	**PT230212**	**PT090212**
Rebaudioside M	96.45	96.23	96.32	96.14	96.60
Rebaudioside D	1.23	1.25	1.25	1.25	1.25
Rebaudioside A	0.03	0.03	0.05	0.06	0.02
Rebaudioside B	1.88	1.88	1.88	1.88	1.87
Stevioside	ND	ND	ND	ND	ND
Rebaudioside F	0.02	0.03	0.02	0.02	0.02
Rebaudioside C	ND	ND	ND	ND	ND
Dulcoside A	ND	ND	ND	ND	ND
Rubusoside	ND	ND	ND	ND	ND
Steviolbioside	ND	ND	ND	ND	ND
Total Steviol Glyosides	**99.61**	**99.41**	**99.52**	**99.35**	**99.75**

ND = Not detected

The steviol glycoside composition of extracts containing approximately 50 to 60% rebaudioside M (Lot Nos. PT170513, PT300714, and PT240714) is also analysed and the results are shown below in Table B.6.2.1-4.

Table B.6.2.1-4 Steviol Glycoside Composition of Extracts Containing Approximately 50% Rebaudioside M

Steviol Glycosides (% w/w on anhydrous basis)	Manufacturing Lot Nos. (%)		
	PT170513	**PT300714**	**PT240714**
Rebaudioside M	55.79	58.55	53.76
Rebaudioside D	33.89	34.19	38.90
Rebaudioside A	4.61	3.59	6.65
Rebaudioside B	0.56	0.49	0.23
Stevioside	0.58	0.11	0.02
Rebaudioside F	ND	ND	ND
Rebaudioside C	0.16	ND	ND
Dulcoside A	ND	ND	ND
Rubusoside	ND	ND	ND
Steviolbioside	ND	ND	ND
Total Steviol Glycosides	**95.59**	**96.93**	**99.56**

ND = Not detected

Collectively the above data demonstrate that steviol glycosides of lower rebaudioside M content are characterised by higher levels of some of the other already recognised steviol glycosides, in particular rebaudiosides D, A, and B. While extracts characterised by a ≥95% content of rebaudioside M contain <5% of rebaudiosides D, A, and B combined, extracts with a lower rebaudioside M content (approximately 50%) may comprise close to 40% rebaudioside D and 7% rebaudioside A. The ranges of steviol glycoside concentrations for extracts containing either approximately 50% rebaudioside M or ≥95% rebaudioside M are presented in Table B.6.2.1-5. As noted above, the total steviol glycoside content (combined content of rebaudioside M and the other already recognised steviol glycosides) of extracts containing rebaudioside M is greater than 95%. Furthermore, while the information presented herein comprises only data for extracts containing either approximately 50% or ≥95% of rebaudioside M, it is reasonable to assume that other extracts of intermediate rebaudioside M content may be produced to provide certain desired sweetness profiles for food and beverage applications. In all such cases, the total content of steviol glycosides also will be ≥95%, with rebaudioside D and rebaudioside A, in addition to rebaudioside M, contributing principally to the overall steviol glycoside content, with lower levels of the other recognised steviol glycosides.

Table B.6.2.1-5 Individual Steviol Glycoside Distribution in Extracts Containing Approximately 50% and ≥95% Rebaudioside M

Steviol Glycoside	Range of Concentrations (%)	
	Approximately 50% Reb M[1]	≥95% Reb M[2]
Rebaudioside M	>50 to <60 (53.76-58.55)	≥95.0 (96.14-96.60)
Rebaudioside D*	>30 to <40 (33.89-38.90)	1 to 2 (1.23-1.25)
Rebaudioside A*	>3 to<7 (3.59-6.65)	<0.1 (0.02-0.06)
Rebaudioside B*	<1 (0.23-0.56)	1 to 2 (1.87-1.88)
Stevioside*	<1 (0.02-0.58)	ND
Rebaudioside F*	ND	<0.05 (0.02-0.03)
Rebaudioside C*	<1 (ND-0.16)	ND
Dulcoside A*	ND	ND
Rubusoside*	ND	ND
Steviolbioside*	ND	ND
Total Steviol Glycosides	**>95**	**>95**

Reb M = Rebaudioside M.
*Recognised steviol glycoside by JECFA (JECFA, 2010).
[1] Ranges in parentheses '()' are the lowest and highest concentration of the particular steviol glycoside identified in the analysed lots (see Table B.6.2.1-4).
[2] Ranges in parentheses '()' are the lowest and highest concentration of the particular steviol glycoside identified in the analysed lots (see Table B.6.2.1-3).

The common name and chemical name of the 9 recognised steviol glycosides are summarized below in Table B.6.2.1-6.

Table B.6.2.1-6	Common Name and Chemical Name of the Steviol Glycosides that are Included in the Joint FAO/WHO Expert Committee on Food Additives (JECFA) specification for "Steviol Glycosides"
Common Name	**Chemical Name**
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
Stevioside	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Rebaudioside B	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid
Rebaudioside C (Dulcoside B)	13-[(2-O-α-L-rhamnopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Rebaudioside D	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester
Rebaudioside F	13-[(2-O-β-D-xylofurananosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Rubusoside	13-β-D-glucopyranosyloxykaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Steviolbioside	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid
Dulcoside A	13-[(2-O-α-L-rhamnopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester

B.6.2.2 Conformance with Existing Steviol Glycoside Specifications

Although rebaudioside M is not presently included as one of the 9 glycosides listed in the specifications according to the JECFA monograph that may be combined to make up the assay value of not less than 95% total steviol glycosides (see Figure B.6.1-1), rebaudioside M shares the same backbone structure as all other already permitted steviol glycosides, and differs only with respect to the number of glycosyl units attached to steviol. Furthermore, as discussed above, the glycoside is already present in low amounts in commercially available stevia extracts; however, by appropriately adjusting the generally recognised production process for steviol glycosides (see Section B.5), extracts rich in rebaudioside M also can be obtained.

High Rebaudioside M Content Preparations

Analysis on 5 non-consecutive lots of high rebaudioside M content preparations (Lot Nos. PT090212, PT100412, PT110212, PT200212, and PT230212) produced according to the manufacturing process described in Section B.5.1 was conducted to determine whether such preparations would meet the current specifications for steviol glycosides in Australia and New Zealand (see Table B.6.2.2-1; for Certificates of Analysis see Appendix C). The data demonstrate that (i) the manufacturing process produces a consistent product and (ii) that, apart from the assay value (rebaudioside M is presently not included as one of the glycosides that may comprise the assay value), such preparations do largely meet all other specification criteria for steviol glycosides.

Table B.6.2.2-1 Batch Analysis for Five (5) Lots of a Steviol Glycoside Preparation Containing Approximately ≥95% Rebaudioside M and Comparison to Current Specification for Steviol Glycosides in Australia and New Zealand

Current Specifications for Steviol Glycosides in Australia and New Zealand[1]		Manufacturing Lot Nos.				
Specification Parameter	**Specification Limit**	**PT090212**	**PT100412**	**PT110212**	**PT200212**	**PT230212**
Assay[a]	Not less than 95% stevioside, rebaudiosides A, B, C, D, and F, steviolbioside, rubusoside and dulcoside A on the dried basis.	99.75	99.61	99.41	99.52	99.35
Description[b]	White to light yellow powder	Conforms	Conforms	Conforms	Conforms	Conforms
	Approximately between 200 and 300 times sweeter than sucrose	See Section 2.2.1[2]	See Section 2.2.1[2]	See Section 2.2.1[2]	See Section 2.2.1[2]	See Section 2.2.1[2]
Identification						
Solubility[c]	Freely soluble in water	Slightly soluble	Slightly soluble	Slightly soluble	Slightly soluble	Slightly soluble
Stevioside and Rebaudioside A	The main peak in the chromatogram obtained by following the procedure in Method of Assay corresponds to either stevioside or rebaudioside A	NA[3]	NA	NA	NA	NA
pH (1% solution)[c]	4.5 to 7.0	6.9	4.97	5.87	5.00	5.14
Purity						
Rebaudioside M, anhydrous basis[a]	None[4]	96.60	96.45	96.23	96.32	96.14
Total ash[c]	Not more than 1 %	0.13	0.60	0.13	0.08	0.08
Loss on drying[c]	Not more than 6 % (105°, 2 hr)	3.70	3.46	3.06	3.02	3.50
Residual solvents[d]	Not more than 200 mg/kg methanol	ND	70	70	60	70
	Not more than 5 000 mg/kg ethanol	370	840	850	830	830
Arsenic[e]	Not more than 1 mg/kg	<0.005	0.014	0.007	0.009	0.010
Lead[e]	Not more than 1 mg/kg	0.212	0.300	0.250	0.213	0.238

NA = not analysed; ND = not detected; ppm = parts per million.
[1] Based on the current specification for steviol glycosides established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2010) and adopted in Australia and New Zealand
[2] A preparation rich in rebaudioside M is approximately 350 times as sweet as sucrose at sucrose equivalency of 5%

(see Section A.2.1.2.1).
[3] Not analysed, but the main peak would correspond to rebaudioside M.
[4] The applicant's limit for this parameter for a product rich in rebaudioside M is not less than 50%.

Methods of analysis

[a] JECFA (2010). Steviol glycosides [Prepared at the 73rd JECFA, 2010) and published in FAO JECFA Monographs 10, 2010)]. In: *Combined Compendium of Food Additive Specifications*. (FAO JECFA Monographs 10). 73rd Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 8-17, 2010, Geneva, Switz. Food and Agriculture Organization of the United Nations (FAO), Joint FAO/WHO Expert Committee on Food Additives (JECFA), Rome, Italy. Available at: http://www.fao.org/ag/agn/jecfa-additives/specs/monograph10/additive-442-m10.pdf.
[b] Sensory evaluation.
[c] FAO/JECFA (2006). *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 1st to 65th JECFA Meetings, 1956–2005. (FAO JECFA Monographs 1). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf [Last updated (Web version): August 2011] (p. 41 for solubility determination; pp. 36-38 for pH determination; pp. 53-54 for ash determination; & p. 61 for loss on drying determination).
[d] USP (2013). <467> Residual solvents. In: *United States Pharmacopeia, 36th edition & National Formulary, 31st edition [Online]*. Rockville (MD): U.S. Pharmacopeia (USP) Convention Inc., pp. 5707-5718. Available at http://www.uspnf.com/ [Subscription Only].
[e] AOAC (2005). *Official Methods of Analysis of the Association of Official Analytical Chemists: Vols. 1&2, 18th edition, Current through Revision 1, 2006)*. Arlington (VA): Association of Official Analytical Chemists (AOAC).

As discussed in greater detail in Section A.2.1.2.2, steviol glycoside preparations rich in rebaudioside M are sweeter (approximately 350 times as sweet as sucrose at 5% sucrose equivalency) than currently available steviol glycoside preparations (which are 200 to 300 times as sweet as sucrose) and also possess an improved sweetness quality. Since use of the currently available steviol glycoside preparations at the presently permitted use-levels (expressed as steviol equivalents) does not provide the adequate level of sweetness in certain categories that would allow for full replacement of sugar (see Section A.2.1.2.2), considering the higher sweetness potency of rebaudioside M, it will be possible to attain an even greater reduction in sugar than presently possible with the existing steviol glycoside preparations.

As with other steviol glycoside preparations that conform to the existing specification parameters, the primary impurities in a steviol glycoside preparation rich in rebaudioside M are the alcohol solvents ethanol and methanol, resulting from their use in the production process during the initial extraction phase and subsequent purification steps (see Section B.5.1). Residual ethanol in a rebaudioside M-rich product has been determined to be present at levels in the range of 370 to 850 mg/kg, which is consistent with the existing specification limit for maximum levels of ethanol according to the JECFA monograph for steviol glycoside preparations (≤5,000 mg/kg). Likewise, residual methanol has been determined to be present in a rebaudioside M-rich product at levels in the range of 60 to 70 mg/kg, which also is consistent with the current specification for steviol glycosides according to the JECFA monograph (maximum levels of methanol ≤200 mg/kg).

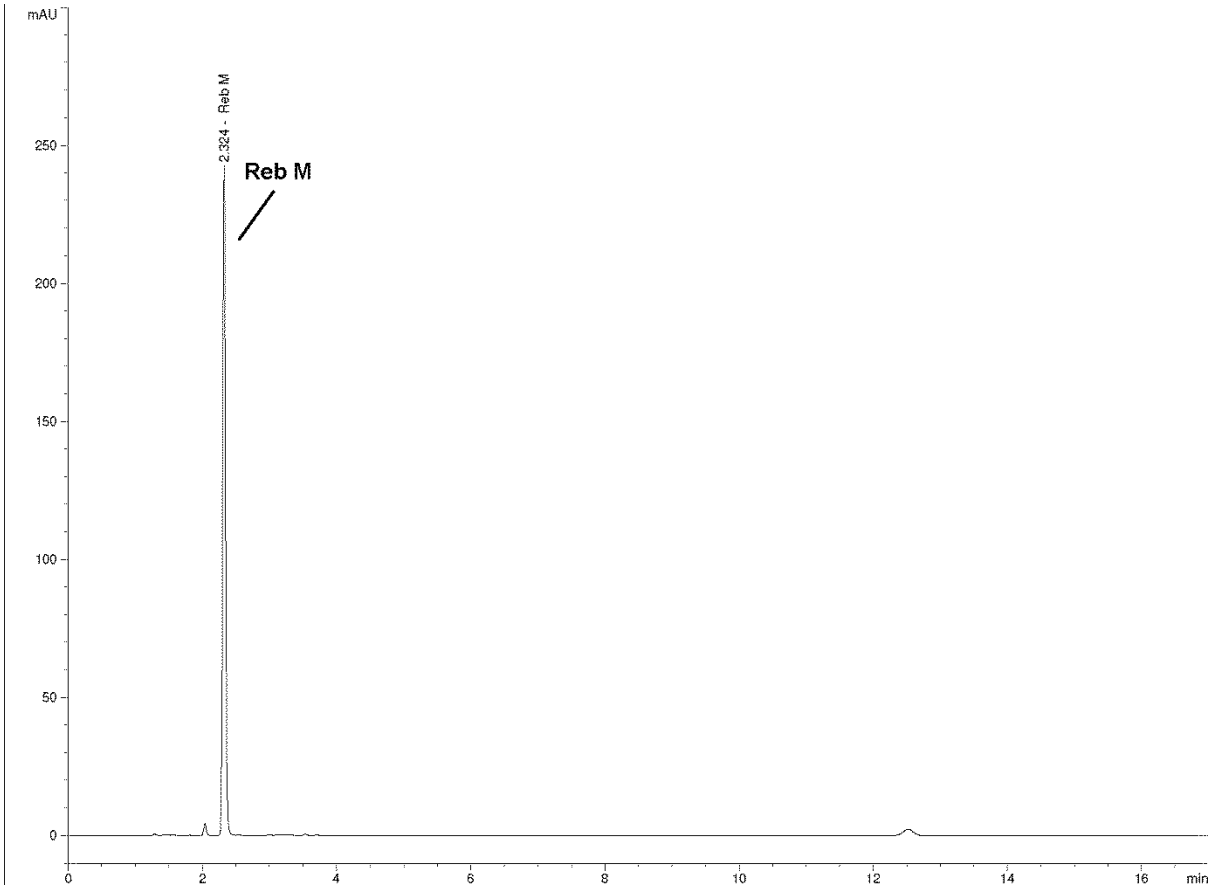
Results for all other parameters [pH, total ash, loss on drying, and heavy metals (also discussed in Section B.4.3)] conformed to the limits set for these parameters as part of the specifications for steviol glycosides according to the JECFA monograph.

As outlined in Table B.6.2.2-1, all analytical methods used to conduct the analysis of the sample lots were either standard JECFA methods (*e.g.*, solubility, pH, assay, identity, loss on drying, total ash content) or alternative, comparable references methods [*e.g.*, the United States Pharmacopeia Method 467 for solvent residue analysis and Association of Official Analytical Chemists (AOAC) methods 993.14 for heavy metal analysis].

While the original JECFA specifications for steviol glycosides specified a limit of not less than 70% for the sum of rebaudioside A and stevioside (JECFA, 2006a), it was subsequently recognised that all steviol glycosides hydrolyse upon ingestion to steviol (see Section C.3.2 for data demonstrating the hydrolysis of steviol glycosides to steviol) and thus it was considered unnecessary to maintain a limit for the sum of rebaudioside A and stevioside (JECFA, 2007a,b).

Finally, stevioside and rebaudioside A may not necessarily be the principal steviol glycosides, as the main peak(s) of a chromatogram of a steviol glycoside preparation could be associated with a steviol glycoside or steviol glycosides other than stevioside or rebaudioside A [*e.g.*, a chromatogram of a high rebaudioside M content preparation (see Figure B.6.2.2-1)].

Figure B.6.2.2-1 Representative HPLC Chromatogram of a High Rebaudioside M Content Preparation Sample



Low Rebaudioside M Content Preparations

In addition to the batch analysis data for extracts containing high levels of rebaudioside M (≥95%) presented above, in order to confirm that products containing rebaudioside M also conform with the existing steviol glycoside specifications [as per JECFA, 2010], further analysis was performed on the 3 sample steviol glycoside extracts containing approximately 50% rebaudioside M. The results for the 3 sample lots (Lot Nos. PT170513, PT240714, and PT300714) are presented in Table B.6.2.2-2. The Certificates of Analysis are attached in Appendix A. The results of the analysis show that products containing 50 to 60% rebaudioside M also meet the existing steviol glycoside specifications.

