

GRAS Notice (GRN) No. 452

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

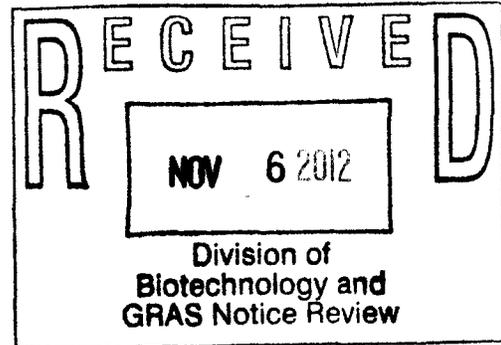
ORIGINAL SUBMISSION



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October 31, 2012

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety (HFS-255)
5100 Paint Branch Parkway
College Park, MD 20740-3835



Attention: Dr. Mary D. Ditto

Re: GRAS Notification – Glucosylated Rebaudioside A

Dear Dr. Ditto:

On behalf of Daepyoung Co., Ltd. of South Korea, we are submitting for FDA review a GRAS notification for Glucosylated Rebaudioside A. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via telephone or email.

We look forward to your feedback.

Sincerely,

(b) (6)

Robert S. McQuate, Ph.D.
CEO & Co-Founder
GRAS Associates, LLC
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Attachment: GRAS Notification for Daepyoung Co., Ltd. – Glucosylated Rebaudioside A



GRAS ASSESSMENT

Of

Glucosylated Rebaudioside A REBATEN PREMIUM

Food Usage Conditions for General Recognition of Safety

for

DAEPYUNG Co., Ltd.

Bundang-Gu, Seongnam-Si, Gyeonggi-Do
Republic of South Korea (463-864)

Evaluation by GRAS Expert Panel

Richard C. Kraska, Ph.D., DABT

Robert S. McQuate, Ph.D.

Robert W. Kapp, Jr., Ph.D., Fellow ATS, ERT (UK)

October 31, 2012



TABLE OF CONTENTS

I. GRAS EXEMPTION CLAIM	4
A. Claim of Exemption from Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1).....	4
B. Name & Address of Notifier.....	4
C. Common Name & Identity of Notified Substance.....	4
D. Conditions of Intended Use in Food.....	4
E. Basis for GRAS Determination.....	5
F. Availability of Information.....	5
II. INTRODUCTION	5
A. Objective.....	5
B. Foreword.....	5
C. Summary of Regulatory History of Stevia & Stevia-Derived Sweeteners.....	6
D. FDA Regulatory Framework.....	10
III. CHEMISTRY & MANUFACTURE OF DAEPYUNG'S REBATEN PREMIUM	11
A. Common or Usual Name.....	11
B. Basic Molecular Structure Considerations for Glucosylated Rebaudioside A.....	11
C. Background Information on Chemistry of Steviol Glycosides	13
D. Accepted Identity Specifications for Food Grade Steviol Glycosides.....	16
E. Manufacturing Process for REBATEN PREMIUM.....	17
1. Scientific & Patent Literature.....	17
2. Daepyeong's Manufacturing Process for REBATEN PREMIUM.....	18
F. Product Specifications & Supporting Methods.....	19
1. Specifications for Daepyeong's REBATEN PREMIUM Starting Material	19
2. Specifications for REBATEN PREMIUM.....	19
3. Analytical Data on REBATEN PREMIUM.....	22
G. Sweetness Intensity of REBATEN PREMIUM.....	22
H. Stability Data on REBATEN PREMIUM	22
IV. INTENDED FOOD USES & ESTIMATED DIETARY INTAKE	24
A. Intended Food Uses.....	24
B. Estimated Daily Intake.....	24
V. SAFETY INVESTIGATIONS FOR REBAUDIOSIDE A	26
A. Safety Data on REBATEN PREMIUM.....	26
B. Summary of Safety Data on Steviol Glycosides & Rebaudioside A: Reviews by Expert Bodies & Other Scientists.....	28
C. Safety Data on Rebaudioside A.....	29
1. Absorption, Distribution, Metabolism & Excretion (ADME) Studies.....	30
2. Subchronic Toxicity Studies.....	31
3. Mutagenicity Studies.....	32
4. Reproduction & Developmental Studies.....	32
5. Clinical Studies on Rebaudioside A.....	34
D. Safety of Enzyme Residues in REBATEN PREMIUM.....	34
E. Safety Data on Steviol Glycosides That Are Predominantly Stevioside & Rebaudioside A.....	35

TABLE OF CONTENTS continued

F. Clinical Studies & Other Reports in Humans on Rebaudioside A & Other Steviol Glycosides.....	35
G. Studies on Principal Metabolite: Steviol.....	35
VI. GRAS CRITERIA & PANEL SAFETY FINDINGS.....	35
A. GRAS Criteria.....	35
B. Panel Findings on Safety Studies of Steviol Glycosides.....	36
1. Discussion of Safety of REBATEN PREMIUM.....	36
2. Discussion of Safety Data of Steviol Glycosides Preparations That Are Predominantly Stevioside & Rebaudioside A.....	37
3. Acceptable Daily Intake for REBATEN PREMIUM.....	39
C. Common Knowledge Elements for GRAS Determinations.....	39
1. Generally Available Information.....	39
2. Scientific Consensus.....	40
VII. CONCLUSIONS.....	42
VIII. REFERENCES.....	44

TABLES

Table 1. FDA's GRAS Notice Inventory on Rebaudioside A & Steviol Glycosides.....	8
Table 2. Components Expected to be Present in Glucosylated Steviol Glycosides Extracts.....	13
Table 3. Chemical Identity of Stevioside & Rebaudioside A	15
Table 4. Specifications for Daepyeong's REBATEN PREMIUM Starting Product (Steviol Glycosides 95% with \geq 80% Rebaudioside A) & Comparison to JECFA Specifications.....	20
Table 5. Levels of Steviol Glycosides by Percent Range in Non-Glucosylated & Glucosylated Steviol Glycosides & Steviol Equivalents of Glucosylated Steviol.....	21
Table 6. Non-Glycoside Impurities Levels in 3 Lots of Starting Material vs. REBATEN PREMIUM Finished Product.....	22
Table 7. Summary of Critical Parameters for Three Lots of Daepyeong Glucosylated Rebaudioside A (REBATEN PREMIUM).....	23
Table 8. Sweetness Intensity of REBATEN PREMIUM	23
Table 9. Summary of REBATEN PREMIUM Stability Tests Completed or in Progress.....	24
Table 10. Food Uses of Steviol Glycosides Reported to JECFA with Calculated Steviol Equivalents.....	25
Table 11. Daily Intake of Sweeteners (In Sucrose Equivalents) & Estimated Daily Intakes of REBATEN PREMIUM.....	26
Table 12. Mutagenicity & Genotoxicity Studies on Rebaudioside A.....	33

FIGURES

Figure 1. Representative Chemical Structures of Glucosylated Stevia	12
Figure 2. Chemical Structures of Various Steviol Glycosides	14
Figure 3. Chemical Structure of Rebaudioside A.....	15
Figure 4. Chemical Structure of Stevioside	16

APPENDICES

APPENDIX 1 Biological & Toxicological Data on Stevia & Steviol Glycosides.....	54
APPENDIX 2 Safety Data on Stevioside & Stevia Extracts That Are Predominantly Stevioside.....	56
APPENDIX 3 Steviol Glycosides Clinical Studies & Other Reports in Humans.....	64
APPENDIX 4 Studies on Principal Metabolite: Steviol.....	67

I. GRAS EXEMPTION CLAIM

A. Claim of Exemption From Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1)¹

Daepyeong Co., Ltd. is Glucosylated Rebaudioside A, also referred to as REBATEN PREMIUM (“RP”), which meets the specifications described in Table 4, is Generally Recognized As Safe (GRAS) in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. Daepyeong made this determination in concert with an appropriately convened panel of experts who are qualified by scientific training and experience. The GRAS determination is based on scientific procedures as described in the following sections. The evaluation accurately reflects the conditions of the stevia-derived sweetener’s intended uses in foods.

(b) (6)

Signed:

Robert S. McQuate, Ph.D.
GRAS Associates, LLC
20482 Jacklight Lane
Bend, OR 97702-3074

Date: October 31, 2012

B. Name Address of Notifier

Daepyeong Co., Ltd.
Leaders B/D 604, #274-4 Seohyeon-Dong
Bundang-Gu, Seongnam-Si, Gyeonggi-Do
Republic of South Korea (463-824)

As the notifier, Daepyeong Co., Ltd. (“Daepyeong”) accepts responsibility for the GRAS determination that has been made for PR as described in the subject notification; consequently, the PR preparation meeting the conditions described herein are exempt from premarket approval requirements for food ingredients.

C. Common Name Identity of Notified Substance

The common or usual name for REBATEN PREMIUM (RP) is glucosylated rebaudioside A; also see Section III.A.

D. Conditions of Intended Use in Food

The RP preparation is intended to be used as a table top sweetener and as a general purpose non-nutritive sweetener for incorporation into foods in general, other than infant formulas and

¹ See 62 FR 18938 (17 April 1997) which is accessible at <http://www.gpo.gov/fdsys/pkg/FR-1997-04-17/html/97-97-9706.htm>.

meat and poultry products, at per serving levels that reflect good manufacturing practices principles in that the quantity added to foods should not exceed the amounts reasonably required to accomplish the intended technical effect.

E. Basis for the GRAS Determination

Pursuant to 21 CFR 170.30, Daepyeong's RP preparation has been determined to be GRAS on the basis of scientific procedures as discussed in the detailed description provided below.

F. Availability of Information

The data and information that serve as the basis for this GRAS notification will be sent to the US Food and Drug Administration (FDA) upon request or will be available for review and copying at reasonable times at the offices of GRAS Associates, LLC, located at 20482 Jacklight Lane, Bend, OR 97702-3074.

II. INTRODUCTION

A. Objective

At the request of Daepyeong, GRAS Associates, LLC ("GA") has undertaken an independent safety evaluation of Daepyeong's RP preparation. The preparation is extracted from the leaves of *Stevia rebaudiana* Bertoni and purified to yield $\geq 95\%$ steviol glycosides with a minimum 80% rebaudioside A content. The stevia-derived starting material is composed primarily of rebaudioside A which is glucosylated using the enzyme, cyclomaltodextrin glucanotransferase (CGTase). The purpose of the evaluation is to ascertain whether the intended food uses of RP as a general purpose non-nutritive sweetener as described in Section IV.A are generally recognized as safe, i.e., GRAS, under the intended conditions of use.

B. Foreword

Daepyeong provided GA with substantial background information needed to enable the GRAS assessment to be undertaken. In particular, the information provided addressed the safety/toxicity of steviol glycosides; the history of use of stevia in food; and compositional details, specifications, and method of preparation of the notified substance. Daepyeong was asked to provide adverse reports, as well as those that supported conclusions of safety. Safety/toxicity studies performed with animals were noted to have value, along with available human testing. Daepyeong was also asked to supply past and present human food use information. Knowing how much steviol glycosides has been safely consumed, i.e., the use levels, is critical in extrapolating to safe exposures for RP when consumed as a food ingredient. The composite safety/toxicity studies on RP and steviol glycosides, in concert with exposure information, ultimately provide the specific scientific foundation for the GRAS determination.

In addition to the product specifications, chemical properties, manufacturing, and safety related information, Daepyeong also provided some consumption/exposure information, along with other related documentation. This was augmented with an independent search of the scientific and regulatory literature extending through September 2012. A GRAS assessment based primarily on the composite safety information, i.e., based on scientific procedures, was undertaken. Those references that were deemed pertinent to the objective at hand are listed in Section VIII.

C. Summary of Regulatory History of Stevia □ Stevia-Derived Sweeteners

Glucosylated steviol glycosides are currently used in human foods in Asia. Glucosylated steviol glycosides have been an approved food additive in Korea since 2000, and enzyme-treated stevia is also listed in the Japan Food Additives Code. Glucosylated stevia was developed by research programs that were initiated in the early 1990s in Japan, Malaysia, Armenia and Canada to enhance taste and improve sweetness qualities over naturally occurring steviol glycosides.

Two GRN notifications (GRN 337 [FDA, 2010]) and GRN 375 [FDA 2011a]) submitted to FDA on similar glucosylated steviol glycosides preparations have both received "no questions" responses from FDA with respect to the conclusions of both expert panels that, indeed, the glucosylated steviol glycosides (GSGs) are GRAS under the intended conditions of use (FDA, 2011b and FDA, 2011c, respectively). In addition, there is limited availability of enzyme-modified steviol glycosides as a dietary supplement in the US.

Between 1989 and 2008, at least two GRAS petitions seeking authorization for the addition of stevioside or steviol glycosides to foods had been submitted to FDA. However, no authorizations had been issued by FDA in response to these filings, and these petitions were withdrawn. It appears that the previously available safety data---including purity considerations---for stevia, stevioside, or steviol glycosides were inadequate.

There is evidence that stevia was "used as a dietary supplement in herbal tea" in the 1980s which resulted in an FDA judicial seizure action against the manufacturer---Sunrider---on the grounds that the products contained stevia, an unapproved food additive (FDA, 1995). Based upon this action, Sunrider marketed stevia solely as a cosmetic ingredient until 1995. With the passage of the Dietary Supplement Health and Education Act of 1994, FDA recognized stevia as a dietary ingredient in August, 1995 (FDA, 1995). These supplements are widely available to consumers in the US through retail outlets and Internet purchases (Al-Achi and Greenwood, 2000).

Stevia-derived sweeteners are permitted as food additives in South America and in several countries in Asia, including China, Japan, and Korea. In recent years, these sweeteners have received food usage approvals in Mexico, Australia, New Zealand, Switzerland, France, Peru, Uruguay, Colombia, Senegal, Russia, Malaysia, Turkey, Taiwan, Thailand, Israel and Hong Kong (EFSA, 2010, and NutraIngredients, 2010).

Based on available information from FDA's GRAS Notice Inventory² website as of September 15, 2012, the agency has filed 23 notices on rebaudioside A or steviol glycosides. A summary of

² <http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing>.

these filings is presented in Table 1. All of these notices have received “no questions” letters from the FDA.

The Joint Expert Committee on Food Additives (JECFA) has reviewed steviol glycosides at its 51st, 63rd, 68th and 73rd meetings. In 2000, JECFA published the original review on steviol glycosides (WHO, 2000). JECFA established a temporary ADI (acceptable daily intake) of 0-2 mg/kg (on a steviol basis) at its 63rd meeting (WHO, 2006). Additionally, JECFA finalized food grade specifications (FAO, 2007a), although they were subsequently updated in 2008 (FAO, 2008) and 2010 (FAO, 2010) (see below). At the 69th meeting, the temporary status of the ADI was removed, and the ADI was raised to 0-4 mg/kg bw/day (on a steviol basis) as a result of the JECFA review of recently completed clinical studies with steviol glycosides (WHO, 2008). In 2009, JECFA published a final monograph addendum on steviol glycosides (WHO, 2009). In 2008, Switzerland’s Federal Office for Public Health (2008) approved the use of stevia as a sweetener citing the favorable actions of JECFA. Subsequently, France published its approval for the food uses of rebaudioside A with a purity of 97% (AFSSA, 2009).

Also in 2008, the Food Standards Australia New Zealand (FSANZ) completed its evaluation of an application for use of steviol glycosides in foods. FSANZ recommended that the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) amend the Australia New Zealand Food Standards Code to allow the use of steviol glycosides in food (FSANZ, 2008). In December 2010, FSANZ recommended accepting the increased usage levels as requested since no public health and safety issues were identified (FSANZ, 2010). Subsequently, FSANZ approved an increase in the maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages and flavored soy beverages up to 200 mg/kg and in plain soy beverages up to 100 mg/kg (FSANZ, 2011).

In early 2009, a number of parties, including the government of Australia and the Calorie Control Council, submitted a request to the Codex Committee on Food Additives in which it was proposed that the JECFA specifications for steviol glycosides should be modified to allow inclusion of Rebaudioside D and Rebaudioside F as specifically named acceptable glycosides that would be considered as part of the minimum 95% steviol glycosides composition (CCFA, 2009). This proposed modification was endorsed by the Codex Alimentarius Committee in July 2009; it was on the agenda for discussion at the JECFA Meeting in June, 2010 (FAO/WHO, 2009), and JECFA recently took final action in approving the modified steviol glycosides specifications to include Rebaudioside D and Rebaudioside F (FAO, 2010; Daepyeong Food Additive Master File 849, Volume 3 - Attachment 1).

In 2010, Food Chemicals Codex (FCC) prepared a monograph with a description and specifications for rebaudioside A. In this monograph, rebaudioside A is described as a white to off-white, hygroscopic fine crystal, granule, or powder having a sweet taste. It is freely soluble in ethanol:water 50/50 (v/v) and is sparingly soluble in water and in ethanol. Rebaudioside A is obtained from the leaves of the *Stevia rebaudiana* Bertoni plant in a multistep separation and purification process. The principal steps of manufacturing include extraction of steviol glycosides from the leaves using an aqueous or aqueous alcoholic (ethanol or methanol) solvent, and purification of rebaudioside A from the resulting mixture of steviol glycosides by resin absorption followed by recrystallization from an aqueous or aqueous alcoholic (ethanol or methanol) solvent. It is primarily composed of rebaudioside A, a glycoside of the *ent*-kaurenoid diterpenoid aglycone known as steviol (FCC, 2010).