Table B.6.2.2-2 Batch Analysis for Three (3) Lots of a Steviol Glycoside Extract Containing Approximately 50% Rebaudioside M and Comparison to the Current Specifications for Steviol Glycosides in Australia and New Zealand

Current Specifications for Steviol Glycosides[1]		Manufacturing Lot Nos.		
Specification Parameter	**Specification Limit**	**PT170513**	**PT240714**	**PT300714**
Assay	Not less than 95% stevioside, rebaudiosides A, B, C, D, E and F, steviolbioside, rubusoside and dulcoside A on the dried basis	95.58[2]	99.56[2]	96.92[2]
Description	White to light yellow powder	Conforms	Conforms	Conforms
	Approximately between 200 and 300 times sweeter than sucrose	Conforms[3]	Conforms[3]	Conforms[3]
Identification				
Solubility	Freely soluble to slightly soluble in water	Conforms[4]	Conforms[4]	Conforms[4]
Stevioside and Rebaudioside A	The main peak in the chromatogram obtained following the procedure in Method of Assay corresponds to either stevioside or rebaudioside A	Pass[5]	Pass[5]	Pass[5]
pH (0.1% solution)	4.5 to 7.0	5.33	5.71	6.61
Purity				
Rebaudioside M, anhydrous basis (%)	NA	55.79	53.76	58.55
Rebaudioside D, anhydrous basis (%)	NA	33.89	38.90	34.19
Total ash (%)	Not more than 1 %	<0.005	<0.005	<0.005
Loss on drying (%)	Not more than 6 %	2.56	3.03	1.16
Residual solvents (mg/kg)	Not more than 200 mg/kg methanol	10	10	10
	Not more than 5 000 mg/kg ethanol	840	830	770
Arsenic (mg/kg)	Not more than 1 mg/kg	<0.005	<0.005	<0.005
Lead (mg/kg)	Not more than 1 mg/kg	<0.005	<0.005	<0.005

NA = Not applicable.
[1] Based on the current specification for steviol glycosides (JECFA, 2010)
[2] Rebaudioside M + 9 steviol glycosides (stevioside, rebaudiosides A, B, C, D, and F, steviolbioside, rubusoside and dulcoside A).
[3] Clean sweet taste with no abnormal odour.
[4] Sparingly soluble.
[5] Absorbance at 370 nm – peak consistent with rebaudioside M.

In addition to testing of the samples for levels of arsenic and lead for which maximum limits are set as part of the specifications for steviol glycosides, the samples also were tested for the potential presence of cadmium and mercury (results are included as part of the Certificates of Analysis of Appendix A). As in the case of arsenic and lead (Table B.6.2.2-2), levels of <0.005 ppm were determined for cadmium and mercury.

B.7 Information for Food Labelling

Steviol glycosides are considered to be intense sweeteners and flavour enhancers when added to various food products. Steviol glycosides have been assigned the INS number of 960. Steviol glycosides will be labelled under its functional class, sweetener, either as sweetener (960) or sweetener (steviol glycosides).

B.8 Analytical Method for Detection

At the time of the initial EFSA evaluation of steviol glycosides (EFSA, 2010), the Committee recognised in-house (validated) methods employing HPLC for the identifications of stevioside, rebaudioside A, and other related steviol glycosides (minor steviol glycoside constituents and degradation products) in food and beverage matrices (EFSA, 2010). Two (2) methods for the determination of steviol glycosides in foods using HPLC also were noted to have been published (Geuns, 2008; Gardana *et al*., 2010).

While PureCircle has not at this time developed any methods for the analysis of rebaudioside M in specific food and beverage matrices, beyond the identification of rebaudioside M in solutions (consistent per stability studies/batch analysis), it is anticipated that any such methods will be based on the same principles (HPLC-based methods) as the already established methods.

B.9 Stability

B.9.1 Stability of the Substance and Reaction and Fate in Food

The stability of steviol glycosides has been previously reviewed by a number of scientific advisory bodies involved in the evaluation of steviol glycoside safety (JECFA, EFSA, and FSANZ) and also is discussed in several published studies (Chang and Cook, 1983; Kroyer, 1999). JECFA evaluated the stability of steviol glycosides under conditions mimicking their use in foods at the 68^{h} meeting (JECFA, 2007a). The Committee noted that steviol glycosides do not undergo browning or caramelisation when heated, and are reasonably stable under elevated temperatures used in food processing. Under acidic conditions (pH 2 to 4), steviol glycosides (approximately 90 to 94% purity), are stable for at least 180 days when stored at temperatures up to 24°C. When exposed to elevated temperatures (80°C, in water, 8 hours), however, 4 and 8% decomposition was observed in solutions of steviol glycosides at pH 4.0 and 3.0, respectively, indicating that the stability of steviol glycosides is pH and temperature dependent. When the temperature was increased to 100°C, expectedly higher rates of steviol glycoside decomposition (10 and 40% at pH 4.0 and 3.0, respectively) were observed. Based on the above findings, as well as additional publicly available stability studies, JECFA concluded that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions.

Although the stability of rebaudioside M was not specifically addressed during any of the previous evaluations, it is expected that the stability of rebaudioside M would be similar to that of other steviol glycosides given the similarities in structure. In order to confirm this, additional studies of rebaudioside M stability were conducted. The studies included evaluations of rebaudioside M stability (i) under normal and exaggerated storage conditions and (ii) at a range of temperatures and pH values. In addition, a forced degradation study was conducted to identify possible degradation products in a steviol glycoside preparation rich in rebaudioside M that may be different from degradation products recognised to form in other steviol glycoside preparations. These studies are summarised in Sections 1.4.2 through 1.4.4 and demonstrate that the stability of rebaudioside M is similar to other steviol glycosides, as previously concluded by JECFA and others.

B.9.2 Stability at Different pH's and Temperatures

Samples of a rebaudioside M-rich preparation (Lot PT200212a; 96.32% rebaudioside M) were stored in sealed amber glass vials for up to 26 weeks either at (1) 25°C and ambient relative humidity (RH) (50 to 55%) or (2) 40°C and 75% RH (see Appendix D for study report). Analyses for steviol glycosides were conducted in accordance with JECFA's assay method (JECFA, 2010) and were conducted upon study initiation, and after 12, 24, and 26 weeks of storage. The storage samples were dissolved into a solution with a concentration of 500 mg/L to determine the product's stability. As summarised in Tables B.9.2-1 and B.9.2-2, minimal degradation (<4%) of rebaudioside M was observed when the samples were stored under either condition. Additionally, minimal changes over the study period were observed in levels of the other steviol glycosides detected in the preparation at study initiation (rebaudioside D, A, and B). Furthermore, the rebaudioside M samples remained in compliance with the assay parameter of not less than 95% steviol glycosides throughout the study.

Table B.9.2-1 Storage Stability of Rebaudioside M (Lot PT200212a) for up to 26 Weeks when Stored at 25°C and Ambient Relative Humidity (50 to 55%) as percent (%) dry basis[a,b]

Duration (weeks)	Rebaudioside D	Rebaudioside M	Rebaudioside A	Rebaudioside B	Steviolbioside
0	1.23	99.93	0.10	0.39	ND
12	1.20	98.22	0.57	0.52	ND
24	1.20	97.31	ND	0.68	0.10
26	1.21	97.91	ND	0.66	ND

ND = not detected
[a] Average of 2 duplicates.
[b] In addition to rebaudioside M, all 9 steviol glycosides (stevioside, rebaudioside A, B, C, D, and F, ducoside A, rubusoside, and steviolbioside were analysed; only the glycosides detected in rebaudioside M (Lot PT200212a) are presented here.

Table B.9.2-2 Storage Stability of Rebaudioside M (Lot PT200212a) for up to 26 Weeks when Stored at 40°C and 75% Relative Humidity as percent (%) dry basis[a,b]

Duration (weeks)	Rebaudioside D	Rebaudioside M	Rebaudioside A	Rebaudioside F	Rebaudioside B
0	1.23	99.93	0.10	ND	0.39
12	1.17	96.50	0.73	ND	1.10
24	1.17	95.53	ND	0.10	1.53
26	1.20	96.00	ND	ND	1.59

ND = not detected
[a] Average of 2 duplicates.
[b] In addition to rebaudioside M, all 9 steviol glycosides (stevioside, rebaudioside A, B, C, D, and F, ducoside A, rubusoside, and steviolbioside were analysed; only the glycosides detected in rebaudioside M (Lot PT200212a) are presented here.

In order to confirm that the stability of rebaudioside M-rich preparations is, like that of other steviol glycoside preparations, a function of time, temperature, and pH, the stability of rebaudioside M (Lot No. PT090212a; 96.32% rebaudioside M) was assessed in 0.1 M phosphate buffer at temperatures ranging from 5 to 40°C and at pH values from 2 to 8 (see Appendix D for study report). Rebaudioside M was added to the solutions at target levels of 500 mg/L and the stability was analysed over 24 weeks under the respective conditions. Tables B.9.2-3 to B.9.2-5 summarise the results for solutions at the different pH values (2 to 8) stored at 5, 25, or 40°C, respectively.

Table B.9.2-3 Stability of Rebaudioside M (Lot No. PT090212a) in Solution (500 mg/mL) with Varying pH Values (2.0 to 8.0) Over 24 Weeks Stored at 5°C

Week	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
0 (initial)	96.53[a]	95.85	96.87	95.44	96.18	97.34	97.84
2	95.39	94.21	95.20	94.71	94.74	98.56	95.61
4	94.28	95.44	95.61	93.99	93.83	98.11	96.62
6	93.42	94.46	95.40	93.79	93.97*	97.48	96.21
7	99.31	100.29	101.27	99.22	99.33	103.37	102.43
9	98.32	99.89	100.88	99.35	99.44	103.85	102.37
10	96.32	98.10	99.24	97.48	97.91	101.91	100.57
12	97.37	95.49	100.62	98.58	99.00	103.39	102.10
14	96.27	99.25	99.70	98.18	98.09	102.80	99.30
16	97.02	99.77	101.12	99.40	99.57	103.67	102.49
18	95.69	99.37	100.15	98.55	99.15	103.00	99.58
20	95.71	99.37	100.47	99.03	98.81	102.90	101.76
22	94.71	99.32	100.88	99.10	99.31	103.61	101.76
24	94.53	98.58	99.84	98.72	98.37	102.80	100.88

[a] Values presented represent a percentage (%) of total steviol glycosides in the finished product (*e.g.*, 437.46 mg/kg rebaudioside M ÷ 453.17 mg/kg total glycosides x 100 = 96.53%).

Table B.9.2-4 Stability of Rebaudioside M (Lot No. PT090212a) in Solution (500 mg/mL) with Varying pH Values (2.0 to 8.0) Over 24 Weeks Stored at 25°C

Week	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
0 (initial)	96.53[a]	95.85	96.87	95.44	96.18	97.34	97.84
2	91.48	95.30	96.77	95.23	95.26	99.31	97.68
4	84.61	94.08	95.49	94.26	95.40	98.60	94.76
6	80.19	93.04	95.29	93.84	94.82	95.48	96.07
7	82.18	98.64	101.16	99.28	100.41	104.04	102.30
9	77.50	97.99	100.93	99.08	99.77	101.76	102.11
10	73.99	95.48	98.84	97.51	97.94	102.05	100.78
12	72.17	96.90	100.79	98.87	99.19	103.44	102.11
14	67.33	95.66	99.78	98.18	98.41	102.75	100.82
16	65.10	95.64	100.28	99.04	99.11	103.99	99.28
18	63.09	94.99	99.89	98.04	98.83	100.63	101.18
20	60.82	95.03	99.76	98.43	99.02	103.30	99.25
22	59.04	95.23	100.76	98.96	99.38	103.48	99.98
24	55.81	93.98	99.70	97.74	98.67	100.83	98.55

[a] Values presented represent a percentage (%) of total steviol glycosides in the finished product (*e.g.*, 437.46 mg/kg rebaudioside M ÷ 453.17 mg/kg total glycosides x 100 = 96.53%).

Table B.9.2-5 Stability of Rebaudioside M (Lot No. PT090212a) in Solution (500 mg/mL) with Varying pH Values (2.0 to 8.0) Over 24 Weeks Stored at 40°C

Week	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
0 (initial)	96.53[a]	95.85	96.87	95.46	96.18	97.34	97.84
2	78.96	91.75	95.94	95.21	96.02	99.67	97.97
4	57.12	90.55	95.73	94.28	95.09	96.48	96.92
6	37.98	84.78	94.80	93.86	94.39	98.14	96.41
7	33.04	87.97	100.11	99.25	100.29	101.59	101.88
9	22.58	84.03	99.32	98.77	100.22	101.47	99.51
10	18.32	80.60	97.26	97.08	98.11	99.63	99.30
12	13.76	79.21	98.32	98.28	99.30	103.11	99.27
14	9.52	75.02	97.08	97.12	98.40	102.20	98.43
16	7.21	72.97	97.64	98.14	99.48	103.56	101.60
18	5.47	69.39	96.44	97.25	98.29	102.00	100.08
20	4.31	66.77	95.95	97.45	98.90	102.36	100.59
22	3.61	64.58	96.39	97.77	99.36	100.83	98.97
24	3.25	62.93	95.38	96.81	98.49	101.55	97.72

[a] Values presented represent a percentage (%) of total steviol glycosides in the finished product (*e.g.*, 437.46 mg/kg rebaudioside M ÷ 453.17 mg/kg total glycosides x 100 = 96.53%).

As with all other steviol glycosides, the extent and rate of rebaudioside M degradation were shown to be dependent on pH, temperature, and time. Consistently, rebaudioside M at any pH level was most stable when stored at 5°C. Over the course of the study, minor degradation (<3%) was observed up to 6 weeks at pH 2, 3, 5, and 6 when stored at 5°C. After 6 weeks of storage, the rebaudioside M content increased slightly. Similar effects were observed at pH

values of 3 and 6 when the solution was stored at 25°C and at pH levels of 4 to 6 when stored at 40°C. As these findings are not consistent, it is likely that the lower values are due to analytical variation and not a reflection on the overall stability of rebaudioside M.

At pH 2 after 2 weeks of storage at both 25 and 40°C, rebaudioside M started to degrade with more than 50% of rebaudioside M degraded after 6 weeks of storage at 40°C, in comparison to only approximately 17 and 3% of rebaudioside M degraded when stored at 25 and 5°C, respectively, at the same time point. After approximately 14 weeks of storage at 40°C, the degradation of rebaudioside M at pH 2 began to plateau, reaching a maximum of approximately 93% of rebaudioside M degraded. Similarly, solutions of rebaudioside M at pH 3 stored at 40°C degraded over the 24 weeks of storage, with approximately 66% of the rebaudioside M sample remaining at study completion. Overall, at higher pH values (3 to 8 at 25°C and 4 to 8 at 40°C), no significant degradation of rebaudioside M was observed over 24 weeks, regardless of the storage temperature of the solution, as previously stated. Similar to other steviol glycosides, the stability of rebaudioside M follows the same degradation pathway and is pH-, temperature-, and time-dependent. Therefore, the conclusions regarding the stability of steviol glycosides made by the scientific advisory bodies can be extended to include rebaudioside M.

B.9.3 Stability at Higher Temperatures

A further study has been conducted to assess the stability of rebaudioside M-containing solutions (0.1 M sodium phosphate buffer; pH 6.5) heated to 100°C, with the temperature maintained for 10 minutes (see Appendix D for study report). The assay was performed with products containing either approximately 50% or ≥95% rebaudioside M (Lot Nos. PT240714 and PT130814, respectively). The study results are presented below in Table B.9.3-1. Following 10 minutes of heating of the solutions at 100°C, no significant differences in the concentrations of individual steviol glycosides or in the total steviol glycoside content were observed. The data presented in Table B.9.3-1 provide support for the stability of rebaudioside M-containing steviol glycoside extracts in solution exposed to higher temperature conditions.