Table 1. FDA's GRAS Notice Inventory on Rebaudioside A & Steviol Glycosides^a

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
1. Merisant	GRN 252	High-Purity Reb A ≥95%	Variety of food categories & table top sweetener
2. Cargill Inc.	GRN 253	High-Purity Reb A >97%	General-purpose sweetener, excluding meat & poultry products
3. McNeil Nutritionals LLC	GRN 275	Purified Steviol Glycosides - Reb A Principal Component	Table top sweetener
4. Blue California	GRN 278	High-Purity Reb A ≥97%	General-purpose & table top sweetener
5. Sweet Green Fields LLC	GRN 282	High-Purity Reb A >97%	General-purpose sweetener, excluding meat & poultry products
6. Wisdom Natural Brands	GRN 287	Purified Steviol Glycosides >95% - Reb A & Stevioside Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas
7. Sunwin USA LLC & WILD Flavors	GRN 303	High-Purity Reb A ≥95% / ≥98%	General-purpose sweetener, excluding meat, poultry products & infant formulas
8. Sunwin USA LLC & WILD Flavors	GRN 304	Purified Steviol Glycosides >95% - Reb A & Stevioside Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas
9. Pyure Brands, LLC	GRN 318	High-Purity Reb A 95% / 98%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
10. PureCircle USA Inc	GRN 323	Purified Steviol Glycosides - Reb A Principal Component	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
11. GLG Life Tech Ltd	GRN 329	High-Purity Reb A ≥97%	General-purpose sweetener, excluding meat & poultry products
12. GLG Life Tech Ltd	GRN 348	High-Purity Stevioside ≥95%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
13. GLG Life Tech Ltd	GRN 349	High-Purity Steviol Glycosides ≥97%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
14. Guilin Layn Natural Ingredients, Corp.	GRN 354	High-Purity Reb A ≥97%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
15. BrazTek International Inc.	GRN 365	Purified Reb A	General-purpose sweetener, excluding meat & poultry products
16. Sinochem Qingdao Co. Ltd.	GRN 367	High-Purity Steviol Glycosides ≥95%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
17. Shanghai Freeman Americas LLC	GRN 369	Purified Reb A	General-purpose sweetener, excluding meat & poultry products

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
18. GLG Life Tech Ltd	GRN 380	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
19. Chengdu Wagott Pharmaceutical	GRN 388	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
20. Chengdu Wagott Pharmaceutical	GRN 389	Steviol Glycosides with Stevioside as the Principal Component	General purpose & table top sweetener, excluding meat & poultry products
21. Daepyung Co., Ltd.	GRN 393	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
22. Daepyung Co., Ltd.	GRN 395	Steviol Glycosides with Reb A & Stevioside as the Principal Components	General purpose & table top sweetener, excluding meat & poultry products
23. MiniStar International, Inc.	GRN 418	Purified Reb A	General-purpose sweetener, excluding meat, poultry products & infant formulas.

^a This table was derived, in part, from McQuate (2011).

On September 18, 2009, based on a review of the international regulation of *Stevia rebaudiana* and the clinical evidence for safety and efficacy, the Canadian Natural Health Products Directorate adopted the same guidelines established by JECFA (Health Canada, 2009). The revised recommendation for the maximum limit for steviol glycosides in NHPs is in accordance with the full ADI of 4 mg steviol/kg bw established by JECFA (WHO, 2008).

As of May 2010, the government of Hong Kong amended its food regulations to allow the use of steviol glycosides as a permitted sweetener in foods (Hong Kong Centre for Food Safety, 2010). This action followed in the aftermath of the detailed safety evaluation and favorable findings as reported by JECFA.

In light of JECFA's 2008 findings and in response to a June 2008 request by the European Commission for European Food Safety Authority (EFSA) to deliver a scientific opinion on the safety of steviol glycosides as a sweetener for use in the food categories specified in the dossiers from three petitioners, EFSA reexamined the safety of steviol glycosides (EFSA, 2010). After considering all the data on stability, degradation products, metabolism and toxicology, the EFSA Panel established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg/bw/day, based upon application of a 100-fold uncertainty factor to the NOAEL for stevioside of 967 mg stevioside/kg bw/day (which corresponds to 388 mg of steviol equivalents/kg/ bw/day from a 2–

year carcinogenicity study in the rat. This is similar to JECFA's determination.³ In addition on May 25, 2011 EFSA published a determination that the daily dietary intake for use of rebaudioside A as a flavoring substance in a variety of foods would be less than the ADI for steviol glycosides (EFSA, 2011b).

The international community continues to exhibit much interest in the food uses of steviol glycosides, with additional advances reported in July, 2011. The Codex Alimentarius Commission has adopted proposed maximum use levels for steviol glycosides in all major food and beverage categories, and this action is expected to favorably influence authorizations of stevia uses in India, Indonesia, Thailand, and the Philippines (FoodNavigator, 2011). Indonesia has current approval for stevia use as a dietary supplement (Stevia Corp, 2012). Furthermore, the International Alliance of Dietary/Food Supplement Associations (IADSA) reported that the Codex Alimentarius Commission agreed to adopt the use of steviol glycosides for addition to chewable food supplements as had been requested by IADSA (NewHope360, 2011). It is anticipated that more details of these actions will be released in the near future. Health Canada Food Directorate has just completed an in-depth review of steviol glycosides for use as a table-top sweetener and as a sweetener in certain food categories. Their review found no safety concerns, hence, the department is proposing to authorize the use of steviol glycosides as a food additive. Health Canada is soliciting comments on its proposal through October 14, 2012. Assuming no issues are brought to the attention of the department, the proposed use of steviol glycosides as a food additive in Canada will be approved sometime after the comment period (Health Canada, 2012).

Lastly, the appropriate European regulatory bodies including the joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA) have now agreed that steviol glycosides are safe for all populations to consume and is a suitable sweetening option for diabetics. Effective December 2, 2011, the EU has approved their use as food additives (EU, 2011).

D. FDA Regulatory Framework

In order to be incorporated into conventional foods, ingredients must undergo premarket approval by FDA as food additives or, alternatively, the ingredients must be determined to be generally recognized as safe (GRAS). The authority to make GRAS determinations is not restricted to FDA. In fact, GRAS determinations may be provided by experts who are qualified by scientific training and experience to evaluate the safety of food and food ingredients under the intended conditions of use.⁴

³ From a historical perspective, it is noted that the UK's Advisory Committee on Novel Foods and Processes for the Ministry of Agriculture, Fisheries and Food on September 24, 1998 rejected an application for use of steviol glycosides as a sweetener in herbal teas because "the applicant had not provided all of the information necessary to enable an assessment to be made." See <http://archive.food.gov.uk/maff/archive/food/novel/980924.htm>. In 1999, the Scientific Committee on Food for the European Commission concluded that "there are no satisfactory data to support the safe use of these stevia plants and leaves" (European Commission, 1999a). In another opinion also dated June 17, 1999, the Committee also reiterated "its earlier opinion that stevioside is not acceptable as a sweetener on the presently available data" (European Commission, 1999b).

⁴ See 21 CFR 170.3(i)(3).

In 1997, FDA altered the GRAS determination process by eliminating the formal GRAS petitioning process. At that time, the petitioning process was replaced with a notification procedure.⁵ While outlining the necessary content to be considered in making a GRAS determination, FDA encouraged that such determinations be provided to FDA in the form of a notification. However, notifying FDA of such determinations is strictly voluntary.

III. CHEMISTRY □ MANUFACTURE OF DAEPYUNG'S REBATEN PREMIUM

A. Common or Usual Name

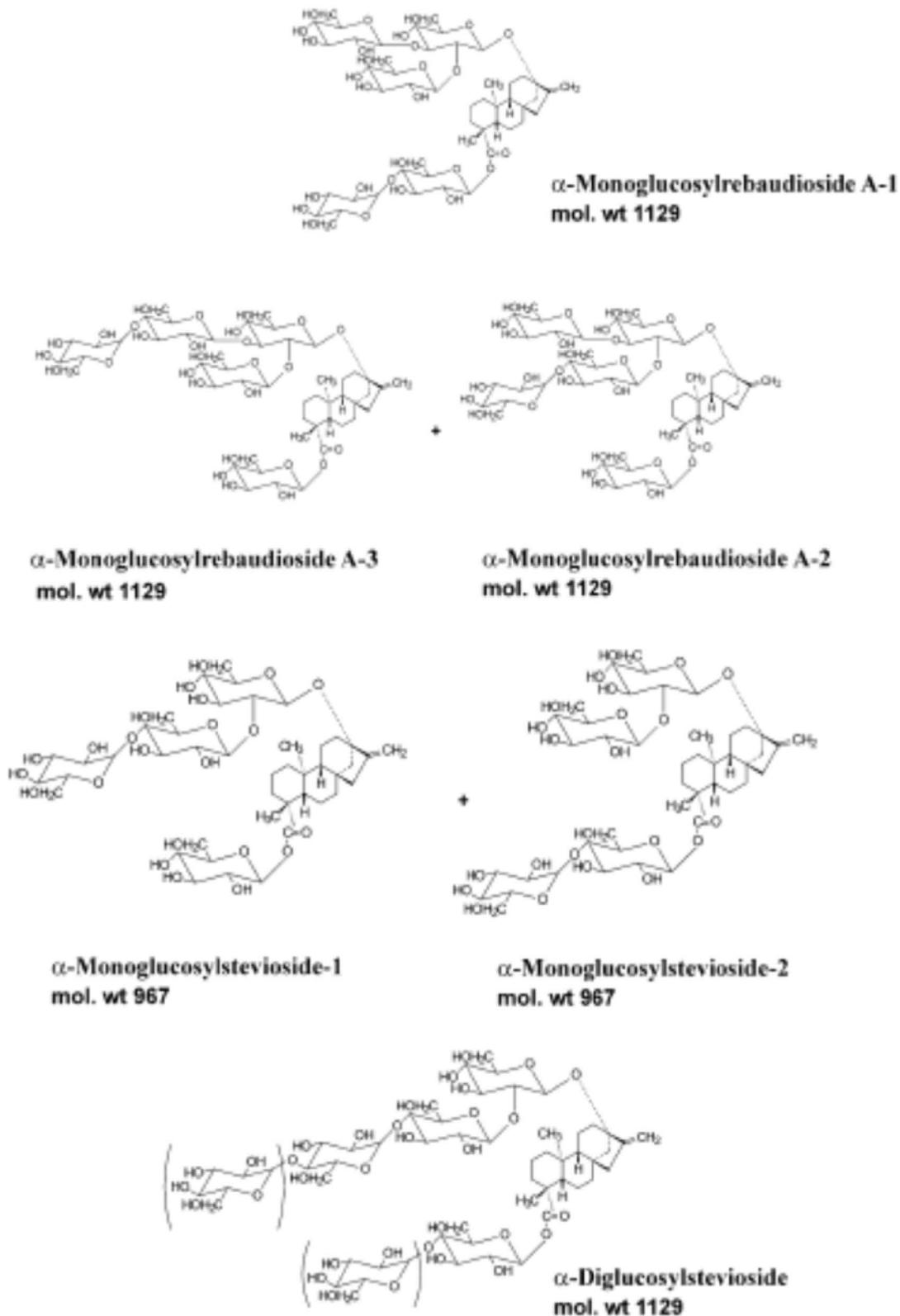
Glucosylated rebaudioside A that is the subject of the GRAS evaluation is the common or usual name of the non-nutritive sweetener derived by enzymatic glucosylation of high purity rebaudioside A. Rebaudioside A is one of the common steviol glycosides found in nature. The compositional features of the subject enzyme-treated rebaudioside A is described in more detail in this Section. REBATEN PREMIUM is the commercial name used by Daepyeong in referring to the notified substance. In the scientific literature, steviol glycosides have been referred to as stevia, stevioside, steviol glycosides, and stevia glycoside. JECFA adopted the term, steviol glycosides, for the family of steviol derivatives with sweetness properties that are derived from the stevia plant. The term, stevia, is used more broadly to describe the plant or crude extracts of the plant, while rebaudioside A is the common name for one of the specific glycosides that is commonly extracted from stevia.

B. Basic Molecular Structure Considerations for Glucosylated Rebaudioside A

Glucosylated rebaudioside A is an α -glucosylated steviol glycoside such that additional glucose moieties is bonded to the original steviol glycoside structure *via* $\alpha(1\text{--}4)$ linkages. α -Glucosylated steviol glycosides are a mixture of α -glucosylated stevioside, rebaudioside A, rebaudioside C, dulcoside A, steviolbioside, rubusoside, rebaudioside B. The chemical composition of glucosylated rebaudioside A is very similar to that found with Reb A and stevioside, where each has the steviol backbone. They each have glucose side chains, as well, but with the glucosylated rebaudioside A constituents, the glucose side chains have been extended, generally with 1 to 3 additional glucose moieties. These additional glucose residues are attached by stereo- and regio-specific 1,4- α -D-glycosidic bonds, whereas the glucose is attached by β -glycosidic bonds in naturally occurring steviol glycosides. The primary constituents of enzymatically treated stevia have been identified (Koyama et al., 2003a) and are described in Table 2, and the chemical structures are shown in Figure 1.

⁵ See 62 FR 18938 (17 April 1997) which is accessible at <http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/ucm083058.htm>.

Figure 1. Representative Chemical Structures of Enzymatically Modified Stevia^a



^a Koyama et al, 2003b

Table 2. Components Expected to be Present in Glucosylated Steviol Glycosides^{a, b}

COMPOUND	MOLECULAR WEIGHT	EMPIRICAL FORMULA	LEVEL OF ENZYME GLYCOSYLATION
Steviolbioside	642	C ₃₂ H ₅₀ O ₁₃	-
Dulcoside A	788	C ₃₈ H ₆₀ O ₁₇	-
Stevioside	804	C ₃₈ H ₆₀ O ₁₈	-
Rebaudioside C	950	C ₄₄ H ₇₀ O ₂₂	-
Rebaudioside A	966	C ₄₄ H ₇₀ O ₂₃	-
Monoglucosyl Rebaudioside B	966	C ₄₄ H ₇₀ O ₂₃	+1
Monoglucosyl Stevioside	966	C ₄₄ H ₇₀ O ₂₃	+1
Monoglucosyl Rebaudioside C	1112	C ₅₀ H ₈₀ O ₂₇	+1
Monoglucosyl Rebaudioside A	1128	C ₅₀ H ₈₀ O ₂₈	+1
Diglucosyl Rebaudioside B	1128	C ₅₀ H ₈₀ O ₂₈	+2
Diglucosylstevioside	1128	C ₅₀ H ₈₀ O ₂₈	+2
Diglucosyl Rebaudioside C	1274	C ₅₆ H ₉₀ O ₃₂	+2
Diglucosyl Rebaudioside A	1290	C ₅₆ H ₉₀ O ₃₃	+2
Triglucosyl Rebaudioside B	1290	C ₅₆ H ₉₀ O ₃₃	+3
Triglucosyl Rebaudioside A	1452	C ₆₂ H ₁₀₀ O ₃₈	+3

^a The level of enzymatic glycosylation indicates the number of glucose units that have been added via enzyme treatment.

^b Data from Koyama et al, 2003b.

C. Background Information on Chemistry of Steviol Glycosides

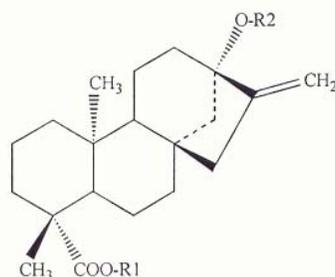
Steviol glycosides (95%)---with a minimum of 80% rebaudioside A---serves as the starting material for enzymatic modification in the production of REBATEN PREMIUM. At its 51st meeting, JECFA reviewed the safety related information on steviol glycosides, including the identity and chemistry of these compounds. The following chemistry related description of steviol glycosides is taken from the original JECFA monograph (WHO, 2000).

Stevioside is a glycoside of the diterpene derivative steviol (ent-13-hydroxykaur-16-en-19-oic acid). Steviol glycosides are natural constituents of the plant *Stevia rebaudiana Bertoni*, belonging to the *Compositae* family. The leaves of *S. rebaudiana Bertoni* contain eight different steviol glycosides, the major constituent being stevioside (triglucosylated steviol), constituting about 5-10% in dry leaves.

Other main constituents are rebaudioside A (tetraglucosylated steviol), rebaudioside C, and dulcoside A. *S. rebaudiana* is native to South America and has been used to sweeten beverages and food for several centuries. The plant has also been distributed to Southeast Asia. Stevioside has a sweetening potency 250-300 times that of sucrose and is stable to heat. In a 62-year-old sample from a herbarium, the intense sweetness of *S. rebaudiana* was conserved, indicating the stability of stevioside to drying, preservation, and storage (Soejarto et al., 1982; Hanson & De Oliveira, 1993).

In the Chemical and Technical Assessment (FAO, 2007b), JECFA identified the sweetener components in stevia extract. They updated the list of common glycosides and their chemical structures, which are slightly different from compounds depicted in older publications (Nanayakkara et al., 1987; Suttajit et al., 1993). They are shown in Figure 2.

Figure 2. Chemical Structures of Various Steviol Glycosides^{a, b}



	Compound name	C.A.S. No.	R1	R2
1	Steviol	471-80-7	H	H
2	Steviolbioside	41093-60-1	H	β -Glc- β -Glc(2→1)
3	Stevioside	57817-89-7	β -Glc	β -Glc- β -Glc(2→1)
4	Rebaudioside A	58543-16-1	β -Glc	β -Glc- β -Glc(2→1) β -Glc(3→1)
5	Rebaudioside B	58543-17-2	H	β -Glc- β -Glc(2→1) β -Glc(3→1)
6	Rebaudioside C (dulcoside B)	63550-99-2	β -Glc	β -Glc- α -Rha(2→1) β -Glc(3→1)
7	Rebaudioside D	63279-13-0	β -Glc- β -Glc(2→1)	β -Glc- β -Glc(2→1) β -Glc(3→1)
8	Rebaudioside E	63279-14-1	β -Glc- β -Glc(2→1)	β -Glc- β -Glc(2→1) β -Glc(3→1)
9	Rebaudioside F	438045-89-7	β -Glc	β -Glc- β -Xyl(2→1) β -Glc(3→1)
10	Rubusoside	63849-39-4	β -Glc	β -Glc
11	dulcoside A	64432-06-0	β -Glc	β -Glc- α -Rha(2→1)

^a From FAO, 2007b.

^b The indicated C.A.S. No. for Rubusoside as reported in the cited reference is incorrect and should be 64849-39-4.

Of the eleven different steviol compounds listed in Figure 2, the two principal sweetener components of stevia extracts have been identified as rebaudioside A and stevioside. The chemical identities and key chemical identifiers for the two major components are presented in Table 3.

Table 3. Chemical Identity of Rebaudioside A

REBAUDIOSIDE A	
Common name	Rebaudioside A
Chemical name	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
Chemical formula	C ₄₄ H ₇₀ O ₂₃
Formula weight	967.03
CAS Number	58543-16-1
STEVIOSIDE	
Common Name	Stevioside
Chemical name	13-[2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Chemical formula	C ₃₈ H ₆₀ O ₁₈
Formula weight	804.88
CAS Number	57817-89-7

The chemical structure of rebaudioside A is presented in Figure 3, and the chemical structure of stevioside is presented in Figure 4.

Figure 3. Chemical Structure of Rebaudioside A

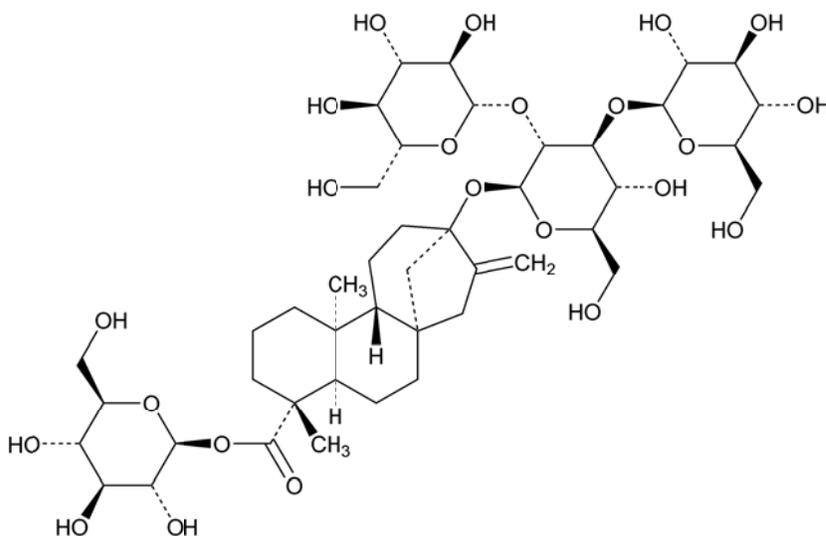
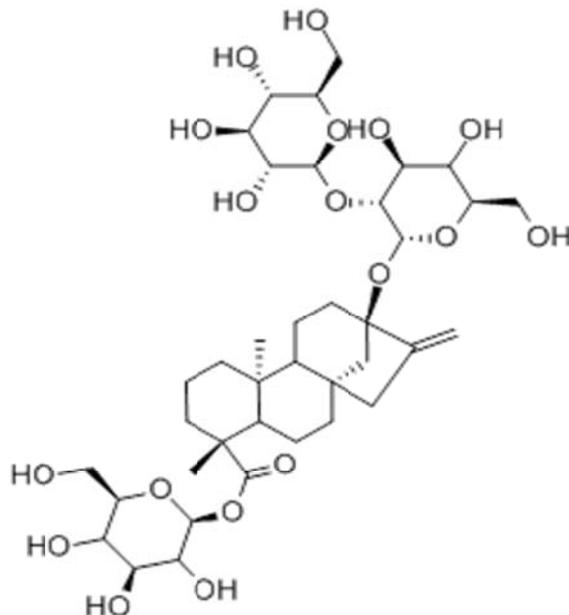


Figure 4. Chemical Structure of Stevioside



In a number of reviews by different authors (Kingham, 2002, Kennelly, 2002, Geuns, 2003), the structures of the components of steviol glycosides have been described. Through a series of chemical reactions and analyses, the structures, stereochemistry, and absolute configurations of steviol and isosteviol were established over a 20-year period after the seminal work of Bridel and Lavielle (1931) in France. The work by Ogawa et al. (1980, cited in Brandle, et al., 1998) on synthetic transformation of steviol into stevioside supported the proposed structures. Two other sweet glycosides, Reb A and B, were obtained from methanol extracts of stevia leaves, along with the major sweet constituent, stevioside, and a minor constituent, steviolbioside, that which was first prepared from stevioside by alkaline hydrolysis by Wood et al. (1955; cited in Brandle et al., 1998). Subsequently, it was suggested that Reb B was an artifact formed from Reb A during isolation (Brandle et al., 1998; Kennelly, 2002). Furthermore, stevioside can be converted both chemically and enzymatically to Reb A. Further fractionation led to the isolation and identification of three other sweet glycosides respectively named Reb C, D, and E. It was reported that Reb A and D could be converted to Reb B by alkaline hydrolysis showing that only the ester functionality differed (Brandle et al., 1998). Dulcosides A and B were also described by Kobayashi et al (1977). Subsequently, dulcoside B and Reb C were shown to be structurally identical.

D. Accepted Identity Specifications for Food Grade Steviol Glycosides

In addition to variations introduced by the manufacturing process, the composition of *Stevia rebaudiana Bertoni* extract depends upon the composition of the harvested leaves, which, in turn, is influenced by soil, climate, etc. (FAO, 2007b). JECFA recommended that food grade specifications for steviol glycosides consist of a minimum of 95% on a dried weight basis of seven specific steviol glycosides (FAO, 2007a), and this has more recently been expanded to include the original seven specific steviol glycosides plus Reb D and Reb F (FAO, 2010; Daeyung Food Additive Master File 849, Volume 3 - Attachment 1). The component glycosides of particular

interest for their sweetening property are stevioside and Reb A. In addition to the more recently added Reb D and Reb F, the other five glycosides that are found at substantially lower levels in the standard preparations of steviol glycosides and recognized by JECFA consist of Reb C, dulcoside A, rubusoside, steviolbioside, and Reb B. JECFA updated the specifications for steviol glycosides in 2010, and they are found in the Daepyoung Food Additive Master File 849, Volume 3 - Attachment 1.

E. Manufacturing Process for REBATEN PREMIUM

Manufacturing processes for RP and other stevia-derived sweeteners have been described in the scientific and patent literature, as noted below. In addition, details of Daepyoung's RP manufacturing process are also presented.

1. Scientific & Patent Literature

The concept of using *Bacillis* was developed by Kitahata et al. (1974) when these researchers isolated a cyclodextrin glucosyltransferase---produced by *Bacillus megaterium*---which efficiently catalyzed the trans- α -D-glucosylation from starch to a 4-hydroxyl group of a glucosyl moiety. It is believed that the enzyme recognizes the 6, 7 or 8 glucose units from the non-reducing end of an amylose molecule, attacks the adjacent α -1,4-linkage, and transfers it to the C-4 position of the non-reducing end to produce α -, β - or γ -cyclodextrin (CD) (Schmid, 1989). Typically, mixtures of α -, β - and γ -CD are formed by the action of CGTases on starch, with the β -form being predominant for thermodynamic reasons. β -Cyclodextrin was determined to be GRAS in 2001 (FDA, 2001).

This regio- and stereo-specific transglucosylation was applied for the improvement of the quality of sweetness of the steviol glycosides. Darise et al., (1984) showed the enzymic transglucosylation of a steviol glycoside---rubusoside---yielded an improvement of its sweetness, disclosing some significance for the structure-sweetness relationship of steviolbisglycosides. Tanaka (1997) reviewed the literature on the sweetness characteristics of steviol glycosides which was significantly improved with modification of sugar moieties by enzymic transglucosylation. Cyclomaltodextrin-glucoamyltransferase (CGTase) efficiently catalyzes intermolecular glucosylation to transfer α -glucosyl units from starch to 4-OH of a glucosyl moiety (trans- α -1,4-glucosylation). Research conducted by Kochikyan et al. (2006) revealed that cyclodextrin glucoamyltransferases (CGTase) produced by mesophilic, thermophilic, alkaliphilic and halophilic bacilli are effective for the transglycosylation of steviol glycosides when using starch as the donor. These authors also found that the method can be used successfully for direct transglycosylation of stevia extract without purification of its individual components.

Steviol glycosides are typically obtained by hot water or alcohols (ethanol or methanol) extraction of *Stevia rebaudiana* Bertoni leaves. This extract is a dark particulate solution containing all the key constituents, soluble polysaccharides, and other impurities. In some processes, the "grease" from the leaves is removed before the extraction by employing solvents such as chloroform or hexane (Kinghorn, 2002). JECFA also cited that the typical manufacture starts with extracting leaves with hot water, and the aqueous extract is then passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is washed with methanol to release the glycosides, and the product is recrystallized with methanol. Ion-exchange resins may be

used in the purification process. The final product is commonly spray-dried. There are several extraction patents for the isolation of steviol glycosides. Kinghorn (2002) has categorized the extraction patents into those based on solvent, solvent plus a decolorizing agent, adsorption and column chromatography, ion exchange resin, and selective precipitation of individual glycosides. In recent patents, methods such as ultrafiltration, metallic ions, supercritical fluid extraction with CO₂, and extract clarification with zeolite have been employed.

At the 68th JECFA meeting in 2007 (FAO, 2007b), steviol glycosides were defined as the products obtained from the leaves of *Stevia rebaudiana* Bertoni. As cited by JECFA, the typical manufacture starts with extracting leaves with hot water, and the aqueous extract is then passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is washed with methanol to release the glycosides, and the product is recrystallized with methanol. Ion exchange resins may be used in the purification process. The final product is commonly spray dried.

2. Daepyeong's Manufacturing Process for REBATEN PREMIUM

For the GMP manufacturing of starting steviol glycosides, Daepyeong employs a fairly typical process that is used in the industry for the production of stevia-derived sweeteners. In order to extract rebaudioside A from the leaves of stevia, Daepyeong has developed a state-of-the-art process. As summarized by flow charts in Daepyeong Food Additive Master File 849, Volume 3 - Attachments 2 – 4, the production of REBATEN PREMIUM (RP) is carried out in three stages. In the first stage, stevia extract powder containing 30% - 60% rebaudioside A is prepared (Attachment 2). The powder obtained from the first step is further purified to obtain 95% steviol glycosides (minimum 80% rebaudioside A) in the second stage (Attachment 3). Lastly, the 95% steviol glycosides (minimum 80% rebaudioside A) is subjected to glucosylation, purification, filtration and is then dried to make the final RP product (Attachment 4).

In the first stage, as illustrated in Daepyeong Food Additive Master File 849, Volume 3 - Attachment 1, dried/crushed leaves from selected varieties of *S. rebaudiana* Bertoni are extracted in water to obtain stevia extract powder containing 30% - 60% rebaudioside A. In order to facilitate the precipitation of the glycosides, ferric chloride and calcium hydroxide are used (see certificates of analysis in Daepyeong Food Additive Master File 849, Volume 3 - Attachments 5 and 6, respectively).

In the third and final stage, the final steviol glycosides 95% intermediate product is dissolved in purified water and is reacted with dextrin using Toruzyme 3.0 L (a cyclomalto-dextrin glucanotransferase enzyme produced by a genetically modified strain of *Bacillus licheniformis*) at 78 ±1°C at pH 5.6 for 24 hours. Toruzyme 3.0 L is manufactured by Novozymes⁶ and is a GRAS substance as defined in 21 CFR 170.30(a) for use as a processing aid in the manufacture of beta cyclodextrins. The Toruzyme 3.0 L performs the glucosylation reaction needed to add glucose moieties to steviol glycosides to form RP. This solution is subsequently deactivated for 1 hour at 90°C, absorbed and desorbed in methanol or ethanol, concentrated, filtered through (See

⁶ Novozymes has provided information that Toruzyme 3.0L is a cyclomalto-dextrin glucanotransferase produced by submerged fermentation of a selected strain of *Bacillus licheniformis*. It is a food grade product and complies with JECFA and FCC recommended specifications for food grade enzymes. See Certification is located in Daepyeong Master File Daepyeong Food Additive Master File 849, Volume 3 - Attachment 7.

certificate of analysis in Daepyeong Food Additive Master File, Volume 3 - Attachment 8), sterilized at 100°C and spray dried to make the final REBATEN PREMIUM product.

The ethanol and methanol used in the purification process complies with Food Chemicals Codex 8th Edition (2012) specifications for these solvents. REBATEN PREMIUM is prepared in accordance with current Good Manufacturing Practices (cGMP). The Korea Food & Drug Administration (KFDA) approved glucosylated steviol glycosides in 2000 as KFDA Notification No 2000-32. In addition, since January 23, 2007 the Daepyeong manufacturing facility is a DHHS/FDA registered food facility (#16411116026 - See Daepyeong Food Additive Master File 849, Volume 3 - Attachment 9).

F. Product Specifications □ Supporting Methods

The Daepyeong RP product is derived from steviol glycosides (95%)---with a minimum of 80% rebaudioside A---which is thoroughly discussed in Section III.C as well as in Daepyeong Food Additive Master File 849, Volume 3 - Attachment 1.

1. Specifications for Daepyeong's REBATEN PREMIUM Starting Material

Daepyeong has adopted product specifications for its purified steviol glycosides starting product that meets or exceeds JECFA recommendations for steviol glycosides as a consumable human food substance. Comparisons of the specifications provided by Daepyeong for REBATEN PREMIUM starting product and those from JECFA and methodologies used are presented in Table 4. Further details for the specifications and certificate of analysis are available in Daepyeong Food Additive Master File 849, Volume 3 - Attachment 12. A test report for analyses of pesticide residues for the intermediate steviol glycosides extract in one production lot is included in the Daepyeong Food Additive Master File 849, Volume 3 - Attachment 10. Attachment 11 also lists the limits of detection for the relevant pesticides noted in the pesticide analyses. These pesticide reports further demonstrate that the substance is well characterized and meets the required purity criteria.

2. Specifications for REBATEN PREMIUM

Starting steviol glycosides materials are stable molecules that have been well described. There are specifications in the scientific literature for stevioside (Chang and Cook, 1983), the JECFA report (FAO, 2010; Daepyeong Food Additive Master File 849, Volume 3 - Attachment 1), and extensive testing for the structurally similar rebaudioside A as presented by Merisant (FDA, 2008a), Cargill (FDA, 2008b) and Sunwin and WILD Flavors (FDA, 2009b).

The typical glycosides content of RP production lots is presented in Table 5. In addition, both non-enzyme-treated and enzyme-treated steviol glycosides, as well as the calculated steviol equivalents, are presented in Table 5. The Daepyeong steviol glycosides starting material was analyzed by high performance liquid chromatography (HPLC) to determine the percent steviol glycosides present. The most abundant steviol glycosides observed were stevioside, rebaudioside A, steviolbioside, rebaudioside C, dulcoside A, rebaudioside B and rebaudioside F. Table 5 identifies the range of steviol glycosides in the Daepyeong glucosylated steviol glycosides. For comparative purposes, the content of steviol glycosides is often expressed as steviol or steviol equivalents. Each component steviol glycoside has a steviol equivalency factor which is

calculated based upon the ratio of molecular weight between steviol and a particular steviol glycoside. As shown in Table 5, the steviol equivalency for RP ranges between 26.6 to 31.9 g steviol/100 g RP. Further details of results of analyses of various production batches or pooled production batches versus the various parameters in the product specifications are provided in Daepyeong Food Additive Master File 849, Volume 3 - Attachment 13.

Table 4. Specifications for Daepyeong's REBATEN PREMIUM Starting Material Product (Steviol Glycosides 95% with 80% Rebaudioside A) Comparison to JECFA Specifications

PARAMETER	JECFA ^a SPECIFICATIONS STEVIOL GLYCOSIDES	FCC ^b SPECIFICATIONS REBAUDIOSIDE A	DAEPYUNG SPECIFICATIONS STEVIOL GLYCOSIDES	METHODS
Appearance	White to light yellow powder	White to off-white, hygroscopic fine crystal, granule, or powder	White powder to off-white	Visual
Sweetness	200-300 times sweeter than sucrose	NA	275-325	Gustatory
Rebaudioside A	NA	NLT 95%	Minimum of 80%	JECFA HPLC
Total Steviol Glycosides	NLT 95%	NA	≥ 95 %	JECFA HPLC
Other Related Steviol Glycosides (such as Stev, Reb A, B, C, Dulc A, Rub & SB) on dry weight basis	NLT 95%	NMT 5% ^c	NS	JECFA, 2007
Residue on Ignition	NS	NS	NS	USP
Moisture (loss on drying)	NMT 6%	NMT 6%	≤ 6%	USP
Ash	NMT 1%	NMT 1%	< 0.2%	JECFA Vol. 4
Optical rotation	NS	NS	NS	USP
Solubility	Freely soluble in water & ethanol	Freely soluble in water:ethanol (50:50)	Freely soluble in water & ethanol	USP
pH (1% solution)	4.5 - 7.0	4.5 - 7.0	4.5-7.0	USP
RESIDUAL SOLVENT LEVELS				
Residual Methanol	NMT 200 mg/kg	NMT 0.02%	< 200 ppm	USP
Residual Ethanol	NMT 5000 mg/kg	NMT 0.5%	< 5000 ppm	USP
HEAVY METALS				
Lead	NMT 1 mg/kg	NMT 1 mg/kg	< 1 ppm	ICP-MS AOAC
Arsenic	NMT 1 mg/kg	NMT 1 mg/kg	< 1 ppm	ICP-MS AOAC
MICROBIOLOGICAL				
Total Plate Count	NA	NA	≤ 1000 cfu/g	AOAC 990.12
Yeast and Mold	NA	NA	≤ 100 cfu/g	AOAC 990.02
Total coliform	NA	NA	NS	
<i>Salmonella</i>	NA	NA	Negative	AOAC 990.02
<i>Escherichia coli</i>	NA	NA	≤ 10 npm/g	AOAC 990.02
<i>Staphylococcus aureus</i>	NA	NA	NS	

^a Prepared at 69th JECFA (WHO, 2008). ^b FCC, 2012. Rebaudioside A monograph. Food Chemicals Codex (87th Ed.)

^c Excludes Reb A but includes additional two glycosides Reb D and Reb F.

Abbreviations: St = Stevioside; Reb A = Rebaudioside A; Reb B = Rebaudioside B; Reb C = Rebaudioside C; Dulc A = Dulcoside A; Rub = Rubusoside; SB = Steviolbioside; NS = not specified; NA = not applicable; NLT = not less than; NMT = not more than.

**Table 5. Levels of Steviol Glycosides by Percent Range in Non-Glucosylated □
 Glucosylated Steviol Glycosides □ Steviol Equivalents of
 Glucosylated Steviol**

COMPONENT	UNTREATED STEVIOL GLYCOSIDES (□) IN STARTING MATERIAL	RANGE OF GLUCOSYLATED STEVIOL GLYCOSIDES (□) IN REBATEN PREMIUM	RANGE OF GLUCOSYLATED STEVIOL EQUIVALENTS □MG ^a
Rebaudioside B	2.0 -3.6	0.3 - 0.9	0.10 - 0.30
Stevioside	0.1 - 0.9	0.4 - 0.8	0.16 - 0.16
Rebaudioside C	0.2 - 0.6	0.1 - 0.5	0.03 - 0.17
Rebaudioside F	0.6 - 1.0	0.1 - 0.2	0.03 - 0.07
Rebaudioside A	93.5 - 96.0	15.7 - 17.4	5.17 - 5.73
Monoglucosyl rebaudioside B m/z 966	ND	0.5 - 1.1	0.16 - 0.36
Monoglucosyl rebaudioside C m/z 1112	ND	0.1 - 0.7	0.03 – 0.20
Monoglucosyl rebaudioside A m/z 1128	ND	27.8 - 30.0	7.84 - 8.46
Diglucosyl rebaudioside C m/z 1274	ND	0.5 - 1.0	0.12 - 0.25
Diglucosyl rebaudioside A m/z 1290	ND	26.0 - 27.4	6.41 - 6.75
Triglucosyl rebaudioside A m/z 1452	ND	15.1 - 16.8	3.31 - 3.68
Unidentified glucosylated m/z >1452	ND	4.4 - 7.4	0.96 - 1.62
Totals			24.33 - 27.74

^a Calculated by multiplying the % of steviol glycosides by the steviol equivalency factor.