Table B.9.3-1 Concentration of Individual Steviol Glycosides in Solutions Prepared with Steviol Glycoside Products Containing Approximately 50 or ≥95% Rebaudioside M

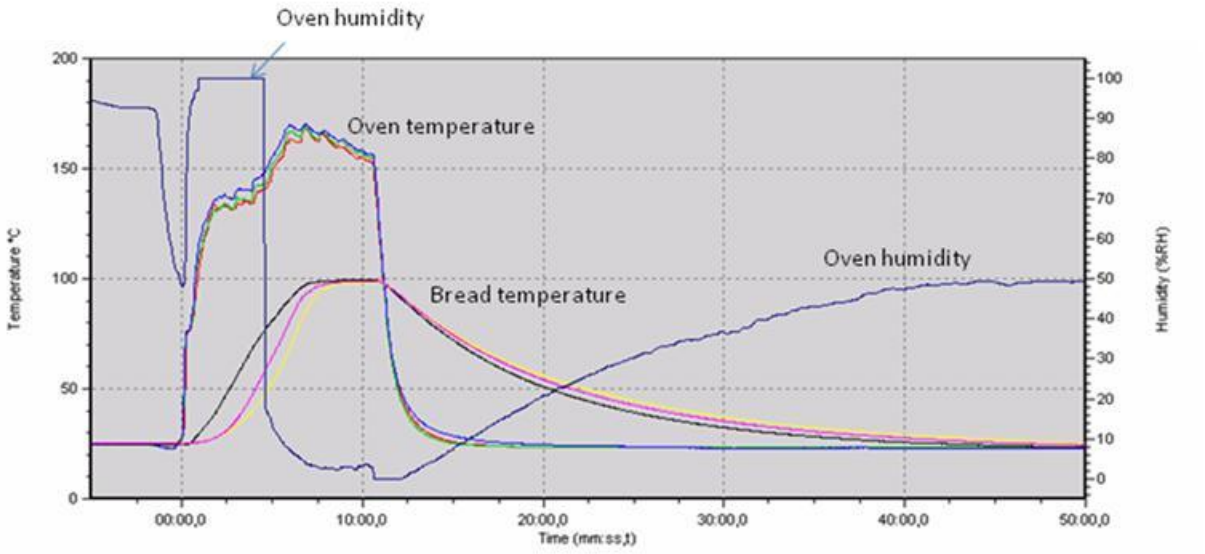
Steviol Glycoside	Concentration (mg/L)			
	50% Reb M[a]		≥95% Reb M[b]	
	Initial	10 min at 100°C	Initial	10 min at 100°C
Rebaudioside M	238.64	240.08	403.12	407.44
Rebaudioside D*	174.74	176.78	10.99	10.89
Rebaudioside A*	29.82	30.68	2.22	2.33
Rebaudioside B*	1.27	1.37	1.10	0.95
Stevioside*	<1	<1	<1	<1
Rebaudioside F*	<1	<1	<1	<1
Rebaudioside C*	<1	<1	<1	<1
Dulcoside A*	<1	<1	<1	<1
Rubusoside*	<1	<1	<1	<1
Steviolbioside*	<1	<1	<1	<1
Total Steviol Glycosides	444.47	448.92	417.43	421.61

*Recognised steviol glycoside under JECFA, 2010
[a] Lot No. PT240714 – rebaudioside M content of 53.76% (see Table B.6.2.1-4).
[b] Lot No. PT130814 – rebaudioside M content of 97.70% (see Appendix A for Certificate of Analysis)

No additional studies have been conducted to assess specifically the stability of steviol glycoside extracts containing rebaudioside M under conditions simulating baking (temperatures >100°C). However, it is noted that while baking involves exposure of baked goods to oven temperatures of >100°C, the internal temperature of baked goods (dough) does not typically exceed 100°C (see Figure B.9.3-1 below) (Zanoni *et al.*, 1993; Lostie *et al.*, 2002; Fehaili *et al.,* 2010; Haegens, 2010). As presented below in Figure B.9.3-1, at the maximum oven temperature of 150°C, the temperature of the dough attained only 100°C.

Considering therefore that samples of rebaudioside M-containing product were shown to be stable in neutral pH solutions (pH 6.5 – pH representative of baked goods) heated to 100°C (as presented above in Table B.9.3-1) and that internal temperatures of food subjected to baking is not expected to exceed 100°C, the results presented above suggest that steviol glycoside extracts containing rebaudioside M also should remain stable upon baking.

Figure B.9.3-1 Bread Dough Temperature at Different Points During Baking and Subsequent Cooling of Rolls, Baked at Approximately 150°C for 10 Minutes (Haegens, 2010)



Furthermore, while based on the data presented to the EFSA Panel evaluating the safety of steviol glycosides it was previously concluded that degradation of steviol glycosides may take place in the presence of high temperatures (*e.g.*, heating, baking) (EFSA, 2010), some additional data on rebaudioside A indicate that steviol glycosides are in fact stable under high-heat conditions.

Specifically, Fry *et al.* (2010) and Fry (2011) examined the stability of rebaudioside A to baking in cookies, pastry, and cakes, prepared using domestic as well as industrial recipes (see Table B.9.3-2). Normal baking processes at oven temperatures up to 205°C resulted in no significant loss of rebaudioside A. This was the case even for the most severe test which involved baking of an empty thin pastry tart shell at 190°C.

Table B.9.3-2 Recovery of Rebaudioside A from Various Baked Goods [Compiled from Fry et al. (2010) and Fry (2011)]

Product	Baking Temperature (°C)	Baking Time (min)	Rebaudioside A Recovered Following Baking (% of initial)
Cookies	190	10	98
Muffins	205	17	100
Banana bread	180	59	96
White cake	170	36	100
Pastry tart shell	190	29	100

Likewise, a recent study by Jooken *et al.* (2012) also corroborates that there are no signs of decomposition of rebaudioside A in dry biscuits baked at a temperature of 185°C for 14 minutes. Furthermore, rebaudioside A also was shown to remain stable following storage of the baked biscuits for 4 weeks at room temperature, which further supports that after baking, rebaudioside A continues to be stable in the finished goods when stored at room temperature (Prakash *et al.*, 2008).

In the case of rebaudioside A degradation, the principal breakdown pathways are confined to changes in the steviol nucleus of the glycoside. More specifically, the changes associated with degradation involve isomerisation or hydration of the C-16 olefin on the steviol nucleus (Prakash *et al*., 2008). Both these processes are mechanistically independent of the attached sugar groups that characterise and define the different glycosides. Accordingly, it is to be expected that degradation involving changes to the steviol nuclei will be the same for all steviol glycosides as for rebaudioside A and that therefore rebaudioside A is a valid model for the stability of other steviol glycosides, including rebaudioside M. As such, the data available on the thermal stability of rebaudioside A may also be relied upon to support the safety of rebaudioside M and extracts thereof.

An additional study to assess the stability of extracts containing only approximately 50% rebaudioside M was conducted (see Appendix D for Study Report). As had been conducted for extracts containing ≥95% rebaudioside M, the stability of the lower rebaudioside M content extract was examined under a series of pH and temperature conditions. Specifically, solutions of pH 2 to 8 were prepared with a steviol glycoside extract containing approximately 50% rebaudioside M (Lot No. PT240714) (0.1 M phosphate buffer) and stored at 5, 25, 37, or 56°C in amber glass vials for up to 3 weeks (see Tables B.9.4-3, B.9.4-4, B.9.4-5, and B.9.4-6 for pH 2, 3, 5, and 7 solutions, respectively). Consistent with the results for the high rebaudioside M content extract, as well as for other steviol glycoside extracts as previously considered by EFSA, the stability of an extract containing rebaudioside M at lower levels (approximately 50%) was shown to be also time-, pH-, and temperature-dependent. At the lowest temperature tested (5°C), minimal changes in the concentrations of the steviol glycosides was apparent in solutions of every pH. At the lower pH levels (pH 2 and 3), considerable degradation of the steviol glycosides occurred at the higher temperatures. At pH 2, degradation of some of the steviol glycosides in the extract (rebaudioside M, D, and A) appeared to be accompanied by rebaudioside B and steviolbioside formation, particularly at 25 and 37°C. In solutions of higher pH (≥4) stored at temperatures of 25°C and 37°C, the total steviol glycoside content remained relatively unchanged over the storage periods (up to 3 weeks). At 56°C, the degree of degradation increased with time, such that decreases of approximately 5 to 15% in the total steviol glycoside content were observed in the pH 4 and greater solutions following 3 weeks of storage.

Table B.9.4-3 Concentration of Individual Steviol Glycosides in Solutions Prepared with a Steviol Glycoside Product Containing Approximately 50% Rebaudioside M – pH 2

Steviol Glycoside	Concentration (mg/L)											
	Initial	5°C			25°C			37°C			56°C		
		Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3
Rebaudioside M	469.73	470.87	471.11	473.42	470.04	387.57	349.81	200.40	101.00	53.12	26.94	8.12	ND
Rebaudioside D*	339.58	340.03	333.04	333.01	298.95	266.78	239.13	122.93	58.33	27.14	1.80	ND	ND
Rebaudioside A*	56.88	57.73	56.78	56.51	52.67	48.29	44.32	27.21	15.84	8.94	7.07	9.57	8.84
Rebaudioside B*	3.54	3.62	3.40	3.91	10.64	13.85	17.57	40.67	37.93	32.31	15.38	7.92	3.82
Rubusoside*	2.87	2.18	2.55	2.19	2.44	3.04	3.14	2.40	2.63	2.98	1.64	1.91	3.01
Steviolbioside*	<1	<1	<1	<1	<1	2.19	4.06	33.70	66.03	99.74	<1	<1	<1
Total SG	872.59	874.43	866.88	869.05	834.75	721.73	658.04	427.30	281.76	224.24	52.83	27.52	15.68

SG = Steviol glycosides.
*Recognised steviol glycoside under JECFA, 2010

Table B.9.4-4 Concentration of Individual Steviol Glycosides in Solutions Prepared with a Steviol Glycoside Product Containing Approximately 50% Rebaudioside M – pH 3

Steviol Glycoside	Concentration (mg/L)											
	Initial	5°C			25°C			37°C			56°C		
		Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3
Rebaudioside M	469.94	474.78	473.35	475.87	473.86	473.59	476.32	472.64	467.20	468.57	439.44	293.01	229.22
Rebaudioside D*	347.37	352.63	346.93	351.32	350.39	344.32	345.64	339.16	322.99	317.20	246.74	186.48	144.46
Rebaudioside A*	58.27	59.55	58.77	58.92	59.51	57.89	57.64	58.32	54.81	54.05	45.78	37.26	30.91
Rebaudioside B*	1.92	1.69	1.44	1.75	2.73	2.16	2.27	5.19	5.95	7.79	31.91	44.25	53.92
Rubusoside*	3.61	2.22	2.61	2.73	1.78	3.28	3.11	2.44	2.66	2.56	1.75	1.99	2.23
Steviolbioside*	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	5.50	15.57	29.47
Total SG	881.11	890.87	883.10	890.59	888.27	881.24	884.98	877.74	853.61	850.17	771.12	578.56	490.21

SG = Steviol glycosides.
*Recognised steviol glycoside under JECFA, 2010

Table B.9.4-5 Concentration of Individual Steviol Glycosides in Solutions Prepared with a Steviol Glycoside Product Containing Approximately 50% Rebaudioside M – pH 5

Steviol Glycoside	Concentration (mg/L)											
	Initial	5°C			25°C			37°C			56°C		
		Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3
Rebaudioside M	471.48	472.00	472.06	475.49	473.06	471.35	475.50	470.87	467.94	470.06	467.05	452.64	451.15
Rebaudioside D*	349.41	353.29	349.18	352.77	352.48	346.44	350.65	349.14	341.74	343.97	344.21	328.23	326.25
Rebaudioside A*	59.36	60.10	59.71	59.06	60.14	58.79	58.94	59.55	57.56	57.06	59.19	56.34	56.34
Rebaudioside B*	2.31	2.04	1.76	1.44	2.28	1.96	2.19	2.86	2.51	2.79	7.93	11.35	15.62
Rubusoside*	3.06	2.88	2.76	3.61	2.83	3.23	3.36	1.40	2.6	3.68	1.07	2.29	1.50
Steviolbioside*	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Total SG	885.63	890.31	885.48	892.37	890.80	881.77	890.64	883.82	872.51	877.57	879.44	850.84	850.86

SG = Steviol glycosides.
*Recognised steviol glycoside under JECFA, 2010

Table B.9.4-6 Concentration of Individual Steviol Glycosides in Solutions Prepared with a Steviol Glycoside Product Containing Approximately 50% Rebaudioside M – pH 7

Steviol Glycoside	Concentration (mg/L)											
	Initial	5°C			25°C			37°C			56°C		
		Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3	Wk 1	Wk 2	Wk 3
Rebaudioside M	469.58	472.95	471.84	475.85	473.53	472.47	475.54	472.11	469.74	472.37	439.17	413.47	391.97
Rebaudioside D*	347.21	352.01	346.90	350.62	351.64	346.32	349.57	349.33	343.02	346.76	323.43	312.55	294.13
Rebaudioside A*	57.84	59.32	58.04	58.41	59.74	58.14	57.90	58.76	57.52	57.33	54.18	54.83	54.42
Rebaudioside B*	2.40	3.10	2.41	2.40	2.64	2.62	2.57	3.18	3.63	4.20	10.26	15.65	22.36
Rubusoside*	3.30	2.81	5.86	5.59	2.99	4.13	4.96	1.60	1.65	1.74	7.92	<1	<1
Steviolbioside*	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Total SG	880.33	890.19	885.05	892.87	890.54	883.68	890.53	884.98	875.57	882.40	834.96	797.45	763.82

SG = Steviol glycosides.
*Recognised steviol glycoside under JECFA, 2010

B.9.4 Forced Degradation

Rebaudioside M (Lot No. VSPC-2973-6B) was subjected to forced degradation conditions, similar to those described above (0.1 M phosphate buffer at pH 2 heated to 80°C for 24 hours), to generate and subsequently identify degradation products. Beyond the identification of rebaudioside M in the sample, 8 degradation products were identified. Five (5) of these degradation products are listed below in Table B.9.4-1 (see Appendix E for Degradant Characterisation Reports).

Table B.9.4-1 Degradation Products

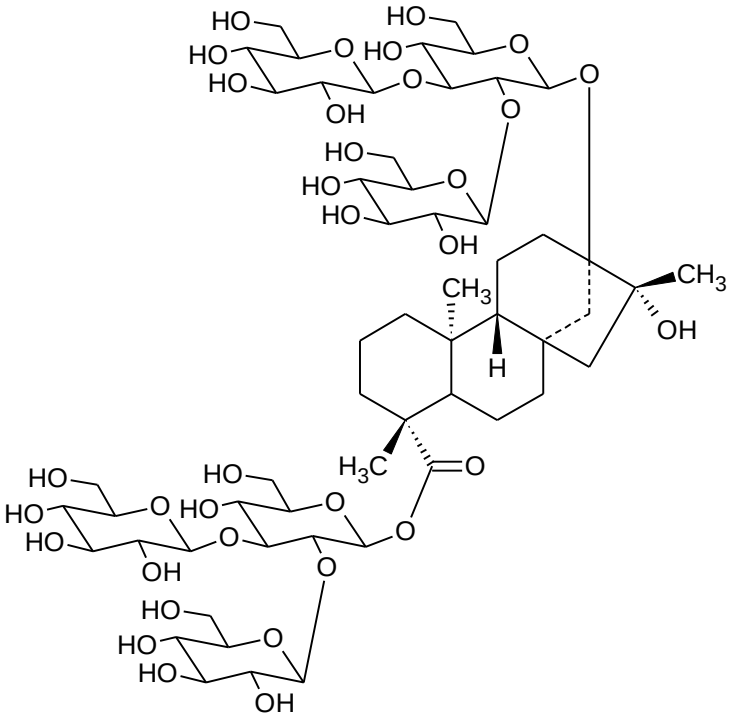
Chemical Name	Chemical Structure
13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-hydroxy-19-oic acid-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester] (CC-00280)	