^b Mass-to-charge ratio.

The purification process for RP removes additional impurities that are present in the starting steviol glycosides. Table 6 shows the reduction in non-glycoside impurity levels comparing 3 different lots of starting materials with the resulting lot of RP. Further details of the results of analyses are provided in Daepyung Food Additive Master File 849, Volume 3 - Attachment 13.

Table 6. Non-Glycoside Impurities Levels in 3 Lots of Starting Material vs. REBATEN PREMIUM Finished Product

LOT	STARTING MATERIAL (IMPURITIES) (□)	REBATEN PREMIUM IMPURITIES (□)
110502	1.6	0.2
110613	1.5	0.3
110711	2.2	0.2

3. Analytical Data on REBATEN PREMIUM

The summary of the results of all specification parameters of three RP product lots is listed in Table 7. A summary of these results appears in Daepyeong Food Additive Master File 849, Volume 3 - Attachment 12. A detailed analytical report of various production batches for the determination of the levels of steviol glycosides and glucosylated steviol glycosides is provided in Daepyeong Food Additive Master File 849, Volume 3 – Attachment 13.

G. Sweetness Intensity of REBATEN PREMIUM

Daepyeong has measured the sweetness intensity of the RP finished product which includes maltodextrin. It has been determined by a taste panel to vary between 120 to 190 times sweeter than sucrose depending upon the concentration in water. The findings are reported in Table 8. Based on these data, the average relative sweetness is estimated to be 150 times that of sucrose.

H. Stability Data of REBATEN PREMIUM

Daepyeong has conducted a battery of RP stability studies in 0, 1 and 10% water at various temperatures. Overall the data indicate that RP is stable under a variety of different pHs and temperatures with minor non-significant changes in RP alone at pH 5.0 and 25°C, while at 40°C it is stable up to six months with 24 month testing planned. In 1% water, RP is again observed to be stable with non-significant changes noted under pH 2, 5 and 8 at 25°C for 2 days with 6 day testing planned. In 10% water, RP is stable; however, there were non-significant changes noted at 50°C when compared to similar solutions at 5°C and 25°C at 20 hours with 60 hour testing planned. Microbiological tests are negative in all tested RP samples at 6 months with 24 month testing planned. These data are presented in Table 9 and can be found in Daepyeong Food Additive Master File 849, Volume 3 - Attachment 14.

Table 7. Summary of Critical Parameters for Three Lots of Daepyung's Glucosylated Rebaudioside A (REBATEN PREMIUM)

SPECIFICATION PARAMETER	SPECIFICATION	RESULT LOT 110502	RESULT LOT 110613	RESULT LOT 110711
Appearance	White to light yellow powder	Passed	Passed	Passed
Odor	Slight characteristic	Passed	Passed	Passed
Taste	Characteristic, sweet	Passed	Passed	Passed
Starting material	> 95% steviol glycosides	98.4%	98.5%	97.8%
Starting material	> 80% rebaudioside A	93.9%	94.0%	93.9%
Total content of α -glucosyl steviol glycosides & unreacted steviol glycosides (dry weight basis)	$\geq 95\%$	96.2%	96.2%	96.1%
Content of α -glucosyl steviol glycosides (dry weight basis)	$\geq 80\%$	87.2%	87.1%	87.1%
Assay (HPLC)	$\geq 95\%$ steviol glycosides & glucosyl rebaudioside A	99.8%	99.7%	99.8%
pH	4.5-6.0	5.4	5.3	5.3
Loss on drying	$\leq 6\%$	3.4%	3.4%	3.5%
Ash	$\leq 1\%$	0.11%	0.12%	0.11%
Solubility	Freely soluble in water & ethanol	Freely soluble	Freely soluble	Freely soluble
Residual methanol	≤ 200 ppm	Passed	Passed	Passed
Residual ethanol	≤ 5000 ppm	Passed	Passed	Passed
Lead	≤ 1 ppm	Passed	Passed	Passed
Arsenic	≤ 1 ppm	Passed	Passed	Passed
Total plate count	≤ 1000 cfu/g	Passed	Passed	Passed
Yeast & Mold	≤ 100 cf/g	Passed	Passed	Passed
<i>Salmonella</i>	Negative	Passed	Passed	Passed
Total <i>E. Coli</i>	≤ 10 npm/g	Passed	Passed	Passed

Table 8. Sweetness Intensity of REBATEN PREMIUM

WATER SOLUTION (□)	REBATEN PREMIUM
0.01%	190
0.02%	180
0.05%	175
0.1%	170
0.2%	160
0.4%	145
0.5%	130
1.0%	120
~Average sweetness	150

Table 9. Summary of REBATEN PREMIUM Stability Tests Completed or in Progress

TEST TYPE	MEASUREMENT	TEMPERATURE (°C)	pH	CYCLE ANALYSIS	PLANNED DURATION
Solid ^a	HPLC	25, 40	5	6 m	24 m
Solution ^b	HPLC	5, 25, 50	5	20 h	60 h
Water Solution ^c	HPLC	25	2, 5 & 8	2 d	6 d

^a REBATEN PREMIUM Product. ^b 10% Water Solution. ^c 1% Water Solution.

TEST TYPE	PARAMETER	SPECIFICATION	CYCLE ANALYSIS	PLANNED DURATION
Microbiology	Total Plate Count	≤ 1000 cfu/g	6 m	24 m
	Yeast & Mold	≤ 100 cfu/g		
	<i>Salmonella</i>	Negative		
	Total <i>E. Coli</i>	≤ 10 mpn/g		

Daepyeong's RP was also tested for shelf-life stability from August 20, 2009 to August 20, 2011. Analyses revealed no changes in purity or moisture over that 2 year period. Details of these tests can be located in Daepyeong Food Additive Master File 849, Volume 3 - Attachment 15.

IV. INTENDED FOOD USES □ ESTIMATED DIETARY INTAKE

A. Intended Food Uses

The subject Daepyeong REBATEN PREMIUM preparation is intended to be used as a table top sweetener and general purpose non-nutritive sweetener as defined in 21 CFR 170.3(o)(19) for use in various foods other than infant formulas and meat and poultry products. As stated in Section III.G, Daepyeong has determined that its RP product containing maltodextrin is 120 - 190 times sweeter than sucrose. The amounts of RP to be added to foods will not exceed the amounts reasonably required to accomplish its intended technical effect in foods as required by FDA regulation.⁷

B. Estimated Daily Intake

The estimated daily intake for steviol glycosides and rebaudioside A has been estimated in a variety of publications and has been provided to FDA in multiple GRAS notifications. The very conservative consumer intake estimates provided by JECFA as shown in Table 10 were utilized to gauge the potential human exposures of rebaudioside A and steviol glycosides and in foods as reported in the US and in other countries. As rebaudioside A is about twice as sweet as the

⁷ Non-nutritive sweeteners: Substances having less than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.

mixed glycosides, these levels can be adjusted accordingly. A summary of the JECFA data (WHO, 2006) is summarized in Table 10.

The intended use levels will vary by actual food category, but the actual levels are self-limiting due to organoleptic factors and consumer taste considerations. However, the amounts of REBATEN PREMIUM to be added to foods will not exceed the amounts reasonably required to accomplish its intended technical effect in foods as required by FDA regulation.⁸ EFSA also calculated the daily intake of steviol glycosides (EFSA, 2010) following the JEFCA guidelines. EFSA (2010) considers that the results of toxicology studies on either stevioside or rebaudioside A are applicable for the safety assessment of steviol glycosides as both rebaudioside A and stevioside are metabolized and excreted by similar pathways, with steviol being the common metabolite for both.

Table 10. Food Uses of Steviol Glycosides Reported to JECFA with Calculated Steviol Equivalents

FOOD TYPE	MAXIMUM USE LEVEL REPORTED ^a (MG STEVIOL GLYCOSIDES /KG OF FOOD)	MAXIMUM USE LEVEL CALCULATED FOR REBAUDIOSIDE A ^b MG REBAUDIOSIDE A /KG OF FOOD	MAXIMUM USE LEVEL CALCULATED FOR REBAUDIOSIDE A ^b MG STEVIOL EQUIVALENTS /KG OF FOOD
Desserts	500	250	83
Cold confectionery	500	250	83
Pickles	1000	500	167
Sweet corn	200	100	33
Biscuits	300	150	50
Beverages	500	250	83
Yogurt	500	250	83
Sauces	1000	500	167
Delicacies	1000	500	167
Bread	160	80	27

^a Reproduced from WHO, 2006.

^b Calculated by Expert Panel assuming twice the sweetness intensity for rebaudioside A and three-fold difference in molecular weight between rebaudioside A and steviol.

An appropriate method for estimating potential intake of sweeteners in general was determined in GRN 301 (FDA, 2009a). In this GRAS notice, the sweetness of their subject product was compared to sucrose by calculating the relative sweetness to sucrose equivalents.

The concept is based upon Renwick (2008) who has published an assessment of intense sweetener intake, including conversion of these intakes to sucrose equivalents. The study was designed to predict dietary exposures for the intense sweetener rebaudioside A. In this study, Renwick collected published data on dietary intakes from a number of countries and converted them to sucrose equivalents using the following estimates of sweetness compared to sucrose: saccharin = 300; aspartame = 180; sucralose = 600; acesulfame = 200; and alitame = 2000. Renwick (2008) provided estimates of both mean and heavy intakes of intense sweeteners, in sucrose equivalents, for the general population, diabetic adults, healthy children, and diabetic children.

⁸ See 21 CFR 182.1(b)(1).

Using this approach, we have calculated the sucrose equivalents of RP and compared those values to existing levels of intake of intense sweeteners in order to establish a common metric, and then determined the amount of RP needed to replace this amount of sucrose equivalence. As noted previously in Section III.G, Table 8, the range of relative sweetness for RP was determined to be 120 - 190 with an average sweetness of 150-fold sweeter than sucrose. Therefore, using the same methodology as previously used in GRN 301, Table 11 presents estimates of both mean and heavy intakes of sweeteners in sucrose equivalents for the general healthy adult population, diabetic adults, healthy children, and diabetic children based on the Renwick (2008) paper. It also shows the calculated amount of RP needed to replace these sweeteners based upon a 150-fold sweetness differential.

Table 11. Daily Intake of Sweeteners (In Sucrose Equivalents)
 □ Estimated Daily Intakes of REBATEN PREMIUM

POPULATION GROUP	INTAKES OF SWEETENERS (G SUCROSE /KG BW /DAY)		INTAKE OF RP (MG /KG BW /DAY) ^a		INTAKE OF RP AS STEVIOL EQUIVALENTS ^b	
	LOW	HIGH	LOW	HIGH	LOW	HIGH
Healthy Population	255	675	1.7	4.5	0.41	1.25
Diabetic Adults	280	897	1.9	6.0	0.46	1.66
Healthy Children	425	990	2.8	6.6	0.68	1.83
Diabetic Children	672	908	4.5	6.1	1.09	1.69

^a Calculated by dividing the sucrose intake by the average RP relative sweetness value of 150.

^b Calculated based on the low (0.256) and high (0.287) percent steviol equivalency of RP based on calculations in Table 5.

The values in Table 11 assume that RP constitutes the entire sweetener market and that would make these estimates extremely conservative since the likelihood of that occurrence is minimal. For the general healthy adult population, the estimated maximum intake of RP is 4.5 mg/kg bw/day or 1.25 mg/kg as steviol equivalents. For healthy children, the estimated maximal intake is 6.6 mg/kg bw/day or 1.83 mg/kg as steviol equivalents.

V. SAFETY INVESTIGATIONS FOR REBAUDIOSIDE A

A. Safety Data on REBATEN PREMIUM

Daepyeong’s RP contains glucosylated rebaudioside A as its major component. As described in Section III, RP is formed by enzymatically adding glucose residues to rebaudioside A. Given the structural similarity to rebaudioside A, stevioside and other steviol glycosides and metabolic considerations, the scientific data on stevia and its other components are relevant to the present

safety assessment. The primary documentation for RP safety consists of a combination of several studies on α -glucosylated steviol glycosides and evidence supporting the safety of rebaudioside A and other steviol glycosides. This includes a number of studies on rebaudioside A and a complete battery of toxicology and clinical studies on steviol glycosides, coupled with evidence of the lack of GI absorption of both glucosylated and unreacted steviol glycosides in the upper GI tract in concert with the conversion of the steviol glycosides to steviol by normal flora of the lower GI tract. Steviol is absorbed but is rapidly converted to glucuronides which are subsequently excreted in the urine or eliminated by the enterohepatic circulation. Because of the increased glucosylation of rebaudioside A, it is reasonable to expect that α -glucosylated rebaudioside A is similarly not absorbed in the GI tract but is converted to steviol by the normal flora of the lower GI tract. Due to the similar metabolic pathway, the acceptable daily intake calculated for steviol glycosides based on steviol equivalents by JECFA and later adopted by FDA applies as an acceptable daily intake of α -glucosylated steviol glycosides.

The safety of α -glucosylated steviol glycosides is supported by both published and unpublished studies on α -glucosylated steviol glycosides and non-enzymatically modified steviol glycosides. Steviol glycosides are not readily absorbed from the upper small intestine as shown in studies by Koyama et al., 2003a and Gardana et al., 2003. Studies performed on a similar α -glucosylated steviol glycosides product in GRN 337 (FDA, 2010) confirmed the lack of absorption.

Koyama et al., 2003b published an *in vitro* study in which α -glucosylated steviol glycosides were degraded by fecal microflora to steviol glycosides. These are subsequently hydrolyzed to the aglycone steviol, demonstrating that the metabolic fate of α -glucosylated steviol glycosides follows that of non-modified steviol glycosides. Due to the similarities in metabolic fate, the safety of α -glucosylated steviol glycosides can be established based on studies conducted with non-modified steviol glycosides. Furthermore, as individual steviol glycosides show similar pharmacokinetics in the rat and humans, the results of toxicology studies on individual steviol glycosides are applicable to the safety of steviol glycosides in general.

Toxicology studies (Hutapea et al., 1997; Geuns et al., 2007) report that human digestive enzymes are not capable of hydrolyzing β -glycosidic bonds, and, thus, steviol glycosides are not digested in the upper gastrointestinal tract. It is believed that the α -glucopyranosyl moiety of α -glucosylated steviol glycosides may be hydrolyzed by digestive enzymes to the non-modified steviol glycosides form due to the presence of an $\alpha(1\rightarrow4)$ glycosidic bond. Therefore, the resulting non-modified steviol glycosides would follow the same metabolic pathway as other steviol glycosides, whereas the cleaved glucose moiety would be absorbed in the intestine and follow normal carbohydrate metabolism pathways. However, the amount of glucose released during hydrolysis in the upper gastrointestinal tract is negligible (0.2 or 0.7 kcal/day for children and adults, respectively).

Specific unpublished α -glucosylated steviol glycosides data were presented in GRN 375 (FDA, 2011a) on a 13-week dietary study in rats receiving 1.25, 2.5 or 5.0% α -glucosylated steviol glycosides (0, 253, 519, or 1,059 mg steviol equivalents/kg bw/day for males and 0, 289, 601, or 1,153 mg steviol equivalents/kg bw/day for females, respectively) (Hooks et al., 1998). The authors concluded that, under the conditions of the study, the NOAEL of dietary exposure to α -glucosylated steviol glycosides for 13 weeks was 1,059 and 1,153 mg/kg bw/day for males and females, respectively. This was consistent with the findings of the subchronic toxicity studies conducted with non-modified steviol glycosides. GRN 375 (FDA, 2011a) also reports a lack of genotoxic activity in both *in vitro* and *in vivo* studies performed by ToyoSugar.

Studies conducted in both rats and humans show that RP starting materials of steviol glycosides (i.e., predominantly rebaudioside A) are metabolized by microflora in the colon to steviol by successive removal of glucose moieties. Steviol is absorbed from the colon, subjected to glucuronidation in the liver, and excreted *via* bile primarily in the feces of rats as steviol glucuronide or the urine of humans. The differences in the route of elimination are due to the lower molecular weight threshold for biliary excretion in rats (325 Da) compared to humans (500 to 600 Da). Although the primary routes of elimination of steviol glucuronide differ between rats and humans, the metabolism of modified and non-modified steviol glycosides and pharmacokinetics are quite similar which confirms that the rat is an acceptable model for risk assessment in humans (Roberts and Renwick, 2008; Wheeler *et al.*, 2008). RP is metabolically converted to steviol as is the case with the naturally occurring steviol glycosides. The naturally occurring steviol glycosides are likely intermediates in the deglycosylation of the higher molecular weight glycosides, which are formed enzymatically in the Daepyeong manufacturing process. Further, RP is a mixed glycosides product which contains unreacted glycosides---primarily rebaudioside A and stevioside.

Since the preponderance of data indicate that non-modified steviol glycosides are predictive of α -glucosylated steviol glycosides, it is worthwhile to consider the toxicity of steviol glycosides extracts. There is an extensive database of literature on steviol glycosides extracts already in the published literature along with in-depth reviews in numerous GRAS submissions. The steviol glycosides data are reviewed in WHO (2008) and also in Daepyeong Food Additive Master File 849, Volume 3 - Attachment 1. In fact, JECFA (WHO, 2008) used studies on $\geq 95\%$ steviol glycosides as a basis for the establishment of the ADI of 4 mg/kg bw expressed as steviol equivalents. In addition, the 23 GRAS notifications for steviol glycosides submitted to FDA since 2008---as listed in Table 1 and described in Section II.C---were all determined to be GRAS by various experts and have received "no questions" letters from FDA.

Glucosylated steviol glycosides submissions were also determined to be GRAS and received "no questions" responses from FDA: GRN 337 (FDA, 2010) repeated the Koyama *et al.* (2003b) studies on a similar product and found no toxicity which supported the conclusion that the JECFA ADI of 4 mg/kg bw/day applies. GRN 375 (FDA, 2011a) referred to toxicology studies that were conducted on a similar product, also finding no toxicity, which supported the finding that the JECFA ADI also applied to their product. RP---the subject of this GRAS notification---is similar to both of the GRN 337 and GRN 375 products, and it is not expected to be toxic at any intended use levels. Further, the JECFA ADI of 4 mg/kg/day applies to RP.

Given these similarities, the results of toxicology studies on stevioside and steviol glycosides can be used to support the safety assessment of α -glucosylated rebaudioside A.

B. Summary of Safety Data on Steviol Glycosides □ Rebaudioside: Reviews by Expert Bodies & Other Scientists

The biological and toxicological data on stevia and steviol glycosides have been evaluated by numerous expert bodies including JECFA, FSANZ and EFSA. Most notably, over the years JECFA has evaluated the safety of stevia and steviol glycosides multiple times (WHO, 2000, 2006, 2007, 2008). FSANZ (2008) also evaluated steviol glycosides for use in food. The JECFA reviews, as well as the other reviews completed before 2008, primarily focused on mixtures of

steviol glycosides typically and were not specific for purified rebaudioside A. A summary of the biological and toxicological data on stevia and steviol glycosides is presented in Appendix 1.

The safety data on naturally occurring steviol glycosides as found in the stevia leaves applies directly to the safety review on RP for the following reasons:

- As discussed in Section V.A, RP is metabolically converted to steviol just as occurs with the naturally occurring steviol glycosides. The naturally occurring steviol glycosides are likely intermediates in the deglycosylation of the higher molecular weight glucosides, which are formed enzymatically in the Daepyeong manufacturing process.
- RP is a mixed glycoside product which contains unreacted glycosides---mainly rebaudioside A---as indicated in Table 5. None of the studies on these intermediates, including studies on rebaudioside A, have shown any safety concerns.
- JECFA's determination of the 4 mg/kg bw/day ADI was established utilizing data from chronic studies conducted on mixed steviol glycosides which included rebaudioside A.

JECFA encouraged the further elucidation of clinical effects on blood pressure and glucose metabolism on hypertensive and diabetic individuals, respectively, in parallel with normal human subjects. By 2006 at its 69th meeting, sufficient data were presented for JECFA to satisfactorily establish a temporary ADI. In 2008, based on additional clinical studies, JECFA finalized the evaluation of steviol glycosides (WHO, 2008) and raised the ADI to 0 – 4 mg/kg bw/day and removed the “temporary” designation. The summary of the Committee’s key conclusions are found in the final toxicology monograph addendum (WHO, 2009).

FSANZ completed a review of the safety of steviol glycosides with the primary constituents being stevioside and rebaudioside A, for use as a sweetener in foods, and a summary is found in the FSANZ Final Assessment Report on Steviol Glycosides as Intense Sweeteners (FSANZ, 2008).

On March 10, 2010, EFSA adopted a scientific opinion on the safety of steviol glycosides. The EFSA opinion states that the results of toxicology studies on either stevioside or rebaudioside A are applicable to the safety assessment of steviol glycosides as both rebaudioside A and stevioside are metabolized and excreted by similar pathways, with steviol being the common metabolite for both. This report can be found in the scientific opinion on the safety of steviol glycosides for the proposed uses as a food additive (EFSA, 2010).

C. Safety Data on Rebaudioside A⁹

Since 2008, several well-designed toxicology studies that followed the current regulatory and other guidelines for such studies have been reported on purified rebaudioside A (although it is uncertain whether or not these studies were considered by JECFA during its 2008 deliberations).

⁹ Questions about the safety of rebaudioside A were previously raised by Huxtable (2002) and Kobylewski and Eckhert (2008). Their respective concerns, as well as opposing views supporting the safety of designated food uses of rebaudioside A expressed by Expert Panels have been outlined in other GRAS notifications that were submitted to FDA. A more detailed account can be found in GRAS notifications 278, 287, 303, and 304.

These investigations included additional subchronic studies in rats and one in dogs, mutagenicity studies, reproduction and developmental studies in rats, and comparative pharmacokinetic studies with stevioside in rats and humans, as well as additional clinical studies.

1. Absorption, Distribution, Metabolism □ Excretion (ADME) Studies

In three recently completed studies, absorption and fate of rebaudioside A was systematically investigated in rats and humans.

For comparative purposes to determine whether toxicological studies conducted previously with stevioside would be applicable to the structurally-related glycoside, rebaudioside A, toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol were examined in rats (Roberts and Renwick, 2008). Orally administered single doses of the radiolabelled compounds were extensively and rapidly absorbed with plasma concentration-time profiles following similar patterns for stevioside and rebaudioside A. Within 72 hours of administration, elimination of radioactivity from plasma was essentially complete. All plasma samples had similar metabolite profiles; the predominant radioactive component in all samples was steviol, with lower amounts of steviol glucuronide(s) and low levels of one or two other metabolites. Rebaudioside A, stevioside, and steviol were metabolized and excreted rapidly, with the majority of the radioactivity eliminated in the feces within 48 hours. Urinary excretion accounted for less than 2% of the administered dose for all compounds in both intact and bile duct-cannulated rats, and the majority of the absorbed dose was excreted *via* the bile. After administration of the compounds to intact and bile duct-cannulated rats, radioactivity in the feces was present primarily as steviol. The predominant radioactive compound detected in the bile of all cannulated rats was steviol glucuronide(s), indicating de-conjugation in the lower intestine. The authors concluded that the overall data on toxicokinetics and metabolism indicate that rebaudioside A and stevioside are handled in an almost identical manner in the rat after oral dosing.

In a randomized, double blind, cross-over study in healthy male subjects, Wheeler et al. (2008) assessed the comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A and stevioside. Following administration of rebaudioside A or stevioside, steviol glucuronide appeared in the plasma of all subjects, with median T_{max} values of 12.00 and 8.00 hours post-dose, respectively. Steviol glucuronide was eliminated from the plasma, with similar $t_{1/2}$ values of approximately 14 hours for each compound. Administration of rebaudioside A resulted in a significantly (approximately 22%) lower steviol glucuronide geometric mean C_{max} value (1472 ng/mL) than administration of stevioside (1886 ng/mL). The geometric mean AUC_{0-t} value for steviol glucuronide after administration of rebaudioside A (30,788 ng*hr/mL) was approximately 10% lower than after administration of stevioside (34,090 ng*hr/mL). Steviol glucuronide was excreted primarily in the urine of the subjects during the 72-hour collection period, accounting for 59% and 62% of the rebaudioside A and stevioside doses, respectively. No steviol glucuronide was detected in feces. Pharmacokinetic analysis indicated that both rebaudioside A and stevioside were hydrolyzed to steviol in the gastrointestinal tract prior to absorption. The majority of circulatory steviol was in the form of steviol glucuronide indicating rapid first-pass conjugation prior to urinary excretion. Only a small amount of steviol was detected in urine (rebaudioside A: 0.04%; stevioside: 0.02%). The investigators concluded that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans with steviol glucuronide excreted primarily in the urine and steviol in the feces. No safety concerns were noted as determined by reporting of adverse events, laboratory assessments of safety or vital signs (Wheeler et al., 2008).

Another pharmacokinetic investigation was done as a toxicokinetic (TK) phase of a dietary study to determine the potential of rebaudioside A toxicity in rats at levels up to 2000 mg/kg bw/day (Sloter, 2008a). Rebaudioside A and total steviol were detected in peripheral blood of rats during daily administration of 2000 mg/kg bw/day of rebaudioside A at extremely low levels, with mean plasma concentrations of approximately 0.6 and 12 ug/mL, respectively. Estimates of absorbed doses for rebaudioside A and total steviol were approximately 0.02% and 0.06%, respectively, based on the amounts measured in urine collected over 24 hours in comparison to daily administered dietary dose to rats. Mean fecal rebaudioside A and measured hydrolysis products expressed as Total Rebaudioside A Equivalents compared to daily administered dose results in an estimate of percent of dose recovered \approx 84%.

2. Subchronic Toxicity Studies

Recently, Curry and Roberts (2008) reported the results of two repeat dose studies of rebaudioside A in Wistar rats. The results of these investigations suggest that administration of rebaudioside A to Han Wistar rats at dietary concentrations of up to 100,000 ppm (9,938 and 11,728 mg/kg bw/day for males and females, respectively) for 4 weeks or 50,000 ppm (4,161 and 4,645 mg/kg bw/day for males and females, respectively) for 13 weeks did not present any evidence of systemic toxicity. In the 4-week study, rebaudioside A (97% purity) was administered at dietary concentrations of 0, 25,000, 50,000, 75,000 and 100,000 ppm to male and female rats. The NOAEL, including an evaluation of testes histopathology, was determined to be 100,000 ppm. In the 13-week study, Wistar rats were fed diets containing rebaudioside A at dietary concentrations of 0, 12,500, 25,000 and 50,000 ppm. In high-dose male and females groups, reductions in body weight gain attributable to initial taste aversion and lower caloric density of the feed were observed. Inconsistent reductions in serum bile acids and cholesterol were attributed to physiological changes in bile acid metabolism due to excretion of high levels of rebaudioside A *via* the liver. All other hepatic function test results and liver histopathology were within normal limits. No significant changes in other clinical pathology results, organ weights and functional observational battery test results were noted. Macroscopic and microscopic examinations of all organs were unremarkable with respect to treatment-related findings. The NOAEL in the 13-week toxicity study was considered to be 50,000 ppm or approximately 4,161 and 4,645 mg/kg bw/day in male and female rats, respectively (Curry and Roberts, 2008).

In another 90-day dietary admix toxicity study, effects of rebaudioside A (99.5% purity) at target exposure levels of 500, 1000 and 2000 mg/kg bw/day were tested in CrI:CD(SD) rats (Nikiforov and Eapen, 2008; Eapen, 2007). Each group consisted of 20/animals/sex. No treatment related effects on clinical observations, food consumption, and functional observational or locomotor activity parameters were noted. There were no treatment-related macroscopic, organ weight or microscopic findings. Significantly lower body weight gains were noted in the 2,000 mg/kg bw/day group in males but not females. At the end of the dosing period, the body weight in males was 9.1% lower than the control group. Due to the small magnitude of difference from the control group value, the investigators did not consider this result to be adverse. The decrease was most likely due to the large proportion of the diet represented by the test material. The NOAEL was determined as \geq 2,000 mg/kg bw/day.

A 6-month dietary toxicity study in Beagle dogs (4/sex/group) was conducted to investigate the potential adverse effects of rebaudioside A (97.5% purity) at dosage levels of 0, 500, 1000 or 2,000 mg/kg bw/day (Eapen, 2008). There were no unscheduled deaths during the course of the

study. No treatment-related clinical observations were noted. Administration of rebaudioside A did not affect home cage, open field observations and functional observations and measurements. No differences in hematology findings, serum chemistry findings, or urinalysis findings between the groups were noted. Additionally, no treatment related gross necropsy observations, alterations in final body weight, alterations in organ weights, or histological changes were noted. The investigators concluded that no systemic toxicity of rebaudioside A was observed at dosage levels up to 2,000 mg/kg bw/day, and the assigned NOAEL was \geq 2,000 mg/kg bw/day.

3. Mutagenicity Studies

In a set of *in vitro* and *in vivo* genotoxicity assays covering mutation, chromosome damage and DNA strand breakage, rebaudioside A consistently and uniformly revealed negative results (Pezzuto et al, 1985; Nakajima, 2000a; Nakajima, 2000b; Sekihashi et al., 2002). These studies were critically reviewed by Brusick (2008). JECFA also reviewed an unpublished chromosome aberration assay of rebaudioside A in cultured mammalian cells (Nakajima, 2000a) and did not find increases in chromosome aberrations.

Additionally, FDA also reviewed three unpublished studies on rebaudioside A including a bacterial mutagenicity study (Wagner and Van Dyke, 2006), a mouse lymphoma study (Clarke, 2006) and a mouse micronucleus study (Krsmanovic and Huston, 2006) submitted by Merisant as part of the GRAS Notification. All three studies demonstrated lack of mutagenic or genotoxic activity. Additionally, Williams and Burdock (2009) also reported lack of genotoxicity in another set of published studies that included *in vitro* mutagenicity assays with *Salmonella*, *E. coli*, and mouse lymphoma cells. These investigators also reported lack of *in vitro* clastogenic effects in Chinese hamster V79 cells and the absence of *in vivo* effects in a mouse micronucleus assay and a rat study for unscheduled DNA synthesis. The key mutagenicity testing results for rebaudioside A are summarized in Table 12.

4. Reproduction □ Developmental Studies

In a two-generation reproductive toxicity study, rebaudioside A (97% purity) at 0, 7,500, 12,500, and 25,000 ppm was administered in the diet to male and female Han Wistar rats (Curry et al., 2008). Administration of rebaudioside A was not associated with any signs of clinical toxicity or adverse effects on body weight, body weight gain, or food consumption. Similarly, administration of rebaudioside A did not affect reproductive performance parameters including mating performance, fertility, gestation lengths, estrous cycles, or sperm motility, concentration, or morphology in either the F₀ or F₁ generations. The survival and general condition of the F₁ and F₂ offspring, their pre-weaning reflex development, overall body weight gains, and the timing of sexual maturation, were not adversely affected by rebaudioside A treatment. The NOAEL for reproductive effects was 25,000 ppm, and the NOAEL for the survival, development, and general condition of the offspring also was considered to be 25,000 ppm or 2,048 to 2,273 mg/kg bw/day (the highest dose tested).

The results from two unpublished studies with rebaudioside A (Sloter 2008a, b) further support the above described findings from published studies. In a two-generation dietary reproduction study, four groups of male and female Crl:CD(SD) rats (30/sex/group) were fed either basal diet or the diet containing rebaudioside A (purity 95.7%) for at least 70 consecutive days prior to mating (Sloter 2008a). For the F₀ and F₁ generations rebaudioside A doses were 0, 500, 1000

and 2000 mg/kg/day. At initiation of study, F₀ animals were approximately 7 weeks of age. The test diet was offered to the offspring selected to become the F₁ generation following weaning

Table 12. Mutagenicity □ Genotoxicity Studies on Rebaudioside A

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
Bacterial Mutagenicity	5 Salmonella strains with & without exogenous metabolic activation system	Reb A	99.5	1.5, 5.0, 15, 50, 150, 500, 1500 & 5000 µg per plate	No mutagenic response	Wagner and Van Dyke (2006)
Bacterial Mutagenicity	4 Salmonella strains & 1 <i>E. coli</i> strain with & without exogenous metabolic activation system	Reb A	95.6	Up to 5000 µg per plate	No mutagenic response	Williams and Burdock (2009)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence & presence of exogenous metabolic activation system	Reb A	99.5	Cloning conc. of 500, 1000, 2000, 3000, 4000 & 5000 µg/mL	No mutagenic or clastogenic response	Clarke (2006)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence & presence of exogenous metabolic activation system	Reb A	95.6	Up to 5000 µg/mL	No mutagenic or clastogenic response	Williams and Burdock (2009)
Chromosome Aberration	Human lymphocytes in absence & presence of exogenous metabolic activation system	Reb A	95.6	Up to 5000 µg/mL	No mutagenic or clastogenic response	Williams and Burdock (2009)
Mouse Micronucleus	Micronucleus study in groups of 5 male & 5 female ICR mice	Reb A	99.5	500, 1000 & 2000 mg/kg bw	No increase in micronuclei formation	Krsmanovic and Huston (2006)
Mouse Micronucleus	Micronucleus study in groups of 5 male & 5 female NMRI mice	Reb A	95.6	Up to 750 mg/kg bw	No increase in micronuclei formation	Williams and Burdock (2009)
Unscheduled DNA Synthesis	Unscheduled DNA synthesis in one group of 4 Wistar rats	Reb A	95.6	Up to 2000 mg/kg bw	No increase in unscheduled DNA synthesis	Williams and Burdock (2009)
DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver	Stevia extract	Stevioside, 52%; Reb A, 22%	250 - 2000 mg/kg bw	Negative ^a	Sekihashi et al. (2002)
Chromosomal aberration	CHL/IU Chinese hamster lung fibroblasts	Reb A	NS	1.2 - 55 mg/mL	Negative ^b	Nakajima (2000a)
Micronucleus formation	BDF1 mouse bone marrow	Reb A	NS	500-2000 mg/kg bw/ day for 2 days	Negative ^c	Nakajima (2000b)
Forward mutation	<i>S. typhimurium</i> TM677	Reb A	NS	10 mg/plate	Negative ^b	Pezzuto et al. (1985)

NS = Not specified.

^a Sacrificed at 3 hours and 24 hours.

^b With or without metabolic activation (source not specified in original monograph).

^c Sacrificed at 30 hours after 2nd administration.

[beginning on postnatal day (PND) 21]. The F₀ and F₁ males continued to receive rebaudioside A throughout mating, continuing through the day of euthanasia. The F₀ and F₁ females continued to receive rebaudioside A throughout mating, gestation and lactation until the day of euthanasia. The authors concluded that there were no effects on reproduction in males or females as evaluated by estrous cycles, mating, fertility, conception or copulation indices, number of days

between pairing and coitus, gestation length, and spermatogenic endpoints. Both for parental systemic and reproductive toxicity a dose level ≥ 2000 mg/kg bw/day (highest dose administered) was assigned to be the NOAEL.

In an embryo/fetal developmental toxicity study in rats (Sloter, 2008b), potential effects of rebaudioside A administered *via* gavage were investigated. Rebaudioside A administration did not affect intrauterine growth and survival, and there were no test article-related fetal malformations or developmental variations at any dosage level. In the absence of maternal or developmental toxicity a dose level ≥ 2000 mg/kg bw/day (highest dose administered) was considered to be the NOAEL for maternal and embryo/fetal developmental toxicity.

5. Clinical Studies on Rebaudioside A

In a four week randomized, double-blind, placebo controlled trial, hemodynamic effects of rebaudioside A at a dose of 1000 mg/day rebaudioside A (97% purity) or placebo in 100 individuals with normal and low-normal systolic blood pressure (SBP) and diastolic blood pressure (DBP) were investigated (Maki et al., 2008a). Subjects were predominantly female (76%, rebaudioside A and 82%, placebo) with a mean age of ~41 (range 18 to 73) years. At baseline, mean resting, seated SBP/DBP was 110.0/70.3 mm Hg and 110.7/71.2 mm Hg for the rebaudioside A and placebo groups, respectively. Compared with placebo, administration of rebaudioside A did not significantly alter resting, seated SBP, DBP, mean arterial pressure (MAP), heart rate (HR) or 24-hour ambulatory blood pressure responses. The investigators concluded that consumption of 1000 mg/day of rebaudioside A produced no clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure.