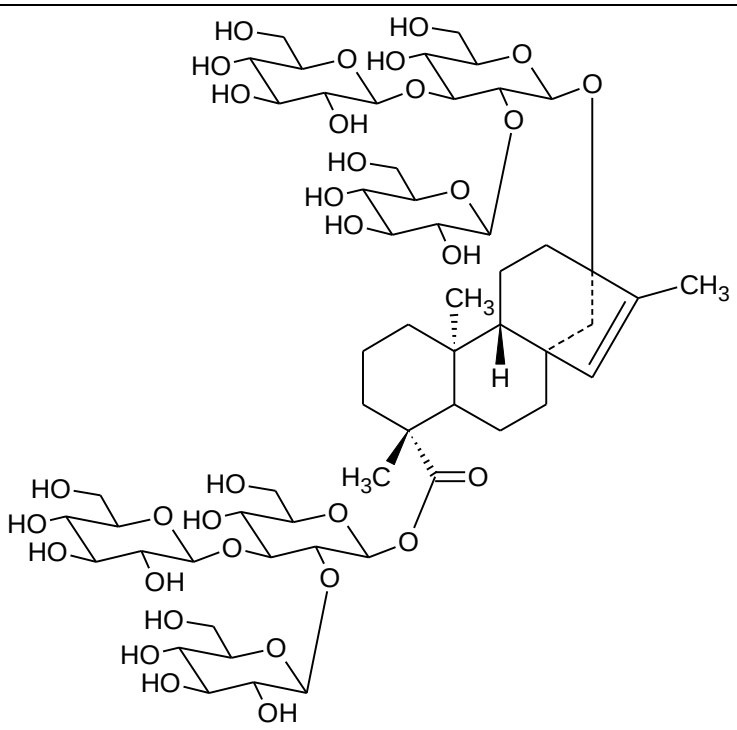
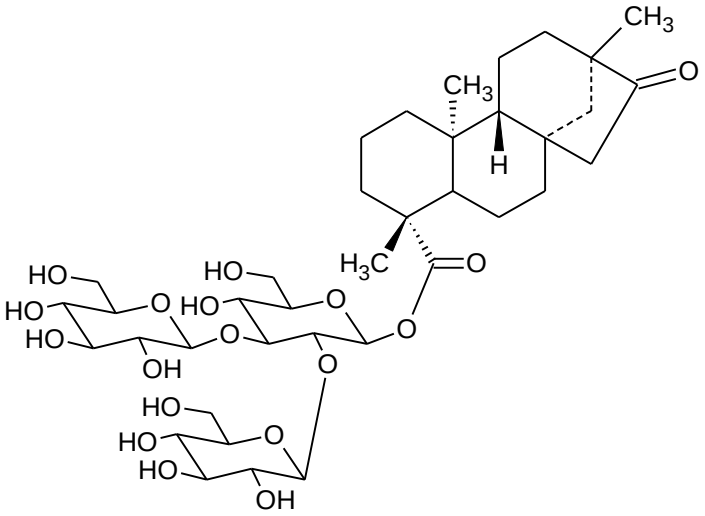
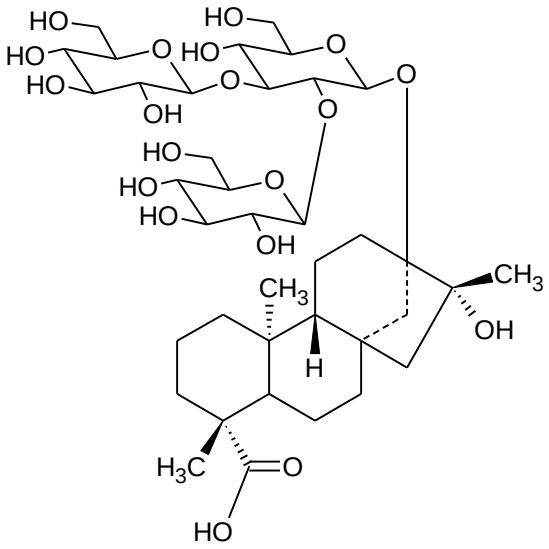
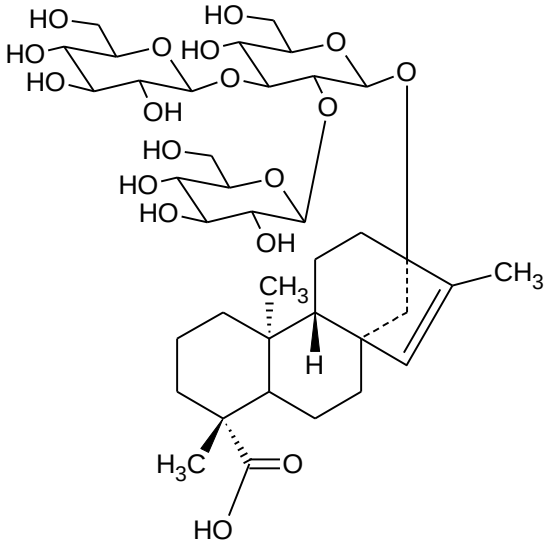
Table B.9.4-1 Degradation Products	
Chemical Name	**Chemical Structure**
13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-15-en-19-oic acid-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester] (CC-00277)	
13-methyl-16-oxo-17-norkauran-19-oic acid-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester] (CC-00281)	

Table B.9.4-1 Degradation Products	
Chemical Name	**Chemical Structure**
13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-16-hydroxy *ent*-kauran-19-oic acid (CC-00209)	
13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-15-en-19-oic acid (CC-00210)	

Degradation products CC-00280, CC-00277, and CC-00281 (molecular weights of 1,309.3, 1,291.3, and 804.9, respectively) were identified at study initiation in very low quantities (<0.5%) and increased as a result of the extreme conditions to approximately 7, 22, and 3%, respectively. Low levels of rebaudioside B also were detected at study initiation (approximately 1%) and increased to approximately 9% at study completion. Following forced degradation, CC-00209 and CC-00210 were found in the samples at levels of up to approximately 10 and 23%, respectively. These products were not identified at study outset. Other substances identified in the samples following the forced degradation that were not present at study outset

included steviol and isosteviol at levels of up to approximately 1 and 7%, respectively, at the end of the study.

The occurrence of new related glycosides following exposure of a steviol glycoside preparation to extreme conditions is not unexpected. As discussed in the EFSA Opinion, preparations that contained not less than 97% rebaudioside A were identified to also contain low amounts of several other related steviol glycosides that shared the same aglycone (steviol) backbone, but were differentiated on the basis of the number of attached sugar units. Additionally, other related substances also were identified that possessed slight structural differences in the aglycone backbone (*e.g.*, presence of an endocyclic double bond or an additional hydroxyl group, or an isosteviol aglycone instead of steviol). While some of these related substances were present in the bulk material, others were only identified in stability studies following certain periods of storage/storage conditions. With respect to the safety of these related substances, EFSA noted that while these have not been assessed in laboratory animals under conditions of traditional toxicological studies, such studies were not needed as the safety could be extrapolated from the presences of these compounds in test materials used in studies conducted to assess the safety of steviol glycosides and/or from these compounds sharing a related structure.

In the case of the rebaudioside M-rich preparations, the results of the forced degradation study also revealed 4 substances, present in the bulk material, to increase with time (*i.e.*, CC-00280, CC-00277, CC-00281, and rebaudioside B). Additionally, 4 compounds were identified that were not present in the starting material: CC-00209, CC-00210, as well as steviol and its structural isomer, isosteviol. Rebaudioside B is presently included as one of the steviol glycosides that may comprise the assay value of not less than 95%. Like the degradation products previously identified for other steviol glycoside preparations, characterisation of CC-00280, CC-00277, and CC-00281 revealed these substances to be related to the parent steviol glycosides, but to be slightly differentiated structurally by virtue of the presence of a hydroxyl group at position C-16, an endocyclic double bond, and an isosteviol backbone, respectively. Likewise, CC-00209 and CC-00210 also were found to be related to the parent steviol glycoside compounds, the differences being again a hydroxyl group at position C-16 and an endocyclic double bond, respectively. Furthermore, both CC-00209 and CC-00210 were identified as degradants of rebaudioside A (Prakash *et al*., 2012). Given the structural similarities of these substances to other steviol glycosides, the identified degradation products are not expected to present any safety concerns.

B.9.5 Possible Interactions with Food

The chemical structure of rebaudioside M is similar to other previously evaluated steviol glycosides. Since no potential for the reaction of steviol glycosides with food was previously identified, and considering the similarities in structure of rebaudioside M to other steviol

glycosides, there is no reason to expect that rebaudioside M would interact with food in ways that are different from other steviol glycosides. The EFSA Opinion (2010) notes the results of one study in which the potential for the interaction of stevioside with water soluble B vitamins and vitamin C, and with other low-calorie sweeteners was considered (Kroyer, 1999). The results of these studies did not provide any evidence for a potential interaction of stevioside with other nutrients or food ingredients. Considering therefore the structural similarities of all steviol glycosides and the existing data, the need for formal stability investigations with rebaudioside M-rich preparations into possible interaction products does not appear to be necessary.

C. INFORMATION ON THE SAFETY OF THE FOOD ADDITIVE

In accordance with Section 3.3.1 – Food Additives of the Food Standards Australia New Zealand *Application Handbook* (FSANZ, 2013) the safety information outlined must be provided for new food additives.

1. Information on the toxicokinetics and metabolism of the food additive and, if necessary, it's degradation products and/or major metabolites

2. Information on the toxicity of the food additive and, if necessary, its degradation products and major metabolites

These points are addressed in the Section that follows.

Section 3.3.1 – Food Additives of the Food Standards Australia New Zealand *Application Handbook* (FSANZ, 2013) states that if available, safety assessment reports prepared by international agencies of other national government agencies should be provided. A summary of the safety assessment reports prepared by international agencies are outlined in the following Section.

C.1 Introduction

The safety of steviol glycosides has already been thoroughly investigated by several advisory scientific bodies and it has been concluded that steviol glycosides, as a group of substances, share a similar structure (*i.e.*, glycosylated derivates of the aglycone steviol with various glucose, xylose, or rhamnose units attached at the R_1 and R_2 positions), and that these substances undergo a common metabolic pathway following ingestion (*i.e.*, hydrolysis of the glucoside units from the steviol backbone). As such, based on their shared metabolic fate, safety data generated for one steviol glycoside is largely applicable to the safety assessment of other steviol glycosides.

Over the last few decades, the safety of steviol glycosides has been considered by several scientific bodies and regulatory agencies, including the FDA, JECFA, the European Commission's Scientific Committee on Food (SCF), EFSA, FSANZ, and Health Canada. Interest in the use of steviol glycosides as sweeteners has encouraged extensive testing of the compounds and as such a large safety database exists. This database includes a thorough examination of the comparative metabolism and pharmacokinetics of steviol glycosides in experimental animals and humans, acute toxicity studies, short- and long-term toxicity and carcinogenicity studies, reproductive and developmental toxicology studies, *in vitro* and *in vivo* mutagenicity/genotoxicity studies, and human studies. Although many earlier studies examining the safety of steviol glycosides were conducted with stevioside as a result of its predominance in *S. rebaudiana* leaves, the database pertaining to the safety of steviol glycosides was

expanded recently following the completion of additional short-term toxicity, reproductive toxicity, *in vitro* and *in vivo* mutagenicity/genotoxicity studies, and human studies on rebaudioside A. Although the majority of toxicity studies have been conducted with either purified stevioside or rebaudioside A, the extensive database on the common metabolic fate of steviol glycosides has permitted the scientific bodies and regulatory agencies to extend their safety opinion to all steviol glycosides, rather than just individual glycosides.

Presented below is a detailed summary of the conclusions and the data deemed pivotal from the scientific bodies and regulatory agencies in determining the safety of steviol glycosides.

C.2 Summary of Safety Opinions by Scientific and Regulatory Authorities

C.2.1 Joint FAO/WHO Expert Committee on Food Additives (JECFA)

The safety of steviol glycosides was reviewed by JECFA at 4 separate meetings (51st, 63rd, 68th, and 69th) in 1998, 2004, 2007, and 2008 (JECFA, 1999, 2006b, 2007c, 2009). At the first of the 4 meetings in 1998, JECFA was asked to specifically review the safety of stevioside. Following review of the available information, the Committee concluded that the data on stevioside were limited and highlighted the need for specifications for commercial materials. An ADI could not be established.

Subsequently in 2004, the Committee determined that the material of commerce for which tentative specifications were developed should be known as “steviol glycosides”. New data as per the requests made at the earlier meeting were provided to the Committee for review. The Committee reviewed the newly available data which demonstrated that stevioside and rebaudioside A were not genotoxic and that the positive *in vitro* results for steviol and its oxidative derivatives were not confirmed *in vivo*. Although the Committee reviewed the results of a developmental study showing adverse effects on fertility following treatment of male rats with a crude aqueous extract of *S. rebaudiana*, the Committee referred back to the studies reviewed at the preceding meeting noting that in studies conducted with higher purity material, no reproductive or developmental effects were observed, and thus, the reproductive effects noted following administration of the crude extract were unlikely to be related to steviol glycosides. Although the Committee did not raise any further questions regarding the potential toxicity of steviol glycosides at this review, the Committee noted that pharmacological effects in patients with hypertension or Type 2 diabetes were observed at doses of 12.5 to 25 mg/kg body weight/day of steviol glycosides (5 to 10 mg/kg body weight/day as steviol equivalents). Consequently, further information regarding the potential effects of steviol glycosides in subjects with diabetes and in normotensive and hypotensive populations was requested. At this time, a temporary ADI of 2 mg/kg body weight/day (expressed as steviol) for steviol glycosides was allocated, based on a no-observed-adverse-effect level (NOAEL) of 970 mg/kg body weight/day

(383 mg/kg body weight/day as steviol) from a 2-year study in rats (Toyoda *et al.*, 1997) and a safety factor of 200 (JECFA, 2006b).

In 2007, the Committee received additional data pertaining to the potential pharmacological effects of steviol glycosides in humans; however, none of these studies were conducted with a material that met the specifications for steviol glycosides. The Committee was made aware however of ongoing human studies that were designed to specifically address the Committee's previous concerns (Maki *et al*., 2008a,b) and thus the temporary ADI was extended until 2008. The specifications were revised and the tentative designation was removed.

At the final safety evaluation of steviol glycosides in 2008, the Committee was presented with new data pertaining to the metabolic fate of steviol glycosides in rats and humans (Roberts and Renwick, 2008; Wheeler *et al*., 2008), subchronic and reproductive/ developmental toxicity of rebaudioside A specifically (Curry and Roberts, 2008; Curry *et al*., 2008; Nikiforov and Eapen, 2008), and the potential pharmacological effects of steviol glycosides in diabetic populations and individuals with normal or low-normal blood pressure (Maki *et al*., 2008a,b). The Committee concluded that the results of the human studies evaluating the effects of steviol glycosides on blood pressure and blood glucose were sufficient to remove the additional safety factor of 2 and establish a full ADI of 4 mg/kg body weight (expressed as steviol) for steviol glycosides. The specifications for steviol glycosides were revised further, requiring not less than 95% of the 7 named steviol glycosides.

During the Committee's 73rd meeting in 2010, JECFA revised the specifications for steviol glycosides to include 2 additional steviol glycosides, rebaudioside D and rebaudioside F, within the purity criteria[2] (JECFA, 2010). Although no specific studies have been conducted with these steviol glycosides individually, their inclusion within JECFA's purity specification further confirms that the safety of steviol glycosides is based on the general recognition that all steviol glycosides are degraded to the aglycone steviol and that the safety demonstrated for one glycoside is relevant to all glycosides in general.

An overview of JECFA's main concerns raised at each meeting at which steviol glycosides were evaluated and the resolution, along with the respective ADI, is presented in Table C.2.1-1.

[2] Not less than 95% of the following 9 steviol glycosides, on a dried weight basis: stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside.

Table C.2.1-1 Summary of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Evaluations of Steviol Glycosides			
Year (meeting)	**Questions/Concerns**	**ADI**	**Reference**
1998 (51st)	• Need for specifications for the material of commerce. • Limited data; additional data required on: - human metabolism; - *in vivo* genotoxicity of steviol; - long-term toxicity and carcinogenicity in other species.	Could not be derived	JECFA (1999)
2004 (63rd)	• Data required on pharmacological effects in patients with Type 2 diabetes and individuals with normal or below-normal blood pressure.	2 mg/kg bw (as steviol) (temporary) • based on a NOAEL of 970 mg/kg bw/d (383 mg/kg body weight/day as steviol) and a safety factor of 200	JECFA (2006b)
2007 (68th)	• Data required on pharmacological effects in patients with Type 2 diabetes and individuals with normal or below-normal blood pressure.	2 mg/kg bw (as steviol) (temporary) • based on a NOAEL of 970 mg/kg bw/d (383 mg/kg body weight/day as steviol) and a safety factor of 200	JECFA (2007c)
2008 (69th)	• No further questions.	4 mg/kg bw (as steviol) (full) • based on a NOAEL of 970 mg/kg bw/d d (383 mg/kg body weight/day as steviol) and a safety factor of 100	JECFA (2009)
2010 (73rd)	• Safety data not evaluated; specifications revised to include 2 additional steviol glycosides within the assay definition.	Not re-evaluated	JECFA (2010)

ADI = acceptable daily intake; bw = body weight; d = day; NOAEL = no observed adverse effect level

C.2.2 Food Standards Australia/New Zealand (FSANZ)

Immediately prior to JECFA's 69th meeting, FSANZ conducted their own evaluation of the safety of steviol glycosides (FSANZ, 2008). In its assessment, FSANZ considered the data previously reviewed by JECFA, as well as supplementary data consisting of published and unpublished studies. FSANZ considered the toxicological database for stevioside to cover a range of toxicological endpoints, and concluded that the supplementary data were sufficient to revise JECFA's temporary ADI to a full ADI of 4 mg/kg body weight/day by removing the additional uncertainty factor of 2.

C.2.3 European Food Safety Authority (EFSA)

In 1985, the European Commission's SCF evaluated stevioside as a sweetener and concluded that its use was "not toxicologically acceptable" due to limited data on metabolism, mutagenicity, long-term, and reproductive and developmental toxicity (SCF, 1985). In a subsequent evaluation, the SCF examined newly available data on metabolism, genotoxicity, and long-term toxicity, but maintained that these data were inadequate to sufficiently assess the safety of

stevioside (SCF, 1999). Specifically, the SCF continued to raise concerns related to the potential reproductive effects of steviol glycosides and recommended that a study in a rat strain other than the F344 rat be conducted [rat strain used in the 2 carcinogenicity studies on stevioside (Yamada *et al*., 1985; Toyoda *et al*., 1997)], since it is not possible to evaluate any potential effects on the testicular system in this strain of rats as it normally seems to develop testicular changes. The SCF (1999) also questioned the relevance of numerous other studies because the composition of the test material was not clearly defined. The potential mutagenic effects of steviol also continued to be a concern (SCF, 1999). Based on the SCF's review of stevioside, the European Commission rejected *Stevia* and stevioside for use as a sweetener (Geuns, 2003). However, in an independent review of the safety data previously reviewed by JECFA at its 69^{h} meeting, EFSA corroborated JECFA's conclusion regarding the safety and concurred with the ADI previously established by JECFA of 4 mg/kg body weight/day for steviol glycosides, expressed as steviol equivalents (EFSA, 2010).

C.2.4 Health Canada

Health Canada conducted its own independent review of the available safety data for steviol glycosides (Health Canada, 2012a). Further corroborating the conclusions by JECFA, FSANZ, and EFSA, Health Canada established an ADI of 4 mg/kg body weight/day for steviol glycosides, expressed as steviol glycosides, based on the NOAEL from the 2-year carcinogenicity study conducted by Toyoda *et al.* (1997) and an uncertainty factor of 100. As previously mentioned, PureCircle has recently submitted a food additive submission to Health Canada to seek the approval for the use of rebaudioside M as a high-intensity sweetener.