In another trial, effects of 16 weeks of consumption of 1000 mg rebaudioside A (97% purity, n = 60) were compared to placebo (n = 62) in men and women (33-75 years of age) with type 2 diabetes mellitus (Maki et al., 2008b). Changes in glucosylated hemoglobin levels did not differ significantly between the rebaudioside A ($0.11 \pm 0.06\%$, mean \pm standard error) and placebo ($0.09 \pm 0.05\%$; $p = 0.355$) groups. Similarly, no significant ($p > 0.05$ for all) changes from baseline for rebaudioside A and placebo, respectively, in fasting glucose (7.5 ± 3.7 mg/dL and 11.2 ± 4.5 mg/dL), insulin (1.0 ± 0.64 μ U/mL and 3.3 ± 1.5 μ U/mL), and Cpeptide (0.13 ± 0.09 ng/mL and 0.42 ± 0.14 ng/mL) were noted. No treatment related changes in blood pressure, body weight, and fasting lipids were noted. Rebaudioside A was well-tolerated, and records of hypoglycemic episodes showed no excess versus placebo. Based on these results, the investigators suggested that chronic use of 1000 mg rebaudioside A does not alter glucose homeostasis or blood pressure in individuals with type 2 diabetes mellitus.

D. Safety of Enzyme Residues in REBATEN PREMIUM

The enzyme used in the manufacturing process for RP is Toruzyme 3.0 L manufactured by Novozymes. It performs the glucosylation reaction needed to add glucose moieties to rebaudioside A to form RP. Novozymes has provided certification information which is located in Daepyeong Food Additive Master File 849, Volume 3 - Attachment 7. Toruzyme 3.0 L is a cyclomaltodextrin glucanotransferase produced by submerged fermentation of a selected strain of *Bacillus licheniformis*. It is a food grade product and complies with JECFA and FCC recommended specifications for food grade enzymes.

E. Safety Data on Steviol Glycosides That Are Predominantly Stevioside □ Rebaudioside A

This section summarizes studies on stevioside or stevia extracts that were identified compositionally as being predominantly stevioside and rebaudioside A, the two most prevalent steviol glycosides isolated from stevia leaves. In some of the published literature, the terms stevia, stevioside, and stevia glycoside are used interchangeably. There are many GRNs and many published articles on steviol glycosides, all of which have shown no toxicity. The details of these safety data are found in Appendix 2.

F. Clinical Studies □ Other Reports in Humans on Rebaudioside A □ Other Steviol Glycosides

A summary of clinical studies on rebaudioside A and steviol glycosides is presented by year in Appendix 3.

G. Studies on Principal Metabolite: Steviol

In a number of studies, steviol, the principal mammalian metabolite of stevioside, has been investigated for its safety. The results of these studies are summarized in Appendix 4.

VI. GRAS CRITERIA □ PANEL SAFETY FINDINGS

A. GRAS Criteria

FDA defines “safe” or “safety” as it applies to food ingredients as:

“...reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use. It is impossible in the present state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any substance.”¹⁰

Amplification is provided in that the determination of safety is to include probable consumption of the substance in question, the cumulative effect of the substance and appropriate safety factors. It is FDA’s operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that:

¹⁰ See 21 CFR 170.3(i).

“...General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food.”

“General recognition of safety through experience based on common use in food prior to January 1, 1958, shall be based solely on food use of the substance prior to January 1, 1958, and shall ordinarily be based upon generally available data and information.”¹¹

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called “common knowledge element,” in terms of the two following component elements:¹²

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and
- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles, textbooks, or compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

The apparent imprecision of the terms “appreciable,” “at the time,” and “reasonable certainty” demonstrates that the FDA recognizes the impossibility of providing absolute safety, in this or any other area (Lu 1988; Renwick 1990).

As noted below, this safety assessment to ascertain GRAS status for REBATEN PREMIUM for the specified food uses meets FDA criteria for reasonable certainty of no harm under the intended conditions of use by considering both the technical and common knowledge elements.

B. Panel Findings on Safety Studies of Steviol Glycosides

1. Discussion of Safety of REBATEN PREMIUM

Based on fundamental toxicological principles, there is a high presumption that RP is safe under the anticipated food use conditions. The natural steviol glycosides, which are enzymatically altered in the manufacturing process for RP, are deemed to be safe as discussed in VI.B.2. The enzymatic process merely adds additional glucose residues to the naturally occurring rebaudioside A molecules that serve as the JECFA-compliant starting material. The larger molecular weight glycosides are not expected to be absorbed from the GI tract *per se* since there is convincing evidence that the smaller glycoside molecules are not absorbed. These additional glucose residues are not expected to be non-enzymatically cleaved in the GI tract because RP is stable under acidic and basic conditions. If these residues were cleaved in the small intestine,

¹¹ See 21 CFR 170.30(a).

¹² See Footnote 1.

the end products would likely be the familiar naturally occurring steviol glycosides encountered from the stevia plant. These naturally occurring glycosides would subsequently be converted to steviol in the large intestine.

Koyama et al. (2003a) has previously demonstrated that any higher molecular weight glycosides that reach the large intestine will slowly be converted to steviol. FDA GRAS submissions GRN 337 (FDA, 2010) and GRN 375 (FDA, 2011a) both extensively examined the safety of very similar enzyme modified steviol glycosides to be used as general purpose non-nutritive sweeteners where the starting material included a high percentage of rebaudioside A. In both of these submissions, the enzyme modified steviol glycosides were found to be GRAS by their respective expert panels and were followed by FDA "no questions" letters (FDA, 2011b; FDA 2011c). The Panel concludes that RP molecules are safe for their intended food uses based on the established metabolic pathway in the GI tract and the large body of data discussed more fully in sections VI.B.2 and VI.B.3 that show that the intended consumption of naturally occurring steviol glycosides is safe.

The Daepyeong RP product identified in the subject notification meets the equivalent of the 95% purity standard comparable to the JECFA specifications for purity. In particular the Panel recognizes that the steviol glycosides mixture that serves as the starting material in the production of RP meets the steviol glycosides JECFA specifications in that the nine specific glycosides constitute 95% or more of the dry weight. Appropriate safety documentation has been supplied regarding pre- and post-enzymatic conversion processes as described in Section V.C. Furthermore, RP is manufactured by a process that complies with FDA Good Manufacturing Practices regulations, and Daepyeong maintains a rigorous set of chemical and microbiological specifications to assure that safe products are generated. The Panel concludes that the RP finished product is a carefully manufactured food grade product.

2. Discussion of Safety Data on Rebaudioside A □ Steviol Glycosides Preparations That Are Predominantly Stevioside □ Rebaudioside A

Steviol glycosides are unique compounds in that they have viable uses as non-nutritive sweeteners in foods.¹³ The series of reviews by JECFA over several years indicate the progression of knowledge on the toxicology and clinical effects of these compounds. The early toxicology studies were largely performed on crude extracts of stevia. Several early concerns were noted, including impairment of fertility and mutagenicity. As more studies were done on purified glycosides, the toxicology profile of steviol glycosides eventually proved to be rather unremarkable.

Since 2008, several well-designed toxicology studies that followed the current regulatory and scientific guidelines for such studies have been reported on rebaudioside A and are detailed in Section V.C. These investigations included subchronic studies in rats and one in dogs, mutagenicity studies, reproduction and developmental studies in rats, and comparative pharmacokinetic studies with stevioside in rats and humans, as well as additional clinical studies. These and some other unpublished studies formed the basis of the two initial GRAS notifications to FDA each by Cargill (GRN 253) and Merisant (GRN 252) (See Table 1). None of these

¹³ It has also been reported that steviol glycosides can impart pharmacological properties which can be utilized in the treatment of certain disease conditions, such as hypertension and type 2 diabetes when administered at elevated levels.

rebaudioside A toxicity studies---published or unpublished---revealed any toxicity concern. The toxicology studies were, in general, adequately designed with appropriately high doses and adequate numbers of animals to maximize the probability of detection of important adverse effects.

As discussed in Section V.B., JECFA reasoned that there were adequate chronic studies in rats--particularly the study by Toyoda et al. (1997)---on which to base a temporary ADI with an adequate margin of safety. The Committee was satisfied that the lack of carcinogenic response in these well-conducted studies justified their conclusion that the *in vitro* mutagenic activity of steviol, buttressed by the evidence of rapid biotransformation and elimination of absorbed steviol, did not present a risk of carcinogenic effects *in vivo*. In addition, they concluded that all common steviol glycosides---including rebaudioside A---share the same basic metabolic and excretory pathways. Therefore, JECFA has concluded that high purity preparations of various steviol glycosides are safe to use as a nonnutritive sweetener. The additional clinical data subsequently presented allowed JECFA to establish a permanent ADI of 0 - 4 mg/kg bw/day (based on steviol equivalents). A detailed reviewed of these toxicology studies is presented in Appendices 2 and 4.

The Panel agrees with this reasoning. It should be noted that, in a 2007 study, DNA damage was reported in a variety of organs in a comet assay in rats maintained on drinking water containing 4 mg/mL steviol glycosides for up to 45 days (Nunes et al., 2007a). Several experts in the field have since questioned the methodology used in this study (Geuns, 2007; Williams, 2007; Brusick, 2008). The Panel has reviewed the cited publications and the responses made by the authors (Nunes et al, 2007b; Nunes et al, 2007c) and concurs with the challenges to the methodology utilized by Nunes et al., 2007a, thereby discounting the validity and importance of this study.

Regarding pharmacological effects in humans, JECFA called for additional studies in individuals that are neither hypertensive nor diabetic (WHO, 2006) to corroborate the observations that the effects of steviol glycosides of reducing blood glucose or blood pressure (or both) only occur in patients who already have these diseases. Data presented to JECFA demonstrated the lack of pharmacological effects of steviol glycosides at 11 mg/kg bw/day in normal individuals. These studies were subsequently published (Barriocanal et al., 2008). In addition, subsequent studies by Maki et al. (2008a, b) showed the lack of pharmacological effects of rebaudioside A. Specifically, the Maki et al. studies showed that rebaudioside A was well-tolerated at chronic use of 1000 mg/person/day rebaudioside A. The studies showed no alterations in glucose homeostasis or blood pressure in individuals with type 2 diabetes mellitus nor were there any clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure. JECFA subsequently adopted an ADI of 4 mg/kg bw/day, derived by applying a 100-fold safety factor to the NOEL of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol) in a 2-year rat study (Toyoda et al, 1997; WHO, 2006). Appendix 3 presents detailed clinical information on steviol glycosides. The Panel has reviewed the clinical studies and concludes that there should be no effects on reducing blood pressure or adversely affecting glucose metabolism in humans at the doses of glucosylated rebaudioside A expected from the proposed use in food as a non-nutritive sweetener based on the analogous metabolic pathways to steviol glycosides.

In addition, a review article by Carakostas et al. (2008) summarized the research on rebaudioside A:

- Steviol glycosides, rebaudioside A, and stevioside are not genotoxic *in vitro*.

- In well-conducted *in vivo* assays, steviol glycosides, rebaudioside A, and stevioside have not been found to be genotoxic.
- A report indicating that stevioside produces DNA breakage *in vivo* appears to be flawed (Nunes, et al., 2007a) and was improperly interpreted as a positive response.
- Steviol genotoxicity in mammalian cells is limited to *in vitro* tests that may be affected by excessive concentrations of the compound.
- The primary evidence for steviol genotoxicity is derived from very specific bacterial tests or purified plasmid DNA that lack DNA repair capabilities.
- Stevioside is not a carcinogen or cancer promoter in well-conducted rodent chronic bioassays.
- The pharmacokinetic similarity between rebaudioside A and stevioside justifies the use of the ADI established by JECFA that was determined on studies employing stevioside as the main component as the ADI for rebaudioside A.
- The dietary levels expected from consumption of rebaudioside A as a total replacement of sugar (Renwick, 2008) are less than the ADI and, therefore, there is no safety concern for consumers.

In summary, the Panel agrees with the conclusion of JECFA and the GRN 252 and GRN 253 Expert Panels that there are a sufficient number of good quality health and safety studies to support the determination that the intended use of purified preparations of steviol glycosides, including rebaudioside A, when added to food at levels up to full replacement of sugar on a sweetness equivalency basis, meets FDA's definition of safe.

3. Acceptable Daily Intake for REBATEN PREMIUM

The Panel concludes that it is reasonable to apply the JECFA ADI of 4 mg/kg bw/day for steviol glycosides (expressed on a steviol basis) to RP. Therefore, with the steviol equivalence values shown in Table 11, the Panel concludes that, for the general population, the estimated maximum daily intake of RP is 6.8 mg/kg bw or 2.13 mg/kg as steviol equivalents and for healthy children, the estimated maximal daily intake is 9.9 mg/kg bw/day or 3.1 mg/kg as steviol equivalents. Based upon these calculations, the intake of RP safely aligns with the 4 mg/kg/day ADI expressed as steviol equivalents as determined by JECFA.

C. Common Knowledge Elements for GRAS Determinations

The first common knowledge element for a GRAS determination is that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing studies published in peer-reviewed scientific journals. The second common knowledge element for a GRAS determination requires that consensus exists among the broader scientific community.

1. Generally Available Information

The key consideration with pharmacokinetic data is the published study that glucosylated steviol glycosides can be converted to steviol in the large intestine by normal anaerobic GI flora as demonstrated by an *in vitro* study in fecal homogenates (Koyama et al., 2003b).

It has been well-established experimentally from various published studies that the smaller steviol glycosides molecules are not absorbed from the GI tract. It would be reasonable to conclude that the larger molecular weight RP molecules would not be absorbed from the GI tract *per se* based on fundamental pharmacokinetic principles. Furthermore, the slow conversion of RP to steviol is comparable to that of naturally occurring steviol glycosides as is noted from the published literature that addresses the safety of the steviol glycosides.

The majority of the studies reviewed on steviol glycosides and rebaudioside A have been published in the scientific literature as reported in Section V. Most of the literature relied upon by JECFA has also been published---most importantly the chronic rat studies on steviol glycosides. JECFA did make limited use of unpublished studies, and they were summarized in the two JECFA monographs. Moreover, JECFA publicly releases the results of their safety reviews, and their meeting summaries and monographs are readily available on their website. Thus, these studies become generally available to the scientific community. JECFA reviewed only a limited number of studies conducted specifically on Reb A. The collection of supporting data on Reb A has been enhanced by the publication of the 2008 studies all of which were cited in Section V.C, and they revealed no safety concerns. The newest clinical studies that address JECFA's concerns about unwanted pharmacological effects with steviol glycosides (Barriocanal et al., 2008) and with Reb A (Maki et al., 2008 a, b) have been published in the scientific literature.

2. Scientific Consensus

The second common knowledge element for a GRAS determination requires that there must be a basis to conclude that consensus exists among qualified scientists about the safety of the substance for its intended use. The Panel maintains that well-qualified scientists would conclude that RP molecules are not absorbed from the GI tract *per se* because smaller steviol glycosides molecules have been well studied and were found to not be absorbed. By virtue of fundamental principles of pharmacokinetics, the majority of scientists would support this determination, and they would likewise concur that RP undergoes a slow conversion to steviol as is known to be the case with the smaller, naturally occurring steviol glycosides. Further, the acceptance of both expert panel conclusions of GRAS Notices GRN 337 (FDA, 2010) and GRN 375 (FDA, 2011a) by FDA resulting in approvals of similar α -glucosylated steviol glycosides is also evidence for scientific consensus of various health agencies worldwide since many follow FDA's lead (FDA, 2011b, c).

Regarding the safety of the naturally occurring glycosides which are converted to RP and then again formed from RP by the action of anaerobic GI flora, the 2008 JECFA final opinion largely meets the scientific consensus test on its own. This is the case because of the well-recognized scientific rigor and broad base of scientific expertise that resides with the prestigious JECFA, which is composed of expert scientists from various regulatory agencies around the world, as well as other scientists chosen because of their specific expertise on various classes of food ingredients. In addition, FDA scientists participate in JECFA deliberations, and EFSA has recently concurred with the JECFA evaluation including the ADI (EFSA, 2010).

The JECFA conclusion has been reviewed and validated by other respected regulatory agencies, including FSANZ and the Switzerland Office of Public Health (FSANZ, 2008 and Switzerland Office of Public Health, 2008). A number of other well-respected scientists have indicated that steviol glycosides are safe for human consumption at doses in the range of the JECFA ADI (Xili et al., 1992; Toyoda et al., 1997; Geuns, 2003; Williams, 2007).

The scientific consensus element has been embellished by the many well-respected scientists that participated in the Cargill-sponsored research conducted on Reb A, most notably David Brusick, Nigel Brown, and Andrew Renwick. An assertion of “general recognition of safety” was also made by Carakostas et al. (2008). We also note that, since December 2008, 23 GRAS notifications have been submitted to FDA for stevia-derived sweetener products as shown in Table 1. Each of these GRAS notifications has precipitated a “no questions” letter from FDA. Two GRN notifications (GRN 337 [FDA, 2010] and GRN 375 [FDA 2011a]) submitted to FDA on GSG preparations have both received “no questions” responses from FDA with respect to the conclusions of both expert panels that, indeed, the GSGs were GRAS under the conditions of use (FDA, 2011b and FDA, 2011c, respectively).

In summary, a compelling case can be made that scientific consensus exists regarding the safety of Reb A and steviol glycosides. The central role of conversion to steviol and subsequent elimination with these naturally occurring steviol glycosides extends to the manner in which RP molecules are metabolized and eliminated from the body. The published work of Koyama et al. (2003a) shows that glucosylated steviol glycosides are subsequently hydrolyzed to the aglycone steviol, demonstrating that the metabolic fate of α -glucosylated steviol glycosides follows that of non-modified steviol glycosides. Due to the similarities in metabolic fate, the safety of α -glucosylated steviol glycosides can be established based on studies conducted with non-modified steviol glycosides. While the scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, the Panel believes that a wide consensus does exist in the scientific community to support a GRAS conclusion as outlined in this evaluation. The scientific community will undoubtedly conclude that concerns expressed by others over the years, e.g., Huxtable, 2002, are likely to be satisfied by newer data on more purified materials and the rigid specifications for purity published by JECFA for steviol glycosides that are applicable to α -glucosylated steviol glycosides.

VII. CONCLUSIONS¹⁴

In consideration of the aggregate safety information available on RP and the naturally occurring steviol glycosides, the Panel concludes that RP is safe for use as a general non-nutritive sweetener in foods. Based on the information that RP will behave pharmacokinetically similarly to the naturally occurring steviol glycosides, the JECFA ADI for steviol glycosides of 4 mg/kg bw/day (as steviol equivalents) can be applied to RP. Based on published dietary exposure data for other approved sweeteners and adjusting for relative sweetness intensity, the intake of α -glucosylated steviol glycosides was estimated for healthy non-diabetic children and adults, and diabetic children and adults.

Given the steviol equivalency calculation for RP as described in Section III and the estimated daily intake calculated in Section IV, the low RP intake for these 4 population groups is estimated to range from 1.7 to 2.8 mg/kg bw/day (0.41 to 0.68 mg/kg bw/day as steviol equivalents) for healthy adults and children, respectively. For these two healthy population groups, the maximum intake is estimated to range from 4.5 to 6.6 mg/kg bw/day (1.25 to 1.83 mg/kg bw/day as steviol equivalents), in healthy adults and children, respectively. The low intake for diabetic adults and children was calculated to be 1.9 and 4.5 mg/kg bw/day (0.46 and 1.09 mg/kg bw/day as steviol equivalents), respectively. The maximum calculated intake for these diabetic groups is estimated to range from 6.0 to 6.1 mg/kg bw/day (1.66 to 1.69 mg/kg bw/day as steviol equivalents), in adults and children, respectively.

Hence, the estimated intakes of α -glucosylated steviol glycosides are all below the ADI of 4 mg/kg bw, expressed as steviol equivalents, established by JECFA. The Panel finds that the dietary levels expected from food consumption will not exceed the ADI when PR is used as a general non-nutritive sweetener.

The Panel finds that the 95% purity specification for RP is sufficient in view of the accepted JECFA specification for 95% purity for naturally occurring steviol glycosides. The Panel also concludes that RP as manufactured by Daepyeong is an appropriate food grade ingredient. The Panel agrees that adverse pharmacological effects are not likely to occur at this designated ADI level and that even high consumers of steviol glycosides are not likely to exceed this level. Therefore, the Panel concludes that RP, when consumed in foods as described within this GRAS notification, is generally recognized as safe (GRAS) within the meaning of the Food, Drug, and Cosmetic Act.

¹⁴ The detailed educational and professional credentials for two of the individuals serving on the Expert Panel can be found on the GRAS Associates website at www.gras-associates.com. Drs. Kraska and McQuate worked on GRAS and food additive safety issues within FDA's GRAS Review Branch earlier in their careers and subsequently continued working within this area in the private sector. Dr. Kapp's curriculum vitae can be accessed on the GRAS Associates website and at <http://www.biotox.net>. All three panelists have extensive technical backgrounds in the evaluation of food ingredient safety. Each individual has previously served on multiple GRAS Expert Panels. Dr. Kraska served as Chair of the Panel.

Daepyeong's REBATEN PREMIUM (glucosylated rebaudioside A) as characterized by the composition and purity specifications established herein by Daepyeong and which is produced in accordance with FDA Good Manufacturing Practices requirements while meeting at a minimum the JECFA purity specifications for steviol glycosides, is Generally Recognized As Safe when consumed as a non-nutritive sweetener in foods other than infant formulas and meat and poultry products within the JECFA ADI of 4 mg/kg bw/day on a steviol equivalent basis. The highest steviol equivalent calculated in any of the four population groups examined is 1.83 mg/kg bw/day on a steviol equivalent basis which is well within the 4 mg/kg bw/day JECFA ADI. In order to remain within the designated ADI, it is important to observe good manufacturing practices principles in that the quantity of a substance added to food should not exceed the amount reasonably required to accomplish its intended technical effect.

This declaration has been made in accordance with FDA's standard for food ingredient safety, i.e., reasonable certainty of no harm under the intended conditions of use.

(b) (6)

Richard C. Kraska, Ph.D., DABT
Chair

(b) (6)

Robert S. McQuate, Ph.D.

(b) (6)

Robert W. Kapp, Jr., Ph.D.
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Date: October 31, 2012

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APPENDIX 1

Biological □ Toxicological Data on Stevia □ Steviol Glycosides

The biological, toxicological and clinical data on stevia and steviol glycosides have been assessed by a number of reviewers (Carakostas et al., 2008; Geuns, 2003; Huxtable, 2002) and most notably through the on-going evaluation by JECFA (WHO, 2000, 2006, 2007, 2008, 2009) and a review by Food Standards Australia New Zealand (FSANZ, 2008) for use in food. Most of these reviews primarily focused on mixtures of steviol glycosides. The early reviews tracked the development of better toxicology studies, and over time increasingly pure samples of steviol glycosides were studied. These reviews followed with keen interest whether effects of concern seen in various toxicology studies---such as the decrease in fertility with crude stevia preparations and the mutagenic activity of the principle metabolite steviol---would be manifest in comprehensive studies using modern test protocols with purer test materials. In addition, JECFA encouraged the further elucidation of clinical effects on blood pressure and glucose metabolism in hypertensive and diabetic individuals, respectively, in comparison to normal human subjects. By 2006, sufficient favorable data were generated for JECFA to yield a temporary ADI which was finalized at an elevated level in 2008.

The key toxicology and clinical data on steviol glycosides (primarily stevioside), more recently developed data on Reb A, and data on the principle metabolite, steviol, as reviewed by JECFA and other reviewers can be summarized as follows:

- Steviol glycosides are not absorbed per se by the GI tract but are converted to steviol in the lower GI tract where steviol is absorbed but rapidly metabolized and excreted.
- Three chronic rat studies basically showed no adverse effects at oral doses as high as 1000 mg/kg bw/day. No evidence of any carcinogenic effects was observed in any of these studies.
- Reproductive studies in several species showed no adverse effects on reproductive outcomes at doses up to 1000 mg/kg bw/day.
- The weight of evidence in over a dozen genetic toxicology studies is that steviol glycosides are not mutagenic or clastogenic *in vitro* or *in vivo*.
- Several clinical studies showed the absence of any effects on healthy adult at doses up to 15 mg/kg bw/day.

More recently, as purified Reb A became available and triggered substantial commercial interest, several studies were conducted on this glycoside to demonstrate safety.

- Pharmacokinetic studies in rats and humans showed that Reb A is not absorbed *per se* from the GI tract and is metabolically handled similar to other steviol glycosides, i.e., converted to steviol in the lower GI tract where steviol is absorbed but rapidly metabolized and excreted.

- Subchronic studies in rats and mice showed the absence of effects at doses of 1000 mg/kg bw/day and higher.
- Reproductive studies in rats and rabbits showed the absence of effects of similar oral dosing regimens.
- Several genetic toxicology assays were performed with none showing any adverse effects.
- Several clinical studies showed the absence of any effects on healthy adults at doses up to 15 mg/kg bw/day.

APPENDIX 2

Safety Data on Stevioside □ Stevia Extracts That Are Predominantly Stevioside

Absorption, Distribution, Metabolism □ Excretion (ADME) Studies

Several studies in rats (Wingard et al., 1980; Nakayama et al., 1986; Koyama et al., 2003a) and other animal models, including chickens (Geuns et al., 2003a), hamsters (Hutapea et al., 1999), and pigs (Geuns et al., 2003b) indicate that stevioside is not readily absorbed from the GI tract. Available evidence from *in vitro* metabolism studies suggests that bacteria in the colon of rats and humans can transform various stevia glycosides into steviol (Gardana et al., 2003). Steviol was shown to be more readily transported with *in vitro* intestinal preparations than various steviosides (Geuns, 2003, Koyama et al., 2003b). Slow absorption of steviol was indicated by detection in the plasma of rats given oral stevioside (Wang et al., 2004). However, Sung (2002) did not detect plasma steviol following oral administration of steviosides to rats. In studies with human and rat liver extracts, Koyama et al. (2003b) demonstrated that steviol can be converted to various glucuronides. Excretion of metabolites of stevioside after oral doses has been shown in urine and feces in rats (Sung, 2002) and hamsters (Hutapea et al., 1999). Oral doses in pigs led to the detection of metabolites in feces but not in urine (Geuns et al., 2003b).

In a human study with 10 healthy subjects, Geuns et al. (2006) measured blood, urine and fecal metabolites in subjects that received 3 doses of 250 mg of purified stevioside (>97%) 3 times a day for 3 days. Urine was collected for 24 hours on day 3 and blood and fecal samples were also taken on day 3. Free steviol was detected in feces but not in blood or urine. Steviol glucuronide was detected in blood, urine and feces. Approximately 76% of the total steviol equivalents dosed were recovered in urine and feces. Based on these measurements, the authors concluded that there was complete conversion of stevioside in the colon to steviol, which was absorbed and rapidly converted to the glucuronide.

In a recent publication, Renwick and Tarka (2008) reviewed studies on microbial hydrolysis of steviol glycosides. The reviewers concluded that stevioside and Reb A are not absorbed directly and both are converted to steviol by gut microbiota in rats and in humans. This hydrolysis occurs more slowly for Reb A than for stevioside. Studies have shown that steviol-16,17 epoxide is not a microbial metabolite. Given the similarity in the microbial metabolism of stevioside and rebaudioside A with the formation of steviol as the single hydrolysis product that is absorbed from the intestinal tract, these investigators concluded that the toxicological data on stevioside are relevant to the risk assessment of rebaudioside A.

Acute Toxicity Studies

The oral LD₅₀ studies of stevioside (purity, 96%) following administration of a single dose to rodents are summarized in Table 2a. No lethality was noted within 14 days after the administration, and no clinical signs of toxicity or morphological or histopathological changes were found, indicating that stevioside is relatively harmless.

Table 2a. Acute Toxicity of Stevioside (Purity 96%) Given Orally to Rodents

SPECIES	SEX	LD ₅₀ (g/kg bw)	REFERENCE
Mouse	Male and Female	>15	Toskulkao et al. (1997)
Mouse	Male	> 2	Medon et al. (1982)
Rat	Male and Female	>15	Toskulkao et al. (1997)
Hamster	Male and Female	>15	Toskulkao et al. (1997)

Subchronic Toxicity Studies

In five published studies, subchronic toxicity of stevioside was investigated in rats following oral administration. In addition, a reproduction study in hamsters included subchronic phases on the F₀, F₁ and F₂ generations. These studies are summarized in Table 2b. One of these studies was particularly important because it served as a range-finding study for two subsequent chronic studies. In this 13-week toxicity study, Fischer 344 rats (10/sex/group) were given doses of 0, 0.31, 0.62, 1.25, 2.5, or 5% in the diet (equivalent to 160, 310, 630, 1300, and 2500 mg/kg bw/day) to determine the appropriate doses for a two-year carcinogenicity study. None of the animals died during the administration period, and there was no difference in body-weight gain between the control and treated groups during administration or in food consumption in the latter part of the study. The activity of lactic dehydrogenase and the incidence of single-cell necrosis in the liver were increased in all groups of treated males. The authors considered these effects to be nonspecific, because of the lack of a clear dose-response relationship, the relatively low severity, and their limitation to males. Other statistically significant differences in hematological and biochemical parameters were also considered to be of minor toxicological significance. The authors concluded that a concentration of 5% in the diet was a suitable maximum tolerable dose of stevioside for a two-year study in rats (Aze et al., 1991).

In earlier 3-month rat studies reviewed by Geuns (2003)---the sample purity, doses, strain of rat were not reported---a no effect level was determined to be in excess of 2500 mg/kg bw/day and 7% of the diet, apparently due to lack of effects at the highest dose tested in both studies (Akashi and Yokoyama, 1975).

In a recently published exploratory subchronic toxicity study, Awney et al. (2010) investigated the effects of 97% pure stevioside on body weight, organ relative weight, hematological and biochemical parameters and enzyme activities in Sprague Dawley rats. In this 12-week toxicity study, groups of male rats (8/group) were given drinking water containing stevioside. The groups were assigned to drink distilled water (control), low-dose stevioside solution (15 mg/kg/day), high-dose stevioside solution (1500 mg/kg/day) or low-dose stevioside (15 mg/kg/day) plus inulin solution for 12 weeks as the sole source of liquid. Fluid intake was recorded daily and levels of test articles were adjusted weekly to receive the appropriate target concentration. Low dose

stevioside (15 mg/kg bw/day) administration without or with inulin for 12 weeks did not reveal any adverse effects on body weight, organs relative weight, hematological and biochemical parameters or enzymes activities. However, treatment with high dose stevioside was reported to cause significant changes in several investigated toxicological parameters. Among the hematological parameters, significant changes were noted in all except WBCs, RBCs, and PCV% and in all clinical chemistry parameters except proteins, total lipids, serum ATL and AST. These data suggest the NOEL of 15 mg/kg/day. However, critical review of the publication reveals that the study was poorly designed and implemented. Design deficiencies include: insufficient numbers of animals, group-housing with the potential for stress-related changes, unreliable access to steviol *via* drinking water resulting in suspect dosing calculations in group-housed cages, no indication of fasting prior to blood collection which affects many chemistry and hematological values, no urine collection and no histopathological evaluations for confirmation of findings beyond the controls. In addition to these study design deficiencies, the report fails to adequately present mean or individual organ weight data and, in general, there appears to be inadequate comparison of study findings against laboratory historical control data. Any one of these oversights could have adversely affected the results and/or interpretation of the hematological and chemistry data.

In addition to the above described parameters, tartrate-resistant alkaline phosphatase (TRAP) levels were measured and found to be significantly decreased (Awney et al., 2010). TRAP is an enzyme that is expressed by bone-resorbing osteoclasts, inflammatory macrophages and dendritic cells. This enzyme was not measured in any previous steviol glycosides studies nor has it been adequately vetted for application in toxicological studies. These investigators did not identify the specific TRAP isomer measured, the methodology employed, the handling of the samples, or any historical data on TRAP levels. The significance and relevance of this poorly documented toxicological endpoint which lacks histopathological confirmation does not appear to have a distinct role in determining the toxicological profile of a material in a test animal. The data presented by Awney et al. (2010) are probably not representative of changes due to the subchronic dietary administration of steviol glycosides because of overall inadequate study design and reliance on the findings of the untested enzyme TRAP. The preponderance of the data from several well designed studies on steviol glycosides suggests that differences noted in hematological and chemistry data are probably random, nonspecific and not toxicologically significant.

Table 2b. Summary of Subchronic Studies on Stevioside

STUDY	ANIMAL MODEL/ GROUP SIZE	TEST MATERIAL/ SAMPLE PURITY	DOSES / DURATION	AUTHOR ASSIGNED NOAEL (mg/kg bw/day)	RESULTS & REMARKS
Aze et al., 1991 ^a	F344 rat/ 10 females and 10 males in each of 6 groups	Stevioside/ Not reported	0, 0.31, 0.62, 1.25, 2.5, 5% in diet/13 weeks	Not reported	No effects observed on mortality, body weight or food consumption. Clinical chemistry investigation revealed increased LDH levels and histopathological investigation indicated increased incidence of single-cell liver necrosis in all male treated groups, but not in a clear dose-response relationship. Investigators did not consider these changes to be treatment related due to the small magnitude and low severity of changes, the lack of a clear dose relationship and the limitation to males only. Organ weights, urine chemistry and gross necropsy not discussed. Authors concluded that 5% stevioside in diet is a tolerable dose for a 2 year study.
Yodyingyuad and Bunyawong, 1991 ^a	Hamster/ four groups of 20 (10 male, 10 female)	Stevioside/ 90%	0, 0.5, 1.0, 2.5 g/kg bw/day/ duration unclear/ 3 months	2500	F ₀ , F ₁ and F ₂ generations in reproductive study were dosed for 90 days. Histological examination showed no effect at any dose. Weights of organs, blood analysis, urine chemistry and gross necropsy not discussed. The F ₁ and F ₂ hamsters continued to receive stevioside (via drinking water for one month, then at same dose as parents).
Mitsuhashi, 1976 ^b	Rat (strain not reported)	Stevioside/ Not reported	Dietary concentrations up to 7%/ 3 months	Not reported	No effects noted at all doses tested. Experimental details such as body weight, organ weight, blood analysis, urine chemistry, gross necropsy and histopathology not discussed.
Akashi and Yokoyama, 1975 ^b	Rat (strain not reported)	Stevioside/ Not reported	Oral doses up to 2500 mg/kg bw/3 months	2500	No effects noted at all doses tested. Experimental details such as body weight, organ weight, blood analysis, urine chemistry, gross necropsy and histopathology not discussed.
Awney et al., 2010	Sprague Dawley rats	Stevioside 97%	Drinking water (15, 1500 mg/kg bw /day)	15	Treatment with high dose stevioside caused significant changes in several investigated toxicological parameters. Among the hematological parameters, significant changes were noted in all except WBCs, RBCs, and PCV% and in all clinical chemistry parameters except proteins, total lipids, ATL and AST.

^a Abstract only. ^b As reported by Geuns, 2003.

Chronic Toxicity Studies

Chronic effects of stevioside have been studied in three separate studies (Table 2c). No treatment-related increase in tumor incidence was seen in any of these studies. In the most recent and well-documented study (additional study details were presented to JECFA in 2006 (WHO, 2006), the apparent no observed adverse effect level (NOAEL) in F344 rats was the dietary level of 2.5% (test sample purity 96%, Toyoda et al., 1997). At 5% of the diet, statistically significant decreases in body weight, percent survival and kidney weight were noted. The author attributed these effects to various factors. The decrease in body weight was attributed to an inhibition of glucose utilization. The decrease in survival seemed to have been caused by an unusual late onset of large granular lymphocyte leukemia in high dose males. The authors reported that this tumor is rather common in F344 rats and that the overall incidence in male rats was actually within the historical control range experienced in the laboratory where studies were

conducted. The authors attributed the decrease in kidney weight as probably due to a decrease in chronic inflammation found in the histopathological examination relative to control animals.

Reproductive □ Developmental Toxicity Studies

The use of *S. rebaudiana* as an oral contraceptive has been reported by Indians in Paraguay (Planas and Kuc, 1968; Schwartzman et al., 1977). In experimental studies in rats, crude stevia leaf extract has been shown to inhibit fertility (Planas and Kuc, 1968). Reproductive toxicity studies have been conducted with orally administered purified stevioside as tabulated in Table 2d, No effect on fertility or reproductive parameters was seen in a three-generation study in hamsters at doses up to 2500 mg/kg/day (Yodyingyuad and Bunyawong, 1991). There was an absence of statistically significant effects at doses up to 3% (equivalent to 3000 mg/kg bw/day; sample purity 96%; Mori et al., 1981). Similar results were observed in an additional rat study that was reviewed by Geuns (2003) where limited information is available in English (Usami et al., 1995).

In a recent study, no effect on pregnancy or developmental parameters were observed in Swiss albino mice with stevioside or aqueous stevia extract at doses up to 800 mg/kg bw/day in female mice (Kumar and Oommen, 2008). Further details on these studies to the extent available are presented in Table 2d.