C.2.5 Search Strategy to Determine Data Published Subsequent to Latest Safety Opinion

In order to identify any new data related to the safety of steviol glycosides published since the latest safety assessment on steviol glycoside by FSANZ (*i.e*.., 2010), an updated search of the scientific literature was conducted on November 19, 2014 (*i.e.*, studies published between 2010 and the date of the search). The database searching tool ProQuest was used to search the following scientific databases: MedLine, ToxFile, Agricola, AGRIS, Allied and Complementary Medicine, Biosis Toxicology, Biosis Previews, Foodline: Science, CAB Abstracts, FSTA, NTIS, EMBASE, and Adis Clinical Trials. Keywords related to steviol glycosides (*i.e.*, rebaudioside M or rebaudioside A or rebaudioside B or rebaudioside C or rebaudioside D or rebaudioside F or dulcoside or steviolbioside or rubusoside or steviol or stevioside) were mapped to the relevant date criteria. No other search limitations were incorporated into the search. Reference lists of review articles were searched to ensure all relevant studies published since 2010 were identified. Any new data retrieved are summarised in the sections that follow.

C.3 Metabolism/Toxicokinetics

C.3.1 Metabolic Fate of Steviol Glycosides

Considering however the importance of the metabolism of individual steviol glycosides in the assessment of their safety, a detailed overview of the general metabolic fate of steviol glycosides is provided below. In order to corroborate previous conclusions regarding the shared metabolic pathway for steviol glycosides, a study examining specifically the microbial metabolism of rebaudioside M in the presence of human faecal homogenates under anaerobic conditions was conducted and also is discussed below. Furthermore, results of a few additional studies designed to assess the metabolic fate of the related steviol glycosides, rebaudiosides D and E, which also have become available since EFSA's review of steviol glycoside safety and which further support a common metabolic pathway for all steviol glycosides also are presented below. Based on the findings of these studies, which confirm rebaudioside M, as well as other related steviol glycosides to be also subject to hydrolysis to steviol prior to absorption, it was deemed appropriate to extend the safety conclusions for steviol glycosides to all glycosylated derivates of the aglycone steviol, including the newly identified rebaudioside M. Specifically, since rebaudioside M shares the metabolic fate of the other already recognised steviol glycosides, the existing ADI of 4 mg/kg body weight for steviol glycosides can be extended to rebaudioside M.

In vitro and *ex vivo* studies, which were previously reviewed by EFSA and JECFA as part of their respective evaluations of the safety of steviol glycosides, demonstrated that steviol glycosides are not hydrolysed by digestive enzymes of the upper gastrointestinal tract and are not absorbed through the upper portion of the gastrointestinal tract (Hutapea *et al*., 1997; Geuns *et al*., 2003, 2007; Koyama *et al*., 2003a). Therefore, steviol glycosides enter the colon intact, where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol (Gardana *et al*., 2003; Renwick and Tarka, 2008). Several *in vitro* studies mimicking the anaerobic conditions of the colon, which were reviewed extensively by Renwick and Tarka (2008), have confirmed the ability of gut microflora from mice, rats, hamsters, and humans to hydrolyse steviol glycosides completely to steviol (Wingard *et al*., 1980; Hutapea *et al*., 1997; Gardana *et al*., 2003; Koyama *et al*., 2003b). Specifically, Koyama *et al*. (2003b) investigated the degradation of a stevia mixture containing rebaudioside A, stevioside, rebaudioside C, and dulcoside A (percent composition not reported) in the presence of human faecal homogenates under anaerobic conditions. Similarly to studies conducted with single steviol glycosides, the stevia mixture was degraded completely to steviol within 24 hours of incubation (Koyama *et al*., 2003b).

Steviol glycosides are hydrolysed sequentially, removing one glucose molecule at a time, with differences in the degradation rates depending on the structural complexities of the steviol glycoside (Wingard *et al*., 1980; Koyama *et al*., 2003b). Stevioside is degraded to

steviolbioside, steviolmonoside, and finally steviol, with glucose released with each sequential hydrolysis, whereas rebaudioside A is first converted to either stevioside (major pathway) or rebaudioside B (minor pathway) prior to being ultimately degraded to steviol (Nakayama *et al*., 1986; Gardana *et al*., 2003; Koyama *et al*., 2003b). The hydrolysis of rebaudioside A to steviol appears to be slower than that of stevioside to steviol partly due to the presence of 1 additional glucose moiety, indicating that microbes hydrolyse steviol glycosides sequentially by removing one glucose molecule at a time. Additionally, the metabolism of differing steviol glycosides appears to be stereoselective such that that degradation of rebaudioside C, which has an α(1>2) rhamnose on the 13-position, is faster than rebaudioside A, which has a β(1>2) glucose on the same position (Koyama *et al.*, 2003b).

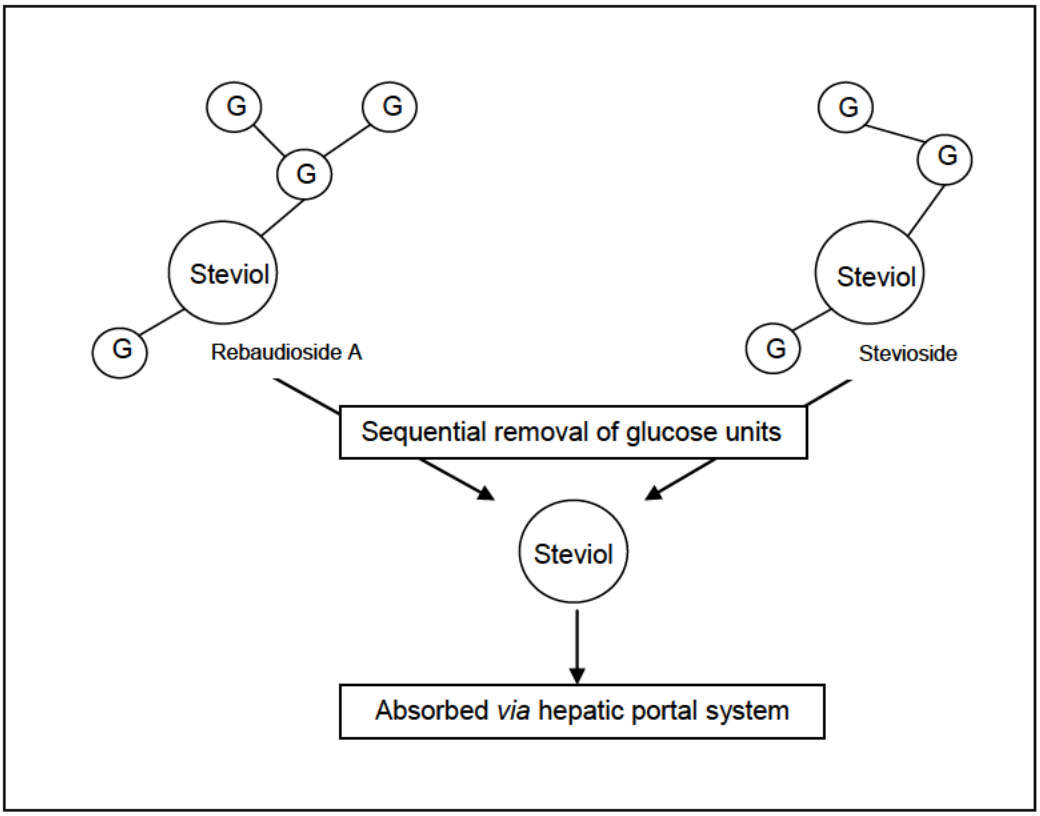
Steviol is absorbed systemically into the portal vein and distributed to a number of organs and tissues, including the liver for additional metabolism, spleen, adrenal glands, fat, and blood (Nakayama *et al*., 1986; Sung, 2002; Koyama *et al*., 2003b; Wang *et al*., 2004; Roberts and Renwick, 2008). Peak concentrations of steviol are detected in the plasma of Sprague-Dawley rats within 15 to 30 minutes of oral steviol administration, whereas maximum levels of steviol were attained approximately 8 hours following oral administration of steviol glycosides to rats, including a mixture of rebaudioside A (28.8%), rebaudioside C (25.2%), stevioside (17.0%), and dulcoside A (10.2%) (Nakayama *et al*., 1986; Koyama *et al*., 2003a; Roberts and Renwick, 2008). Generally, the delay between the time of administration of steviol glycosides and the occurrence of steviol levels in the plasma is attributed as being due to the fact that glycosides are first cleaved to steviol before absorption (Koyama *et al*., 2003a).

Following absorption from the colon, steviol primarily undergoes conjugation with glucuronic acid to steviol glucuronide in the liver. In rats, free steviol (82 to 86% of chromatographed radioactivity), steviol glucuronide (10 to 12% of chromatographed radioactivity), and 2 unidentified metabolites (5 to 6% of chromatographed radioactivity) were identified in the plasma 8 hours after oral administration with either rebaudioside A or stevioside (Roberts and Renwick, 2008). Similarly, steviol glucuronide was detected in the plasma following ingestion of stevioside or rebaudioside A in humans, with maximal concentrations detected 8 and 12 hours after administration, respectively (Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Wheeler *et al*., 2008). The differences in the time to reach maximum steviol glucuronide plasma concentrations between stevioside and rebaudioside A are due to the simpler structure and faster bacterial degradation of stevioside (Wheeler *et al.,* 2008). Moreover, significant inter-individual variability in maximum plasma steviol glucuronide levels, and in the time required to reach peak plasma levels, was noted in study participants following stevioside ingestion (Geuns *et al*., 2007). Such variations can likely be attributed to differences in the time required to release steviol from the glycoside in the gut as a result of inter-individual variability in the microflora composition or gastric emptying.

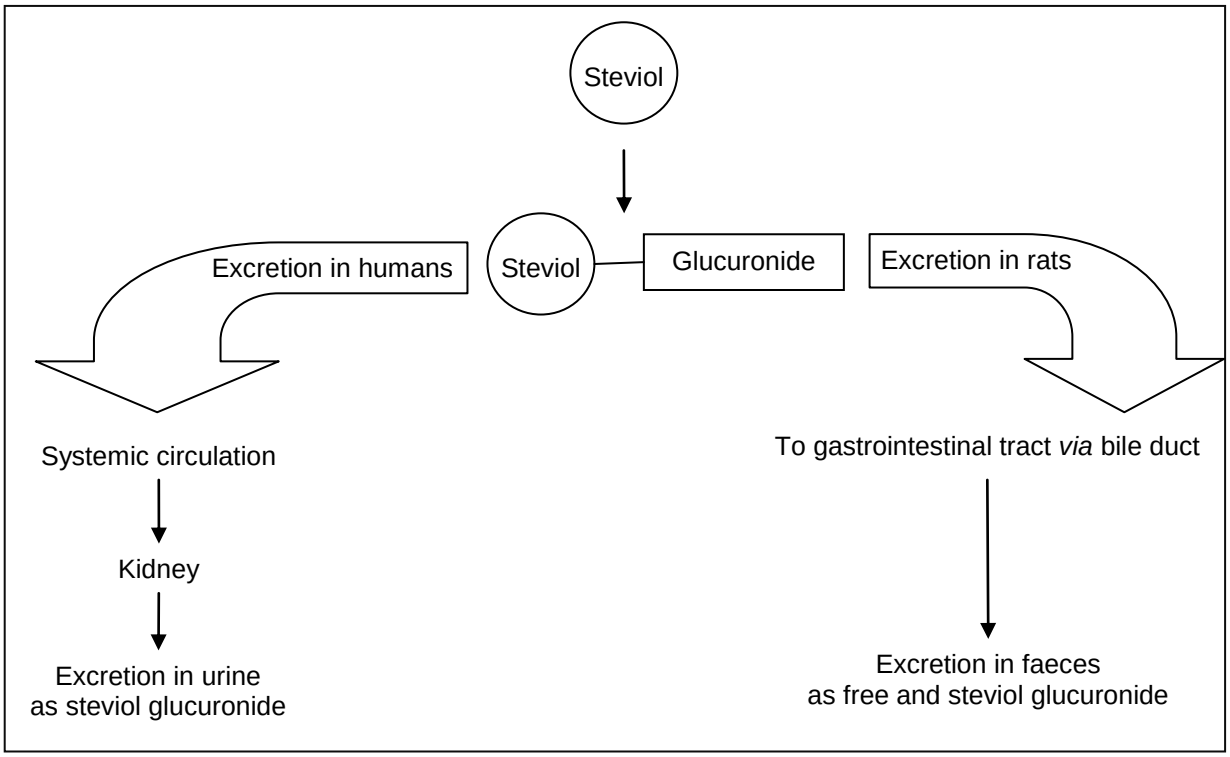
In rats, free and conjugated steviol, as well as any unhydrolysed fraction of the administered glycosides, are excreted primarily in the faeces *via* the bile (generally within 48 hours), with smaller amounts appearing in the urine (less than 3%) (Wingard *et al*., 1980; Nakayama *et al*., 1986; Sung, 2002; Roberts and Renwick, 2008). Two (2) steviol conjugates were identified by Nakayama *et al*. (1986) in the bile of Wistar rats, one which was hydrolysed by a weak acid and another which was hydrolysed by a weak acid and β-glucuronidase; therefore, steviol is available to be released again from its conjugated form by the action of the microflora and may enter enterohepatic circulation following elimination in the bile. In contrast, elimination of steviol glycosides, primarily as steviol glucuronide with very small amounts of the unchanged glycoside or steviol, in humans occurs *via* the urine (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). Relative to amounts recovered in urine, larger amounts of steviol (unabsorbed steviol released from steviol glycosides in the colon or from small amounts of steviol glucuronide secreted back into the gut *via* the bile) also were eliminated in the faeces, (Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Wheeler *et al*., 2008). A general schematic of the metabolism of steviol glycosides is provided in Figure C.3.1-1, below.

Figure C.3.1-1 Schematic Diagram of Metabolic Fate of Steviol Glycosides

In the colon – breakdown of steviol glycosides to steviol occurs:



In the liver of humans and rats:



Adapted from Carakostas *et al.* (2008)

C.3.2 Results of Recent Metabolism Studies Examining the Metabolic Fate of Steviol Glycosides Other Than Rebaudioside A and Stevioside, Including Rebaudioside M

As discussed in Section C.3.1, EFSA and JECFA previously evaluated several studies related to the metabolic fate of steviol glycosides; however, with the exception of the study by Koyama *et al*. (2003b) in which the microbial degradation of a stevia mixture was examined, all other studies available at the time of the evaluations were limited to investigations of rebaudioside A and stevioside metabolism. Since then, a few other studies have become available which were designed to evaluate the metabolic fate of a number of related steviol glycosides, including that of rebaudioside M (Nikiforov *et al*., 2013; Prakash Chaturvedula and Prakash, 2013; Purkayastha *et al*., 2014).

Nikiforov *et al*. (2013) conducted a series of studies to evaluate the metabolism and toxicity of rebaudioside D specifically and compare the metabolic fate and toxicity profile of rebaudioside D to rebaudioside A (for discussion of the toxicology study, refer to Section C.4.2). Results from the *in vitro* assays confirmed that rebaudiosides A and D were generally stable when incubated in simulated gastrointestinal and intestinal fluids and as such are not expected to be extensively metabolised in the stomach or intestine. Specifically, each steviol glycoside was incubated with simulated gastrointestinal and intestinal fluids with and without enzyme (pepsin and pancreatin, respectively). Over a 240-minute incubation period, the only statistically significant decrease in rebaudioside A concentration (approximately 10%) occurred in the gastric fluid in the absence of

enzyme (pepsin). In the case of rebaudioside D, its concentration was statistically significantly lower at the end of the incubation period, but only in the gastric and intestinal fluids without the respective enzymes (reductions of approximately 20 and 8.8%, respectively). In the case of rebaudioside A, the authors noted the presence of stevioside in the gastric fluid samples, whereas in the case of rebaudioside D, rebaudioside A was detected in some of the samples (gastric or intestinal not specified). In no samples was the presence of steviol reported. When incubated with rat liver microsomes for 90 minutes, neither the concentration of rebaudioside A nor rebaudioside D was observed to decrease. Conversely, when incubated with rat caecal contents, the concentration of each steviol glycoside decreased rapidly to levels below the limit of detection over a period of 90 minutes. The rapid decreases in the concentrations of the steviol glycosides were accompanied by increases in steviol, as well as stevioside. Considering the sequential hydrolysis of the glycosides as described above, formation of stevioside as a result of either rebaudioside D or A hydrolysis was expected.

Toxicokinetic evaluations were included as part of the 28-day repeat-dose rat [Crl:CD(SD)] feeding studies which were conducted by Nikiforov *et al*. (2013) to assess the potential toxicity of rebaudioside D and rebaudioside A (see Section C.4.2). Specifically, blood samples were obtained for a period of 24 hours on days 1 and 21/22 for the evaluation of levels of the parent compound (rebaudioside A or D), as well as potential hydrolysis and conjugation products from animals [3 rats/sex/group] provided either steviol glycoside in the diet at concentrations aimed to provide target dose levels of 2,000 mg/kg body weight/day. Plasma levels of the parent compounds and unconjugated/free steviol were reported to be measurable at all sampling time-points (over the 24-hour time period) and on both sampling days, but were generally very low following dietary consumption of either rebaudioside A or rebaudioside D. In the case of animals administered rebaudioside D in the diet, rebaudioside A also was identified in the plasma. At all sampling times, plasma levels of steviol glucuronide were the highest (≤40 µg/mL), followed by levels of free steviol (≤12 µg/mL), and the parent compound (≤1.5 µg/mL). Overall the results of this study provide further support that the systemic absorption of intact steviol glycosides is minimal and that steviol glycosides are predominantly degraded in the colon and from there, absorbed in low amounts as steviol, which is conjugated and excreted as steviol glucuronide. Furthermore, direct comparison of the metabolic fate of rebaudioside A and D shows that both glycosides share similar pathways, with the noted exception that the hydrolysis of rebaudioside D to steviol is prolonged by the presence of an additional glucose unit.

In another study conducted to compare the metabolism of several related steviol glycosides including that of rebaudioside M, 0.2 mg/mL of rebaudioside M, rebaudioside A, rebaudioside B, rebaudioside D, and steviolbioside (a metabolic intermediate) were incubated individually with pooled faecal homogenates from 6 healthy male and 6 healthy female volunteers for up to 24 hours at 37°C under anaerobic conditions (Purkayastha *et al*., 2014). Additionally, the microbial degradation of higher concentrations of rebaudiosides B, D, and A (2 mg/mL) also was

examined. The hydrolysis of rebaudioside M and steviolbioside was not examined at the higher concentration as both glycosides precipitated out of solution at this concentration. Rebaudioside M was rapidly hydrolysed to steviol, with up to approximately 83 and 91% of rebaudioside M degraded after 8 hours of incubation with male and female faecal homogenates, respectively. Within 16 hours of incubation, all of the rebaudioside M was degraded to steviol. Similarly, at the lower concentration (0.2 mg/mL), up to 99 and 100% of rebaudioside A was hydrolysed to steviol by male and female faecal homogenates, respectively, within 8 hours of incubation. Although some variability among the different pooled samples was noted at 8 hours with respect to the degree of hydrolysis attained (51.6 to 99.1% in males and 89 to 100% in females), degradation of ≥91% was attained after 24 hours in all samples. Similar results were obtained for rebaudioside D (0.2 mg/mL), with ≥85% hydrolysis noted in all samples after 8 hours. Rebaudioside B was metabolised somewhat more slowly than the other steviol glycosides (*i.e.*, 71.5 to 87.3% and 58.1 to 89.7% hydrolysis in males and females, respectively after 8 hours); however, near complete metabolism (≥89%) was reported after 24 hours of incubation. When the rebaudiosides (A, B, and D) were incubated at the higher concentration with the faecal samples, the hydrolysis was observed to be slower, with rebaudioside B again showing the lowest degree of hydrolysis at any time point in comparison to the other rebaudiosides. Considering that rebaudioside B does not possess a glucose unit at the R1 position, the study authors noted that its hydrolysis may have been in fact predicted to be faster than that of rebaudioside A; however, the opposing results may be reflective of the fact that it is not only the number of the sugar moieties that determines the degree and rate of hydrolysis but also the orientation of the molecule. In the case of the metabolic intermediate, steviolbioside (0.2 mg/mL), hydrolysis of approximately 80% to steviol was attained at 8 hours, with little change thereafter (up to 24 hours). This lower than expected result was suggested to have been due to the higher dilution factor used in the preparation of the test samples rather than the efficiency of the microbial degradation of steviolbioside. When the results of this study are taken into consideration with the results from other faecal homogenate studies as previously reviewed by EFSA, the study confirms that rebaudioside M, like all other steviol glycosides, is hydrolysed to steviol within 24 hours. Additionally, the study conducted by Purkayastha *et al*. (2014) corroborates the differences in degradation rates of steviol glycosides which may be related to the structural variations among the molecules. The slight increase in the rate of hydrolysis for rebaudioside A in comparison to rebaudioside M was determined to be the result of the structural differences between the 2 steviol glycosides, with rebaudioside A having 4 glucose moieties in comparison to the 6 glucose moieties within rebaudioside M.

In addition to the results obtained for rebaudioside M specifically (Purkayastha *et al*., 2014), results of other recent studies examining the metabolic fate of rebaudioside E, D, and B also provide further confirmation of a shared metabolic pathway for all related steviol glycosides (Nikiforov *et al*., 2013; Prakash Chaturvedula and Prakash, 2013). Therefore, based on the overall similarities in the microbial metabolism of steviol glycosides to steviol, including rebaudioside M, following incubation with intestinal microflora within the faecal homogenates,

the comparable rates of hydrolysis, and the negligible changes in microflora produced after prolonged incubation with steviol glycosides previously reported in the literature, permits the extrapolation of the toxicological data on stevioside and rebaudioside A to support the safety of steviol glycosides in general and rebaudioside M specifically (Renwick and Tarka, 2008; Purkayastha *et al*., 2014).

C.3.3 Summary

Steviol glycosides pass undigested through the upper portion of the gastrointestinal tract and enter the colon intact where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol. All steviol glycosides, including stevioside and rebaudioside M are hydrolysed to steviol prior to absorption. Studies comparing the metabolic fate of rebaudioside A and stevioside demonstrate that both glycosides have similar pharmacokinetics in the rat; they are both metabolised in the gut to steviol prior to absorption followed by glucuronidation in the liver and excretion in the faeces *via* the bile. In both rats and humans, steviol was shown to be metabolised to steviol glucuronide following absorption. 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, including rebaudioside M 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.

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) 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.

C.4 Pre-Clinical Data

C.4.1 Acute Toxicity

There is no safety concern with respect to the acute toxicity of steviol glycosides based on the LD_{50} of >15 g/kg body weight of stevioside reported in rats, mice, and hamsters as reported in the EFSA opinion (EFSA, 2010).

C.4.2 Sub-Chronic Toxicity

Three additional studies related to the sub-chronic toxicity of steviol glycosides were identified and are summarised below.

A 12-week study was identified in which weanling (21-day-old) male Sprague-Dawley rats (8 rats per group) were administered stevioside (97% pure) in drinking water corresponding to dose levels of 0, 15 (low dose), or 1,500 mg stevioside/kg body weight/day (high dose) (Awney *et al*., 2011). An additional group was administered stevioside (15 mg/kg body weight/day), together with inulin (dose not reported) for the same time-period. Animals were monitored for signs of adverse effects throughout the study period, while body weights were recorded weekly, and food and fluid intake recorded daily. Standard haematological and biochemical/enzyme activity parameters were analysed at the start and end of the study period, including lipid levels. Organ weights were measured; however, macro- and microscopic evaluations were not reported to have been conducted.

No mortality or signs of adverse effects were observed in any of the dosed animals. The average body weight gains of animals in the high-dose stevioside and stevioside plus inulin groups were statistically significantly decreased compared to the control group animals. Food intakes were statistically significantly decreased in the high-dose stevioside group compared to controls during the second 6-week study period. In the high-dose group, the relative weights of testes and epididymis, kidney, and brain tissues were significantly increased compared to control animals, while relative liver weights were significantly decreased. With regards to haematological parameters, mean corpuscular volume (MCV) was significantly decreased, while mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were significantly elevated in the high-dose stevioside group compared to control animals. No significant differences in the parameters measured above were noted in the low-dose stevioside group. Blood glucose, alkaline phosphatase (ALP), acid phosphatase, and tartrate-resistant acid phosphatase (TRAP) were significantly decreased, whereas, urea, creatinine, and bilirubin levels were significantly increased in the high-dose stevioside group compared to the control group. Total lipid levels were significantly decreased in the low-dose stevioside group compared to the control group, although a similar effect was not observed in the high-dose stevioside group. Serum cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels were statistically significantly increased in the high-dose

stevioside group compared to the control group. TRAP levels also were significantly decreased in the low-dose steviol group. No significant changes in alanine aminotransferase (ALT) or aspartate aminotransferase (AST) activity were reported in either of the dosed groups.

As previously discussed by EFSA (2010), reduced body weights and decreased food intakes in animals following administration of high-intensity sweeteners are mostly related to palatability and reduced nutritional quality of the feed and not indicative of adverse effects. The brain, kidney, and testes/epididymis weights were considered to be increased only relative to body weights, and therefore, were also likely a consequence of the reduced body weights in the high-dose group. With regards to the haematology parameters variations, the study investigators considered the decrease in MCV, and the elevations in MCHC, MCH, and haemoglobin to be a consequence of stevioside exposure; however, in a critical review of the study findings, Carakostas (2012) noted that the levels of these parameters in all animals, including controls, were below reference values for rats (for example, haemoglobin levels are typically between 11 and 19 g/dL compared to 7.32 to 9.78 g/dL reported for these test animals [Sharp and La Regina, 1998]). Consequently, clinical signs/symptoms normally associated with anaemia would be expected in such rats if indeed such variations in these haematological parameters occurred. As no symptoms of anaemia were reported in the animals, it was suggested by Carakostas (2012) that the variability was likely the result of analytical errors in the data evaluation/collection. In a rebuttal to the editor, the study authors argued that the haematological variability was related to the rats' age and is not uncommon. It should also be noted that in comparison to references values (Sharp and La Regina, 1998), similar discrepancies are identified for bilirubin and protein levels. Awney *et al.* (2011) also suggested that the decreased ALP and TRAP values may be reflective of stevioside-related adverse effects on bone metabolism (reduced osteoclast activity). Carakostas (2012) also discusses the fact that not only was the colorimetric measurement method used by the investigators out-dated (more reliable assays are currently in use), but also that the methodology is not specific to bone enzyme activity. Furthermore, as discussed by Waddell (2011) in another critical review of the study findings, chronic toxicity studies conducted with other high-purity steviol glycosides do not corroborate the biochemical findings reported by Awney *et al.* (2011). Finally, the high-dose of stevioside administered to animals in this study of 1,500 mg/kg body weight/day exceeds the dose of 970 mg/kg body weight/day which was considered to be the NOAEL for stevioside based on the results of a 2-year study (Toyoda *et al.*, 1997).

In a further 28-day study, male Wistar albino rats (6/group) were administered a *S. rebaudiana* dried leaf aqueous extract by gavage at dose levels of 0, 500, 1,000, or 2,000 mg/kg body weight/day (low-, mid-, and high-dose groups, respectively) (Ramanathan and Sellappan, 2010). The purity of the extract used in this study was however not identified by the authors in the publication. No significant differences in clotting time, or red or white blood cell counts were observed between any of the treated groups. Bleeding time was significantly increased in the low- and mid-dose groups compared to the control group (increased, but not significantly in the

high-dose group), while AST levels were significantly reduced in the mid-dose group. The high-dose group presented elevated AST levels, although the difference compared to controls was not statistically significant (233 *vs.* 270.7 U/L). ALT levels were not affected by the administration of the extract, while ALP levels were significantly reduced in the low- and high-dose groups compared to controls. The investigators reported that liver samples revealed evidence of hepatic vacuolar degeneration in all treated groups. The severity was reported to be "mild" in the mid-dose group, and "moderate" in the high-dose group. Histological effects reported included, perivascular collection of mononuclear cells (low-dose group), stray areas of sinusoidal congestion and periportal collection of inflammatory cells (mid-dose group), and multifocal collection of round cells and congestion of blood vessels and mild proliferation of epithelium lining of the bile duct (high-dose group). In spite of the identified histopathological findings, the study authors concluded that no evidence of toxicity was observed as a result of the administration of the aqueous extract. Irrespective of the interpretation of the study results, the poor study design and overall low study quality, combined by the fact that the purity of the test article used in this study cannot be ascertained, renders the usefulness of the data as being questionable when evaluating the safety of steviol glycosides of high-purity.

Most recently, a 28-day repeat-dose toxicity study [Good Laboratory Practice (GLP)-, OECD-, and FDA Redbook-compliant study; for toxicokinetic evaluation refer to Section C.3.2] was conducted to specifically assess the potential subchronic toxicity of rebaudioside D and provide direct comparison with rebaudioside A. Specifically, Sprague-Dawley rats (10 animals/sex/group) were administered a control diet and either rebaudioside D at dietary concentrations providing target dose levels of 500, 1,000, or 2,000 mg/kg body weight/day or rebaudioside A at a dietary concentration providing a target dose level of 2,000 mg/kg body weight/day (Nikiforov *et al*., 2013). In the case of rebaudioside D, mean intakes of 506 and 495, 1,027 and 1,012, and 2,042 and 2,016 mg/kg body weight/day were calculated for males and females respectively at each of the target dose levels respectively. Males and females provided rebaudioside A in the diet were estimated to achieve intakes of 2,034 and 1,965 mg/kg body weight/day, respectively. Animals were monitored for mortality and morbidity twice daily, clinical examinations occurred daily, while detailed physical examinations and food intake and body weight measurements were conducted once weekly. All animals were housed individually. Additional evaluations included functional observational battery (FOB) and locomotor activity, clinical pathology (*i.e.*, blood and urine), macro- and microscopic examinations, and histopathology. Examination of survival, clinical observations, body weights, food consumption, and organ weights, as well as results of the macro- and microscopic evaluations, haematology, serum chemistry, and urinalysis revealed no adverse effects related to the administration of either steviol glycoside. Evaluation of locomotor activity showed female rats administered rebaudioside D at all 3 dose levels to have significantly lower total and ambulatory activity counts. This however was not observed in the rebaudioside D-treated males and was considered by the investigators to be related to faster habituation, which was noted as typically not a treatment-related effect. Based on the absence of any adverse effects at the highest dose

level tested, the NOAEL for rebaudioside D under the conditions of this study was concluded by investigators to be 2,042 and 2,016 mg/kg body weight/day in males and females, respectively, which was the highest dose level tested.

C.4.3 Chronic Toxicity and Carcinogenicity

The chronic toxicity and carcinogenicity of steviol glycosides has been previously addressed in safety opinions of many authoritative and regulatory agencies. No new data were identified in relation to this endpoint.

C.4.4 Developmental and Reproductive Toxicity

The reproductive and developmental toxicity of steviol glycosides has been previously addressed in safety opinions of many authoritative and regulatory agencies. No new data were identified in relation to this endpoint.

C.4.5 Genotoxicity

The *in vitro* and *in vivo* genotoxicity of steviol glycosides and steviol were previously addressed in the safety opinions of many authoritative and regulatory agencies and it was concluded that these substances are not genotoxic.

A comprehensive review of the genotoxicity was conducted by Urban *et al.* (2013). The conclusions derived in this review of potential steviol glycoside genotoxicity, complement those previously made by EFSA.

C.5 Human Safety Data

C.5.1 Human Studies

The effects of steviol glycosides in humans have been previously addressed in safety opinions of many authoritative and regulatory agencies. No new data were identified in relation to this endpoint.

C.5.2 Immunotoxicity, Hypersensitivity/Allergy, and Food Intolerance

The allergenicity potential of steviol glycosides has been previously addressed in the safety opinions of many authoritative and regulatory agencies. No new data were identified in relation to this endpoint.

C.5.3 Special Studies

Various investigative or mechanistic studies designed to assess potential pharmacological effects of steviol glycosides have been conducted and previously reviewed by other authoritative bodies. Additional studies that were identified in the updated literature review are briefly presented below.

C.5.3.1 Potential Effects on Lipids and Glycaemia

In a study conducted by Geeraert *et al.* (2010), 12-week-old double knock-out leptin and LDL-receptor deficient mice (14 animals/group; sex not reported) were administered stevioside (99.9% purity) by gavage at a dose of 10 mg/kg body weight/day for 12 weeks. In comparison to a saline control group, there were no significant differences in body weights, triglyceride levels, or glucose tolerance. Stevioside administration did lower blood glucose, insulin, and cholesterol levels, while also increasing insulin signalling, glucose transport, and antioxidant status in adipose tissue and the aorta. The plaque volume of the aortic arch also was reduced with evidence of stabilisation of the plaque.

Wang *et al.* (2012) reported that the administration of stevioside (>98% purity) at a dose of 10 mg/kg body weight/day for 1 month to mice fed high-fat diets resulted in improved whole-body insulin sensitivity, fasting glucose, and glucose tolerance compared to high-fat diet control animals. In adipose tissues, stevioside administration decreased the expression of inflammatory cytokines in adipose tissues, and inhibited the nuclear factor-*kappa* b (NF-kB) signalling pathway.

In NMRI-Haan mice, the administration of stevioside at a dose of 20 mg/kg body weight/day (purity not reported) by gavage for 7 days significantly reduced glycaemia after adrenaline load compared to pre-treatment (Cekic *et al*., 2011). Additionally, stevioside administration for 12 days prior to alloxan administration significantly reduced glycaemia compared to pre-treatment; however, the reduction was not significantly different from the placebo group.

C.5.3.2 Potential Effects on Reactive Oxygen Species (ROS) Scavenging

The anti-oxidant properties of steviol glycosides were previously reviewed by EFSA (2010). An additional *in vitro* study was identified in which stevioside was evaluated in hydroxyl, superoxide, and hydrogen peroxide radical assays and was reported to be superior in scavenging properties to other sugars, and sugar derivatives with regards to hydroxyl and superoxide radical scavenging (Stoyanova *et al*., 2011).

In an additional study, Awney (2011) administered stevioside (97.8% pure) in drinking water to immature Sprague-Dawley rats for 12 weeks at concentrations resulting in intakes of approximately 0, 15, or 1,500 mg/kg body weight/day (low- and high-dose groups, respectively)

to evaluate the effects of stevioside administration on oxidative stress biomarkers. Superoxide dismutase and catalase activities were significantly decreased in liver and kidney tissues in the high-dose group compared to the control group. No significant differences in the levels of these enzymes in liver or kidneys were observed in the low-dose group or in serum in either group compared to the control group. Glutathione reductase and reduced glutathione levels were significantly decreased in liver, kidney, and serum in both groups. With regards to thiobarbituric acid reactive substance (TBARS) levels, significant reductions in the liver and kidney tissues were reported in the low-dose group, but significant increases were reported in the high-dose group. No significant differences in TBARS levels in serum were measured in either group. Notably, however, the high dose of 1,500 mg stevioside/kg body weight/day employed in this 12-week study is greater than the NOAEL determined in a chronic toxicity study with stevioside (*i.e*., 970 mg/kg body weight/day; Toyoda *et al*., 1997) which formed the basis of the current ADI for steviol glycosides. Although similar biochemical parameters were not specifically assessed in the study by Toyoda *et al*. (1997), the absence of any adverse effects or any other physiological perturbations in the chronic toxicity study indicates absence of any effects on these parameters at dose levels of up to 970 mg/kg body weight/day.

The pre-treatment of Wistar rats with 250 mg stevioside/kg body weight (purity not reported) prior to administration of scopolamine, significantly reduced memory impairment, attenuated brain acetylcholinesterase activity, and reversed the rise in brain oxidative stress markers (*i.e.*, reduced TBARS levels, and increased glutathione levels) (Sharma *et al*., 2010).

C.5.3.3 Potential Effects on Immune Systems

Yingkun *et al.* (2013) reported that the pre-treatment of BALB mice with stevioside (12.5, 25, or 50 mg/kg body weight) prior to intranasal instillation of lipopolysaccharide (LPS) resulted in significant attenuation of LPS-induced histological alterations in the lung, and inhibited production of pro-inflammatory cytokines compared to dexamethasone. The investigators suggested that the anti-inflammatory properties of stevioside may be related to inhibition of the NF-kB signalling pathway.

C.5.4 Toxicity of Related Steviol Glycosides and Degradation Products

As discussed in Section B.9.4, the degradation products identified in rebaudioside M-rich preparations are related substances, and while they may be characterised by slight structural differences, their presence is not expected to be of a safety concern as all are expected to share pathways of metabolism that are similar to the parent glycosides.

D. INFORMATION ON DIETARY EXPOSURE TO THE FOOD ADDITIVE

In accordance with Section 3.3.1 – Food Additives of the Food Standards Australia New Zealand *Application Handbook* (FSANZ, 2013) the following dietary exposure information must be provided:

1. A list of the foods or food groups proposed to contain the food additive.
2. The maximum proposed level and/or concentration range of the food additive for each food group or food.
3. The percentage of the food group in which the food additive is proposed to be used or the percentage of the market likely to use the new food additive.
4. Information relating to the use of the food additive in other countries.

Each point is addressed in turn in the Section that follows.

D.1 Current Permitted Food Uses and Use Levels of Steviol Glycosides

FSANZ approved the use of steviol glycosides in specific food-uses at specified use levels as shown below in Table D.1-1 (FSANZ, 2014c). PureCircle intends to market steviol glycoside preparations rich in rebaudioside M for use as intense sweeteners under the same conditions as those presently approved for steviol glycoside preparations; see Table D.1-1).

Table D.1-1 Summary of Currently Permitted Uses and Use Levels for Steviol Glycosides in Australia/New Zealand

Category No	Food Description	Steviol Glycoside Concentration (mg/kg) as Steviol Equivalents
1.1.2	Liquid milk products and flavoured milk	115
1.2.2	Fermented milk products and rennetted milk products	176
3	Ice cream and edible ices	200
	Ice confection sold in liquid form	115
	Reduced and low fat ice cream and edible ices	208
4.3.2	Fruits and vegetables in vinegar, oil, brine, or alcohol	160
4.3.4	Fruit and vegetable spreads including jams, chutneys, and related products	-
	Low joule chutneys, low joule jams, and low joule spreads	450
4.3.6	Fruit and vegetable preparations including pulp	210
5	Confectionary	-
5.1	Chocolate and cocoa products	550
5.2	Sugar confectionary	1100
	Low joule chewing gum	1100
6.3	Processed cereal and meal products	250
7	Breads and bakery products	-
7.1	Breads and related products	-
	Fancy breads	160
7.2	Biscuits, cakes, and pastries	160
11.4	Tabletop sweeteners	GMP
11.4.1	Tabletop sweeteners - liquid preparation	GMP
11.4.2	Tabletop sweeteners – tablets or powder or granules packed in portion sized packages	GMP
13.3	Formula meal replacements and formulated supplementary foods	175
13.4	Formulated supplementary sports foods	175
14.1.2.1	Fruit and vegetable juices	50
14.1.2.2	Fruit and vegetable juice products	-
	Soybean beverage (plain)	100 (plain)
	Soybean beverage (flavoured)	200 (flavoured)
	Low joule fruit and vegetable juice products	125
14.1.3	Water based flavoured drinks	200

Table D.1-1 Summary of Currently Permitted Uses and Use Levels for Steviol Glycosides in Australia/New Zealand		
Category No	**Food Description**	**Steviol Glycoside Concentration (mg/kg) as Steviol Equivalents**
14.1.3.1	Brewed soft drink	160
14.1.4	Formulated beverages	200
14.1.5	Coffee, coffee substitutes, tea, herbal infusions, and similar products	100
20.2	Food other than beverages	-
	Custard mix, custard powder, and blancmange powder	80
	Jelly	260
	Dairy and fat based desserts, dips, and snacks	150
	Sauces and toppings (including mayonnaises and salad dressings)	320

As the current steviol glycoside concentrations are provided in terms of steviol equivalents, the conversion factor for rebaudioside M will need to be provided and added to the conversion factor table. Presently, as indicated in *Standard 1.3.1 – Food Additives* of the Standard Code, the conversion factor is provided for each of the 9 permitted steviol glycosides (see Table D.1-2). In order to determine the steviol equivalent of rebaudioside M, the conversion factor of 0.25 is required and will need to be added to *Standard 1.3.1 – Food Additives* of the Standard Code.

Table D.1-2 Conversion Factors of Steviol Glycosides In Order to Determine Steviol Equivalents	
Column 1	**Column 2**
Steviol glycoside	**Conversion factor**
Dulcoside A	0.40
Rebaudioside A	0.33
Rebaudioside B	0.40
Rebaudioside C	0.33
Rebaudioside D	0.28
Rebaudioside F	0.34
Rubusoside	0.50
Steviol	1.00
Steviolbioside	0.50
Stevioside	0.40

Adapted from *Standard 1.3.1 – Food Additives* of the Standard Code (FSANZ, 2014c)

D.2 Exposure Data

Since rebaudioside M is intended for use as an intense sweetener in food-uses under the same conditions of use as those presently authorised for steviol glycosides, intakes of rebaudioside M (as steviol equivalents) will be the same as for steviol glycosides, which are already available in Australian/New Zealand marketplace. Accordingly, a separate intake assessment for rebaudioside M specifically was not performed for the purpose of this food additive application. It should be further noted that the use-levels for steviol glycosides are expressed as steviol equivalents and as such are not specified for any one particular steviol glycoside, but rather are based on the total content of the aglycone, steviol, in the final food product resulting from the addition of any steviol glycoside product meeting the appropriate specifications.

REFERENCES

Awney HA (2011). Oxidative stress biomarkers in young male rats fed with stevioside. Afr J Biochem Res 5(11):333-340.

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.

Carakostas M (2012). [Comment on] 'Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing rats' [including reply from Hala A. Awney]. J Appl Toxicol 32(2):149-152.

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

CCFA (2011). Steviol glycosides. In: *Joint FAO/WHO Food Standards Programme Codex Alimentarius Commission. Thirty fourth Session Geneva, Switz., 4-9 July 2011 / Report of the Forty-Fourth Session of the Codex Committee on Food Additives Xiamen, China 14 – 18 March 2011*. (REP11/FA). Rome, Italy: Codex Committee on Food Additives (CCFA), Codex Alimentarius Commission (CAC). Available at: http://www.codexalimentarius.org/input/download/report/759/REP11_FAe.pdf.

Cekic V, Vasovic V, Jakovljevic V, Mikov M, Sabo A (2011). Hypoglycaemic action of stevioside and small a, Cyrillic barley and brewer's yeast based preparation in the experimental model on mice. Bosn J Basic Med Sci 11(1):11-16.

Chang SS, Cook JM (1983). Stability studies of stevioside and rebaudioside A in carbonated beverages. J Agric Food Chem 31(2):409-412.

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

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

Das S, Das AK, Murphy R A, Punwani IC, Nasution MP, Kinghorn AD (1992). Evaluation of the cariogenic potential of the intense natural sweeteners stevioside and rebaudioside A. Caries Res 26(5):363-366.

DuBois GE, Walters DE, Schiffman SS, Warwick ZS, Booth BJ, Pecore SD et al. (1991). Concentration-response relationships of sweeteners. A systematic study. In: Walters DE, Orthoefer FT, DuBois GE, editors. *Sweeteners: Discovery, Molecular Design, and Chemoreception*. Symposium Sponsored by the Division of Agricultural and Food Chemistry at the 199th National Meeting of the American Chemical Society, Apr. 22-27, 1990, Boston, Mass. (ACS Symposium Series no 450). Washington (DC): American Chemical Society (ACS), pp. 261-276.

EFSA (2010). EFSA Panel on Food Additives and Nutrient Sources (ANS); Scientific Opinion on safety of steviol glycosides for the proposed uses as a food additive (question no EFSA-Q-2007-071, EFSA-Q-2008-387, EFSA-Q-2008-401, adopted on 10 March 2010 by European Food Safety Authority). EFSA J 8(4):1537. [85 pp.]. doi:10.2903/j.efsa.2010.1537. Available at: http://www.efsa.europa.eu/fr/efsajournal/pub/1537.htm.

European Commission (2011). Commission Regulation (EU) No 1131/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council with regard to steviol glycosides. Off J Eur Union 54(L295):205-211. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:295:0205:0211:EN:PDF.

FCC (2014). *Food Chemicals Codex, 9th edition*. Rockville (MD): United States Pharmacopeial Convention (USP), pp. 194-195, 434-435, and 781-782.

Fehaili S, Courel M, Rega B, Giampaoli P (2010). An instrumented oven for the monitoring of thermal reactions during the baking of sponge cake. J Food Eng 101:253-263.

Ferlow K (2005). Stevia - The sweetest substance on Earth. NutraCos 4(2, Suppl.):10-11.

Fry JC (2011). Rebiana in baked goods. Cereal Foods World 56(5):191-194.

Fry JC, Christensen TA, Yurttas N, Furlano BM (2010). Rebiana, a natural high-potency sweetener, is stable in baking consumer-acceptable, reduced-sugar cookies and muffins. Cereal Foods World 55 (Suppl. 4):A23 [abstract].

FSANZ (2008). *Final Assessment Report: Application A540: Steviol Glycosides as Intense Sweeteners*. Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ). Available at: http://www.foodstandards.gov.au/code/applications/documents/FAR_A540_Steviol_glycosides.pdf.

FSANZ (2013). *Food Standards Australia New Zealand Application Handbook. 1 September 2013 (amended 18 September 2013).* Canberra, Australia / Wellington, NZ: Food Standards Australia New Zealand (FSANZ). Available at: http://www.foodstandards.gov.au/code/changes/pages/applicationshandbook.aspx.

FSANZ (2014a). Chapter 1—General Food Standards. Part 1.3— Substances Added to Food. Standard 1.3.4—Identity and Purity. In: *Australia New Zealand Food Standards Code*. Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ). Available at: http://www.comlaw.gov.au/Details/F2014C01187 [Prepared 30 Oct 2014].

FSANZ (2014b). Chapter 1—General Food Standards. Part 1.3— Substances Added to Food. Standard 1.3.3—Processing Aids. In: *Australia New Zealand Food Standards Code*. Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ). Available at: http://www.comlaw.gov.au/Details/F2014C01186 [Prepared 30 Oct 2014].

FSANZ (2014c) Chapter 1—General Food Standards. Part 1.3—Substances Added to Food. Standard 1.3.1—Food Additives [Vol 1 and Vol. 2]. In: *Australia New Zealand Food Standards Code*. Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ). Available at: http://www.comlaw.gov.au/Details/F2014C01335 [Prepared 04 Dec 2014].

FSSAI (2012). *Minutes of National Workshop on Regulatory and Enforcement Procedures – reg.* [File No. 1(48) 2011/Seminar/FSSAI]. New Delhi, India: Food Safety and Standards Authority of India (FSSAI), Ministry of Health & Family Welfare, Government of India. Available at: http://www.fssai.gov.in/Portals/0/Pdf/Workshop_Minutes(14-05-2012).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(22):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.

Geeraert B, Crombé F, Hulsmans M, Benhabilès N, Geuns JM, Holvoet P (2010). Stevioside inhibits atherosclerosis by improving insulin signaling and antioxidant defense in obese insulin-resistant mice. Int J Obes 34(3):569-577.

Geuns JMC (2003). Stevioside. Phytochemistry 64(5): 913-921.

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.

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

Geuns JM, Augustijns P, Mols R, Buyse JG, Driessen B (2003). Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. Food Chem Tox 41(11):1599-1607.

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.

Haegens N (2010). 2.3. Baking. In: *Bread and the Technology of Bread Production*. Available at: http://www.classofoods.com/page2_3.html [Last accessed 13th May 2014].

Health Canada (2012a). *Information and Consultation Document on Health Canada's Proposal to Allow the Use of the Food Additive Steviol Glycosides as a Table-Top Sweetener and as a Sweetener in Certain Food Categories.* Ottawa (ON): Health Canada, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch. Available at: http://www.hc-sc.gc.ca/fn-an/consult/steviol/document-consultation-eng.php [Date Modified: 2012-11-30].

Health Canada (2012b). *Notice of Modification to the Lists of Permitted Food Additives to Enable the Use of Steviol Glycosides as a Table-Top Sweetener and as a Sweetener in Certain Food Categories*. Ottawa (ON): Health Canada, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch. Available at: http://www.hc-sc.gc.ca/fn-an/consult/nom-adm-0002/index-eng.php [Date Modified: 2012-11-30].

Hutapea AM, Toskulkao C, Buddhasukh D, Wilairat P, Glinsukon T (1997). Digestion of stevioside, a natural sweetener, by various digestive enzymes. J Clin Biochem Nutr 23(3):177-186.

Japan Food Chemical Research Foundation (2014). *List of Existing Food Additives* [Complied and published by the Ministry of Health and Welfare on April 16, 1996]. Tokyo, Japan: Japan Food Chemical Research Foundation. Available at: http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/list-exst.add [Effective from January 30, 2014, Last update: 06/30/2014].

JECFA (1999). Stevioside. In: *Safety Evaluation of Certain Food Additives*. 51st Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 9-18, 1998. (WHO Food Additives Series, no 42). Geneva, Switz.: World Health Organization (WHO) / International Programme on Chemical Safety (IPCS), pp. 119-143. Available at: http://www.inchem.org/documents/jecfa/jecmono/v042je07.htm.

JECFA (2006a). Steviol glycosides (Tentative) [New tentative specifications prepared at the 63rd JECFA (2004), published in FNP 52 Add 12 (2004)]. In: *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 1st to 65th JECFA Meetings, 1956-2005. (FAO JECFA Monographs 1). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/.

JECFA (2006b). 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. 117-144, 638. Available at: http://whqlibdoc.who.int/publications/2006/9241660546_eng.pdf.

JECFA (2007a). Steviol glycosides [Prepared at the 68th JECFA (2007) and published in FAO JECFA Monographs 4 (2007), superseding tentative specifications prepared at the 63rd JECFA (2004)]. In: *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 68th Meeting, June 19-28, 2007, Geneva, Switz. (FAO JECFA Monographs 4). Rome, Italy: Food and Agriculture Organization of the United Nations. Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA), pp. 61-64. Available at: ftp://ftp.fao.org/docrep/fao/010/a1447e/a1447e.pdf.

JECFA (2007b). Steviol glycosides. In: *68th JECFA - Chemical and Technical Assessment (CTA)*. [Sixty-eighth meeting held June 17-26, 2008]. (Revised by Paul M. Kuznesof, Ph.D., Original prepared by Harriet Wallin for the 63rd JECFA). Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: http://www.fao.org/fileadmin/templates/agns/pdf/jecfa/cta/68/Steviol_glycosides.pdf.

JECFA (2007c). 3.1.9 Steviol glycosides. *Evaluation of Certain Food Additives and Contaminants*. Sixty-eighth Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 19-28, 2007, Geneva, Switz. (WHO Technical Report Series, no 947). Geneva, Switz.: World Health Organization (WHO), pp. 50-54, 206, 215. Available at: http://whqlibdoc.who.int/publications/2007/9789241209472_eng.pdf.

JECFA (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. 183-219. Available at: http://whqlibdoc.who.int/publications/2009/9789241660600_eng.pdf.

JECFA (2010). Steviol glycosides [Prepared at the 73rd JECFA, 2010) and published in FAO JECFA Monographs 10, 2010)]. In: *Combined Compendium of Food Additive Specifications*. (FAO JECFA Monographs 10). 73rd Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 8-17, 2010, Geneva, Switz. Food and Agriculture Organization of the United Nations (FAO), Joint FAO/WHO Expert Committee on Food Additives (JECFA), Rome, Italy. Available at: http://www.fao.org/ag/agn/jecfa-additives/specs/monograph10/additive-442-m10.pdf.

Jooken E, Amery R, Struyf T, Duquenne B, Geuns J, Meesschaert B (2012). Stability of steviol glycosides in several food matrices. J Agric Food Chem 60(42):10606-10612.

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

Koyama E, Sakai N, Ohori Y, Kitazawa K, Izawa O, Kakegawa K et al. (2003b). Absorption and metabolism of glycosidic sweeteners of stevia mixture and their aglycone, steviol, in rats and humans. Food Chem Toxicol 41(6):875-883.

Kraemer T, Maurer HH (1994). On the metabolism of the sweetener stevioside in humans. Eur J Pharm Sci 2(1/2):103 [Abstract No. FC12].

Kroyer GT (1999). The low calorie sweetener stevioside: stability and interaction with food ingredients. Lebensm Wiss Technol 32(8):509-512.

Lemus-Mondaca R, Vega-Gálvez A, Zura-Bravo L, Ah-Hen K (2012). *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: a comprehensive review on the biochemical, nutritional and functional aspects. Food Chem 132(3):1121-1132.

Lewis WH (1992). Early uses of *Stevia rebaudiana* leaves as sweetener in Paraguay. Econ Bot 46(3):336-337.

Lostie M, Peczalski R, Andrieu J, Laurent M (2002). Study of sponge cake batter baking process. Part I: Experimental data. J Food Eng 51:131-137.

Maki KC, Curry LL, Reeves MS, Toth PD, McKenney JM, Farmer MV et al. (2008a). Chronic consumption of rebaudioside A, a steviol glycoside, in men and women with type 2 diabetes mellitus. Food Chem Toxicol 46(7, Suppl.):S47-S53.

Maki KC, Curry LL, Carakostas MC, Tarka SM, Reeves MS, Farmer MV et al. (2008b). The hemodynamic effects of rebaudioside A in healthy adults with normal and low-normal blood pressure. Food Chem Toxicol 46(7, Suppl.):S40-S46.

Marie S (1991). Sweeteners. In: Smith J, editor. *Food Additive User's Handbook*. Glasgow, Scotland: Blackie / New York (NY): AVI, pp. 47-74.

Nakayama K, Kasahara D, Yamamoto F (1986). Absorption, distribution, metabolism and excretion of stevioside in rats. Shokuhin Eiseigaku Zasshi [J Food Hyg Soc Jpn] 27(1):1-8.

Nikiforov AI, Eapen AK (2008). A 90-day oral (dietary) toxicity study of rebaudioside A in Sprague-Dawley rats. Int J Toxicol 27(1):65-80.

Nikiforov AI, Rihner MO, Eapen AK, Thomas JA (2013). Metabolism and toxicity studies supporting the safety of rebaudioside D. Int J Toxicol 32(4):261-273.

Ohta M, Sasa S, Inoue A, Tamai T, Fujita I, Morita K et al. (2010). Characterization of novel steviol glycosides from leaves of Stevia rebaudiana Morita. J Appl Glycosci 57: 199-209.

Prakash Chaturvedula VS, Prakash I (2013). Structural characterization and hydrolysis studies of rebaudioside E, a minor sweet component of *Stevia rebaudiana*. Eur Chem Bull 2(5):298-302.

Prakash I, Dubois GE, Clos JF, Wilkens KL, Fosdick LE (2008). Development of rebiana, a natural, non-caloric sweetener. Food Chem Toxicol 46(Suppl 7):S75-S82.

Prakash I, Clos JF, Prakash Chaturvedula VS (2012). Stability of rebaudioside A under acidic conditions and its degradation products. Food Res Int 48(1):65-75.

Prakash I, Chaturvedula VSP, Markosyan A (2013). Isolation, characterization and sensory evaluation of a hexa (*beta*)-D-glucopyranosyl diterpene from *Stevia rebaudiana*. Nat Prod Commun 8(11):1523-1526.

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.

Ramanathan P, Sellappan RS (2010). Safety study of aqueous leaves extract of *Stevia Rebaudiana* leaves in albino rats. Intl J Pharm Sci 2(1):477-481.

Renwick AG (2007). Toxicokinetics [section on elimination: excretion via the gut]. In: Hayes W, editor. *Principles and Methods of Toxicology* (5th edition). 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.):S70-S74.

Roberts A, Renwick AG (2008). Comparative toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol in rats. Food Chem Toxicol 46(7, Suppl.):S31-S39.

SCF (1985). Reports of the Scientific Committee for Food concerning sweeteners (Opinion expressed by the SCF on 14 September 1984) [Stevioside]. In: *Food Science and Techniques*. (Reports of the Scientific Committee for Food (16th series; EUR 10210 EN). Brussels, Belgium: Commission of the European Communities (EEC), Health & Consumer Protection Directorate-General, Scientific Committee for Food (SCF). Available at: http://aei.pitt.edu/40825/1/16th_food.pdf.

SCF (1999). *Opinion on Stevioside as a Sweetener (Adopted on 17/6/99)*. (CS/ADD/EDUL/167 Final). Brussels, Belgium: European Commission, Health & Consumer Protection Directorate-General, Scientific Committee on Food (SCF). Available at: http://ec.europa.eu/food/fs/sc/scf/out34_en.pdf.

Sharma D, Puri M, Tiwary AK, Singh N, Jaggi AS (2010). Antiamnesic effect of stevioside in scopolamine-treated rats. Indian J Pharmacol 42(3):164-167.

Sharp PE, La Regina MC, 1998. Important biological features (chapter 1). In: The Laboratory Rat. (Laboratory Animal Pocket Reference Series). CRC Press Inc., Boca Raton, pp. 1-19 [See p. 16].

Simonetti P, Gardana C, Bramati L, Pietta PG (2004). Bioavailability of stevioside from Stevia rebaudiana in humans: preliminary report. In: Geuns JMC, Buyse J, editors. *Safety of Stevioside*: Proceedings of the First Symposium Sponsored by KULeuven, April 16, 2004, Leuven, Belgium. Heverlee, Belgium: Euprint ed., pp. 51-62.

Stoyanova S, Geuns J, Hideg E, Van Den Ende W (2011). The food additives inulin and stevioside counteract oxidative stress. Int J Food Sci Nutr 62(3):207-214.

Sung LH (2002) [unpublished]. *Unpublished report on pharmacokinetic (PK) studies of T100 sunstevia 95% stevioside in rats*. Unpublished report from Singapore, Singapore: Sunlabel Pte Ltd. Submitted to Geneva, Switz.: World Health Organization (WHO) by Japan: Ministry of Health and Welfare, Japan. Cited In: JECFA, 2006b.

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.

U.S. FDA (2008a). *Agency Response Letter GRAS Notice No. GRN 000252 [Rebaudioside A purified from Stevia Rebaudiana (Bertoni) Bertoni]*. Submitted by Chicago (IL): Whole Earth Sweetener Company LLC (a wholly owned subsidiary of Merisant Company) to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=252 [December 17, 2008].

U.S. FDA (2008b). *Agency Response Letter GRAS Notice No. GRN 000253 [Rebaudioside A purified from Stevia Rebaudiana (Bertoni) Bertoni]*. Submitted by Wayazata (ST): Cargill, Inc. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=253 [December 17, 2008].

U.S. FDA (2009a). *Agency Response Letter GRAS Notice No. GRN 000275 [Purified Steviol Glycosides With Rebaudioside A as the Principal Component]*. Submitted by Fort Washington (PA): McNeil Nutritionals, LLC to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=275 [June 11, 2009].

U.S. FDA (2009b). *Agency Response Letter GRAS Notice No. GRN 000278 [Rebaudioside A Purified From Stevia Rebaudiana (Bertoni) Bertoni]*. Submitted by Rancho Santa Margarita (CA): Blue California to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=278 [July 20, 2009].

U.S. FDA (2009c). *Agency Response Letter GRAS Notice No. GRN 000282 [Rebaudioside A Purified From the Leaves of Stevia Rebaudiana (Bertoni) Bertoni]*. Submitted by Bellingham (WA): Sweet Green Fields, LLC to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=282 [August 11, 2009].

U.S. FDA (2009d). *Agency Response Letter GRAS Notice No. GRN 000287 [Purified Steviol Glycosides With Rebaudioside A and Stevioside As the Principal Components]*. Submitted by Gilbert (AZ): Wisdom Natural Brands to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=287 [August 28, 2009].

U.S. FDA (2010a). *Agency Response Letter GRAS Notice No. GRN 000303 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni]*. Submitted by Frisco (TX) / Erlanger (KY): Sunwin USA, LLC / WILD Flavors to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=303 [March 22, 2010].

U.S. FDA (2010b). *Agency Response Letter GRAS Notice No. GRN 000304 [Purified steviol glycosides with rebaudioside A and stevioside as the principal components]*. Submitted by Submitted by Frisco (TX) / Erlanger (KY): Sunwin USA, LLC / WILD Flavors to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=304 [March 22, 2010].

U.S. FDA (2010c). *Agency Response Letter GRAS Notice No. GRN 000318 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni]*. Submitted by Naples (FL): Pyure Brands, LLC to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=318 [May 15, 2010].

U.S. FDA (2010d). *Agency Response Letter GRAS Notice No. GRN 000323 [Purified steviol glycosides with rebaudioside A and stevioside as the principal components]*. Submitted by Oak Brook (IL): PureCircle USA, Inc. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=323 [July 9, 2010].

U.S. FDA (2010e). *Agency Response Letter GRAS Notice No. GRN 000329 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni]*. Submitted by Vancouver (BC): GLG Life Tech, Ltd. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=329 [September 9, 2010].

U.S. FDA (2011a). *Agency Response Letter GRAS Notice No. GRN 000337 [Enzyme-modified steviol glycosides preparation (EMSGP)].* Submitted by Bloomingdale (IL): NOW Foods to to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=337 [June 17, 2011].

U.S. FDA (2011b). *Agency Response Letter GRAS Notice No. GRN 000348 [Stevioside purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni (stevioside)]*. Submitted by Vancouver (BC): GLG Life Tech, Ltd. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=348 [July 14, 2011].

U.S. FDA (2011c). *Agency Response Letter GRAS Notice No. GRN 000349 [Purified steviol glycosides with rebaudioside A and stevioside as the principal components]*. Submitted by Vancouver (BC): GLG Life Tech, Ltd. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=349 [July 14, 2011].

U.S. FDA (2011d). *Agency Response Letter GRAS Notice No. GRN 000354 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni (rebaudioside A)]*. Submitted by Guilin, China: Guilin Layn Natural Ingredients, Corp. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=354 [July 15, 2011].

U.S. FDA (2011e). *Agency Response Letter GRAS Notice No. GRN 000365 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni (rebaudioside A)].* Submitted by El Dorado Hills (CA): BrazTek International Inc., d/b/a Rio Natural) to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=365 [August 18, 2011].

U.S. FDA (2011f). *Agency Response Letter GRAS Notice No. GRN 000367 [Purified steviol glycosides with rebaudioside A and stevioside as the principal components]*. Submitted Quingdao, China: Sinochem Qingdao Co., Ltd. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=367 [July 8, 2011].

U.S. FDA (2011g). *Agency Response Letter GRAS Notice No. GRN 000369 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni].* Submitted by Piscataway (NJ): Zhucheng Haotian Pharm Co., Ltd., C/O Shanghai Freemen Americas, LLC to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=369 [October 11, 2011].

U.S. FDA (2011h). *Agency Response Letter GRAS Notice No. GRN 000375 [Enzyme modified steviol glycosides, Tokyo, Japan: Toyo Sugar Refining Co., Ltd./Tokyo, Japan: Nippon Paper Chemicals Co., Ltd.].* College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=375 [September 2, 2011].

U.S. FDA (2011i). *Agency Response Letter GRAS Notice No. GRN 000380 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni (rebaudioside A)].* Submitted by Vancouver (BC): GLG Life Tech Corporation to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=380 [November 28, 2011].

U.S. FDA (2012a). *Agency Response Letter GRAS Notice No. GRN 000388 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni (rebaudioside A)].* Submitted by Chengdu, Sichuan, China: Chengdu Wagott Pharmaceutical to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=388 [January 9, 2012].

U.S. FDA (2012b). *Agency Response Letter GRAS Notice No. GRN 000389 [Steviol glycosides with stevioside as the principal component].* Submitted by Chengdu, Sichuan, China: Chengdu Wagott Pharmaceutical to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=389 [January 18, 2012].

U.S. FDA (2012c). *Agency Response Letter GRAS Notice No. GRN 000393 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni (rebaudioside A)].* Submitted by Republic of South Korea: Daepyung Co., Ltd., to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=393 [January 23, 2012].

U.S. FDA (2012d). *Agency Response Letter GRAS Notice No. GRN 000395 [Steviol glycosides with rebaudioside A and stevioside as the principal components].* Submitted by Republic of South Korea: Daepyung Co., Ltd., to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=395 [January 24, 2012].

U.S. FDA (2012e). *Agency Response Letter GRAS Notice No. GRN 000418 [Rebaudioside A purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni (rebaudioside A)].* Submitted by City of Industry (CA): MiniStar International, Inc., to Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=418 [June 7, 2012].

U.S. FDA (2013a). *Agency Response Letter GRAS Notice No. GRN 000473 [Purified steviol glycosides with rebaudioside X as the principal component].* Submitted by Oak Brook (IL): PureCircle Ltd., to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=473 [December 2, 2013].

U.S. FDA (2013b). *Agency Response Letter GRAS Notice No. GRN 000448 [Enzyme-modified steviol glycosides].* Submitted by Republic of South Korea: Daepyung Co., Ltd., to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=448 [May 3, 2013].

U.S. FDA (2013c). *Agency Response Letter GRAS Notice No. GRN 000452 [Enzyme-modified steviol glycosides].* Submitted by Republic of South Korea: Daepyung Co., Ltd., to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=452 [July 1, 2013].

U.S. FDA (2013d). *Agency Response Letter GRAS Notice No. GRN 000456 [Rebaudioside D purified from the leaves of Stevia rebaudiana (Bertoni) Bertoni (rebaudioside D)].* Submitted by Oak Brook (IL): PureCircle USA, Inc., to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=456 [July 1, 2013].

U.S. FDA (2013e). *Agency Response Letter GRAS Notice No. GRN 000461* [Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)]. Submitted by Bangkok, Thailand: Almendra Limited to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=461 [August 14, 2013].

U.S. FDA (2013f). *Agency Response Letter GRAS Notice No. GRN 000467 [*Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni]. Submitted by Shangdong, China: Qufu Xiangzhou Stevia Products Co., Ltd., to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=467 [November 25, 2013].

U.S. FDA (2013g). *Agency Response Letter GRAS Notice No. GRN 000493 [High purity steviol glycosides (minimum purity 95%)]*. Submitted by Vancouver (BC): GLG Life Tech Corporation to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=493 [Date of filing: December 23, 2013; Date of closure / FDA's Letter: May 30, 2014].

U.S. FDA (2014a). *Agency Response Letter GRAS Notice No. GRN 000516 [Steviol glycosides with rebaudioside A and stevioside as the principal components, Pluakdaeng, Thailand: Almenda (Thailand) Limited].* Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=516 [Date of filing: April 28, 2014; Status as of November 8, 2014: Pending].

U.S. FDA (2014b). *Agency Response Letter GRAS Notice No. GRN 000512 [High purity Rebaudioside M, Vancouver (BC): GLG Life Tech Corporation].* Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=512 [Date of filing: 2014; Status as of November 8, 2014: Pending].

U.S. FDA (2014c). *Agency Response Letter GRAS Notice No. GRN 000536 [High purity rebaudioside C, Vancouver (BC): GLG Life Tech Corporation].* Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=536 [Date of filing: August 18, 2014; Status as of November 8, 2014: Pending].

Urban JD, Carakostas MC, Brusick DJ (2013). Steviol glycoside safety: is the genotoxicity database sufficient? Food Chem Toxicol 51:386-390.

Waddell WJ (2011). [Comment on] 'Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing rats' [including reply from Hala A. Awney]. J Appl Toxicol 31(6):595-598.

Wang LZ, Goh BC, Fan L, Lee HS (2004). Sensitive high-performance liquid chromatography/ mass spectrometry method for determination of steviol in rat plasma. Rapid Commun Mass Spectrom 18(1):83-86.

Wang Z, Xue L, Guo C, Han B, Pan C, Zhao S et al. (2012). Stevioside ameliorates high-fat diet-induced insulin resistance and adipose tissue inflammation by downregulating the NF-(*kappa*)B pathway. Biochem Biophys Res Commun 417(4):1280-1285.

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.

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

Yamada A, Ohgaki S, Noda T, Shimizu M (1985). Chronic toxicity study of dietary stevia extracts in F344 rats. Shokuhin Eiseigaku Zasshi [J Food Hyg Soc Jpn] 26(2):169-183.

Yingkun N, Zhenyu W, Jing L, Xiuyun L, Huimin Y (2013). Stevioside protects LPS-induced acute lung injury in mice. Inflammation 36(1):242-250.

Zanoni B, Peri C, Pierucci S (1993). A study of the bread-baking process. I: A phenomenological model. J Food Eng 19:389-398.