Table 2c. Summary of Chronic Toxicity Studies on Stevioside

STUDY	ANIMAL MODEL/ GROUP SIZE	TEST MATERIAL/ SAMPLE PURITY	DOSES / DURATION	AUTHOR ASSIGNED NOAEL (mg/kg bw/day)	RESULTS AND REMARKS
Toyoda et al., 1997	F344 rat/ 50 per sex per group	95.6% Stevioside	<i>Ad libitum</i> 0,2.5, 5% of diet/~24 months (104 weeks)	Author did not assign a NOAEL. (Mid-dose calculates to 970 in males; WHO, 2006)	A significant decrease in survival rates in males receiving 5%. General condition, body weight, food intake, mortality, hematological, histopathological and organ weights were observed. Body weight gains dose-dependently decreased in both sexes. Kidney weights were significantly lower in 5% males and ovary, kidney and brain weights were significantly increased in 5% females. Tumors and non-neoplastic lesions found in all groups, and were not correlated to treatment. Conclusion was that stevioside is not carcinogenic under these experimental conditions.
Xili et al., 1992 ^a	Wistar rat/ 45 per sex per group	85% Stevioside	0, 0.2, 0.6, 1.2 % of diet/24 months	794 (high dose)	After 6, 12 and 24 months five rats from each group were sacrificed for analysis. No effects observed on growth, food utilization, general appearance, mortality or lifespan. No changes in hematological, urinary or clinical biochemical values. Histopathological analysis showed that the neoplastic and non-neoplastic lesions were unrelated to the level of stevioside in the diet.

Yamada et al., 1985	F344 rat/ 70 per sex per group, 30 per sex per group in low-dose	95.2% Steviol glycosides (75% stevioside; 16% Reb A)	0.1, 0.3, 1% of diet/22 months for males, 24 months for females	550 (high dose)	At 6 and 12 months, 10 males and 10 females were sacrificed for analysis. General behavior, growth and mortality were same among groups throughout the experiment. At 6 months, protein urea was significantly increased in females, and blood glucose was increased in both sexes, although urinary glucose not detected. Weights of liver, kidney, heart, prostate and testes were increased in males at 6 months, and weight of ovaries was decreased in females in dose-dependent manner. Histopathological examination showed differences in various organs at 6 months that were unrelated to stevioside dose. These differences were not found at 12 months. Authors concluded that there were no significant changes after 2 years.
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^a Only abstract available.

Table 2d. Summary of Reproductive Toxicity Studies on Steviol Glycosides

STUDY	ANIMAL MODEL/ GROUP SIZE	TEST SAMPLE PURITY STEVIOSIDE (UNLESS OTHERWISE NOTED)	DOSES / DURATION	AUTHOR ASSIGNED NOAEL (mg/kg bw/day)	RESULTS AND REMARKS
Kumar and Commen, 2008	Swiss albino mice/ 4 groups of 5 females	Not reported	500 and 800 mg/kg bw/15 days	800	Stevioside and stevia extract (purity and composition not reported) did not have any effect on reproductive parameters in mice when administered to female mice before or during pregnancy. No changes seen in number of implantations or uterine resorptions. No gross anatomical or histopathologic effects seen in 16-day embryos.
Usami et al., 1995 ^a	Wistar Rat/4 groups of 25 or 26 pregnant rats	95.6% ^b	0, 250, 500, 1000 mg/kg bw/10 days	1000	Pregnant rats given doses of stevioside by gavage once a day on days 6-15 of gestation and were sacrificed on day 20 of gestation. Fetuses were examined for malformations in addition to maternal and fetal body weight, number of live fetuses, sex distribution, and numbers of resorptions or dead fetuses. No treatment-related effects observed. Authors concluded that orally administered stevioside is not teratogenic in rats.
Yodyingyuad and Bunyawong, 1991	Hamster/ 10 male, 10 female per group (40 total)	90%	0, 500, 1000, 2500 mg/kg bw/day/ duration unclear/ 3 months	2500	Males from each group were mated to females from respective dose group. Each female was allowed to bear 3 litters during the course of experiment. Stevioside had no effect on pregnancies of females at any dose. The F ₁ and F ₂ hamsters continued to receive stevioside (via drinking water for one month, then at same dose as parents); showed normal growth and fertility. Histological examination showed no effect on reproductive organs at any dose.
Oliveira-Filho et al., 1989 ^a	Rat/number not reported	Not reported (Dried Stevia Leaves)	0 or 0.67 g dried leaves /ml, 2 ml twice per day/ 60 days	Not reported	Prepubertal rats (25-30 days old) tested for glycemia; serum concentrations of thyroxine; tri-iodothyroxine; available binding sites in thyroid hormone-binding proteins; binding of ³ H-methyltrienolone (a specific ligand of androgen receptors) to prostate cytosol; zinc content of prostate, testis, submandibular salivary gland, and pancreas; water content of testes and prostate; body-weight gain; and final weights of testes, prostate, seminal vesicle, submandibular salivary gland, and adrenal. Only difference due to treatment was seminal vesicle weight, which fell to 60% compared to control.
Mori et al., 1981	Rat/11 male, 11 female per group (44 total)	96%	0, 0.15, 0.75 or 3 % of feed/60 days	2000	Males given stevioside dose in diet for 60 days before and during mating with females who received same diet (as mated male) 14 days before mating and 7 days during gestation. No effect due to treatment on fertility or mating performance, and no effect of fetal development. Rats of each sex had slightly decreased body weight gain at highest dose with non-significant increase in number of dead and resorbed fetuses at highest dose.
Planas and Kuc, 1968 ^c	Rat/14 per group (28 total)	Not reported (Crude stevia extract)	0 or 5% Crude stevia extract /18 days	Not reported	Extract given orally to adult female rats for 12 days, who were mated with untreated males during the last 6 days. Fertility reduced to 21% of fertility in control rats and remained reduced in a 50-60 day recovery. Histological examination, weights of organs, blood analysis, urine chemistry and gross necropsy not discussed.

^a Only abstract available. ^b As reported by European Commission, 1999b.

Mutagenicity □ Genotoxicity Studies

In a series of studies mutagenic and genotoxic effects of stevia and stevioside were investigated. These studies are summarized in Table 2e. All studies were negative with the exception of a comet assay done in rats (Nunes et al., 2007a). The methodology used in this study and the resulting conclusions have been questioned by Geuns, 2007, Williams, 2007 and Brusick, 2008 and responded to by the authors (Nunes et al., 2007b, Nunes et al., 2007c.)

Table 2e. Mutagenicity □ Genotoxicity Studies on Stevia Extracts □ Stevioside

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
<i>In Vitro</i>						
Reverse mutation	<i>S. typhimurium</i> TA97, TA98, TA100, TA102, TA104, TA1535, TA1537	Stevioside	83	5 mg/plate ^a 1 mg/plate ^b	Negative	Matsui et al. (1996)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Stevioside	99	50 mg/plate	Negative ^c	Suttajit et al. (1993)
Forward mutation	<i>S. typhimurium</i> TM677	Stevioside	83	10 mg/plate	Negative ^c	Matsui et al. (1996)
Forward mutation	<i>S. typhimurium</i> TM677	Stevioside	NS	10 mg/plate	Negative ^c	Pezzuto et al. (1985)
Forward mutation	<i>S. typhimurium</i> TM677	Stevioside	NS	Not specified	Negative ^c	Medon et al. (1982)
Gene mutation	Mouse lymphoma L5178Y cells, TK- locus	Stevioside	NS	5 mg/mL	Negative ^{c,d}	Oh et al. (1999)
Gene mutation (umu)	<i>S. typhimurium</i> TA1535/pSK1002	Stevioside	83	5 mg/plate	Negative ^c	Matsui et al. (1996)
Gene mutation	<i>B. subtilis</i> H17 rec+, M45 rec-	Stevioside	83	10 mg/disk	Negative ^c	Matsui et al. (1996)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	83	8 mg/mL 12 mg/mL	Negative	Matsui et al. (1996)
Chromosomal aberration	Human lymphocytes	Stevioside	NS	10 mg/mL	Negative	Suttajit et al. (1993)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	85	12 mg/mL	Negative ^a	Ishidate et al. (1984)
<i>In Vivo</i>						
DNA damage (comet assay)	Wistar rats; liver, brain and spleen	Stevioside	88.62	4 mg/L (estimated to be 80 - 500 mg/kg bw/day) in drinking water for 45 days	Positive in all tissues examined, most notably in liver	Nunes et al. (2007a)
DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver	Stevia extract	Stevioside, 52; Reb A, 22	250 - 2000 mg/kg bw	Negative ^e	Sekihashi et al. (2002)
DNA damage (comet assay)	Male ddY mouse stomach, colon, liver, kidney, bladder, lung, brain, bone marrow	Stevia	NS	2000 mg/kg bw	Negative ^e	Sasaki et al. (2002)
Micronucleus formation	ddY mouse bone marrow and regenerating liver	Stevioside	NS	62.5 - 250 mg/kg bw	Negative	Oh et al. (1999)
Mutation	<i>D. melanogaster</i> Muller 5 strain	Stevioside	NS	2% in feed	Negative	Kerr et al. (1983)

NS = Not specified. ^a Without metabolic activation. ^b As calculated by Williams, 2007. ^c With and without metabolic activation (source not specified in original monograph). ^d Inadequate detail available. ^e Sacrificed at 3 hours and 24 hours.

APPENDIX 3

Steviol Glycosides Clinical Studies on Other Reports in Humans

In several studies, pharmacological and biochemical effects of crude extracts of stevia leaves and purified steviol glycosides have been investigated. The effects noted included glucose uptake, insulin secretion, and blood pressure (Geuns, 2003a). In South America, stevioside is used as a treatment for Type II diabetes. These effects were key concerns for JECFA. In 2006, JECFA summarized the available clinical studies of stevioside and further studies were recommended (WHO, 2006). Subsequently, several studies were conducted, and in 2009, JECFA reviewed these new studies (WHO, 2009). JECFA's summaries of the key studies are included below.

Studies Summarized in 2006

In a study by Curi et al. (1986), aqueous extracts of 5 g of *S. rebaudiana* leaves were administered to 16 volunteers at 6 hour intervals for three days, and glucose tolerance tests were performed before and after the administration. Another six volunteers were given an aqueous solution of arabinose in order to eliminate possible effects of stress. The extract increased glucose tolerance and significantly decreased plasma glucose concentrations during the test and after overnight fasting in all volunteers.

In a multi-center randomized, double-blind, placebo-controlled trial of hypertensive Chinese men and women (aged 28–75 years), 60 patients were given capsules containing 250 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 750 mg of stevioside per day (equivalent to 11 mg/kg bw/day as calculated by FSANZ, 2008) and followed up at monthly intervals for one year. Forty-six patients were given a placebo. After 3 months, systolic and diastolic blood pressure in men and women receiving stevioside decreased significantly, and the effect persisted over the year. Blood biochemistry parameters, including lipids and glucose, showed no significant changes. Three patients receiving stevioside and one receiving the placebo withdrew from the study as a result of side effects (nausea, abdominal fullness, dizziness). In addition, four patients receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved, and the patients remained in the study (Chan et al., 2000).

In a follow-up multi-center randomized, double-blind, placebo-controlled trial was conducted in hypertensive Chinese men and women (aged 20–75 years), 85 patients were given capsules containing 500 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 1500 mg of stevioside per day (equivalent to 21 mg/kg bw/day, as calculated by FSANZ, 2008). Eighty-nine patients were given a placebo. During the course of study, three patients in each group withdrew. There were no significant changes in body mass index or blood biochemistry parameters throughout the study. In the group receiving stevioside, mean systolic and diastolic blood pressures were significantly decreased compared with the baseline, commencing from about 1 week after the start of treatment. After 2 years, 6 out of 52 patients (11.5%) in the group receiving stevioside had left ventricular hypertrophy compared with 17 of 50 patients (34%) in the group receiving the placebo ($p < 0.001$). Eight patients in each group reported minor side effects (nausea, dizziness and asthenia), which led two patients in each group to withdraw from the study. Four patients in the group receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved and the patients remained in the study (Hsieh et al., 2003).

In a paired cross-over study, 12 patients with Type II diabetes were given either 1 g of stevioside (stevioside, 91%; other stevia glycosides, 9%) or 1 g of maize starch (control group), which was taken with a standard carbohydrate-rich test meal. Blood samples were drawn at 30 minutes before and for 240 minutes after ingestion of the test meal. Stevioside reduced postprandial blood glucose concentrations by an average of 18% and increased the insulinogenic index by an average of 40%, indicating beneficial effects on glucose metabolism. Insulin secretion was not significantly increased. No hypoglycemic or adverse effects were reported by the patients or observed by the investigators. Systolic and diastolic blood pressure was not altered by stevioside administration (Gregersen et al., 2004).

In a randomized, double-blind trial designed, 48 hyperlipidemic volunteers were recruited to investigate the hypolipidemic and hepatotoxic potential of steviol glycoside extract. The extract used in this study was a product containing stevioside ($73 \pm 2\%$), rebaudioside A ($24 \pm 2\%$) and other plant polysaccharides (3%). The subjects were given two capsules, each containing 50 mg of steviol glycoside extract or placebo, twice daily (i.e., 200 mg/day, equivalent to 3.3 mg/kg bw/day assuming an average body weight of 60 kg), for 3 months. One subject from placebo group and three from treatment group failed to complete the study for personal reasons, not related to adverse reactions. At the end of the study, both groups showed decreased serum concentrations of total cholesterol and of low-density lipoproteins. Analyses of serum concentrations of triglycerides, liver-derived enzymes and glucose indicated no adverse effects. The authors questioned the subjects' compliance with the dosing regimen, in view of the similarity of effect between treatment and placebo (Anonymous, 2004a). In a follow-up study, 12 patients were given steviol glycosides extract in incremental doses of 3.25, 7.5 and 15 mg/kg bw/day for 30 days per dose. Preliminary results indicated no adverse responses in blood and urine biochemical parameters (Anonymous, 2004b).

Studies Summarized in 2009

In a short term study of stevioside in healthy subjects, 4 male and 5 female healthy volunteers (aged 21–29 years) were provided with capsules containing 250 mg stevioside (97% purity) to be consumed 3 times per day for 3 days (Temme et al., 2004). Doses, expressed as steviol, were 288 mg/day or 4.4 mg/kg bw/day for females and 3.9 mg/kg bw/day for males. Twenty-four hour urine samples were taken before dosing on day 1 and after dosing on day 3. Fasting blood samples were taken before dosing on day 1, and six samples were taken at different time points on day 3 after dosing. Fasting blood pressure measurements were taken before the first capsule and at six different time intervals after the first dose. Urine was analyzed for creatinine, sodium, potassium, calcium, and urea. Blood was analyzed for plasma glucose, plasma insulin, alkaline phosphatase, ALT, GPT, creatine kinase, and lactate dehydrogenase. The clinical analyses of blood, blood pressure, and urine showed no differences between samples taken before or after dosing.

In an unpublished double-blind, placebo-controlled trial study reviewed at the sixty-eighth JECFA meeting, 250 mg of a product containing 91.7% total steviol glycosides, including 64.5% stevioside and 18.9% rebaudioside A, was administered to groups of type 1 ($n = 8$) and type 2 diabetics ($n = 15$) and non-diabetics ($n = 15$) 3 times daily for 3 months. Control groups with the same number of subjects received a placebo. After 3 months, there were no significant changes in systolic or diastolic blood pressure, glycated haemoglobin (HbA1c), blood lipids, or renal or hepatic function. No adverse effects were reported. This study was approved by the local ethics committee and met the requirements of the Declaration of Helsinki (Barriocanal et al., 2006,

2008). The Committee previously noted that this product did not meet the proposed specification of “not less than 95% steviol glycosides” and that the study was conducted in a small number of subjects.

A study of antihypertensive effects was conducted in previously untreated mild hypertensive patients with crude stevioside obtained from the leaves of *S. rebaudiana*. Patients with essential hypertension were subjected to a placebo phase for 4 weeks and then received either capsules containing placebo for 24 weeks or crude stevioside at consecutive doses of 3.75 mg/kg bw/day (7 weeks), 7.5 mg/kg bw/day (11 weeks) and 15 mg/kg bw/day (6 weeks). Comparison of patients receiving stevioside with those on placebo showed neither antihypertensive nor adverse effects of stevioside. This study was approved by the local ethics committee and met the requirements of the Declaration of Helsinki (Ferri et al., 2006). The product in this study also did not meet the proposed specification.

In a long-term, randomized, double blinded, placebo-controlled study, Jeppesen et al. (2006) investigated the efficacy and tolerability of oral stevioside in patients with type 2 diabetes. In this study, 55 subjects received 500 mg stevioside (purity unspecified) or placebo (maize starch) 3 times daily for 3 months. Compared with the placebo, stevioside did not reduce the incremental area under the glucose response curve and maintained the insulin response and HbA1c and fasting blood glucose levels. HbA1c is an indicator of mean glucose levels and is used in identifying effects on the control of diabetes. No differences in lipids or blood pressure were observed. It is not clear whether this study was approved by the local ethics committee or met the requirements of the Declaration of Helsinki (Jeppesen et al., 2006).

A placebo-controlled double-blind trial was carried out in 49 hyperlipidemic patients (aged 20–70 years, number of males and females not supplied) not undergoing treatment. The study was approved by the local ethics committee and complied with the principles of the Declaration of Helsinki. Individuals were divided into two groups, with 24 subjects receiving placebo capsules and 25 receiving capsules containing a dose of 50 mg steviol glycosides (70% stevioside, 20% Rebaudioside A), equivalent to 1.04 mg steviol/kg bw/day, using the mean body weight of the treatment group, 72.7 kg. Two capsules were taken before lunch and two before dinner each day for 90 days. Six subjects withdrew from the study, four in the placebo group and two in the test group. Self-reported adverse reactions were recorded, and fasting blood samples were taken at the end of the study and analyzed for ALT, aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low density lipoprotein (VLDL), and triglycerides. No effects of treatment on ALT, AST, or GGT were found. Decreases in the total cholesterol and LDL were observed in both the stevioside group and the placebo group, which were not treatment related. No adverse effects were observed (Cavalcante da Silva et al., 2006). The Committee noted at its sixty-eighth meeting that the product used in this study did not meet the proposed specification

APPENDIX 4

Studies on Principal Metabolite: Steviol

Studies on Principal Metabolite: Steviol

In a number of studies, steviol, the principal mammalian metabolite of stevioside, has been investigated for its safety. The results of these studies are summarized in the following sections.

Acute Toxicity Studies

The oral LD₅₀ of steviol (purity, 90%) in male and female mice and rats was reported to be > 15 g/kg bw. In this study, only one of 15 animals died within 14 days of administration. The LD₅₀ values in hamsters given steviol orally were 5.2 g/kg bw in males and 6.1 g/kg bw in females. Histopathological examination of the kidneys revealed severe degeneration of the proximal tubular cells, and these structural alterations were correlated with increased serum blood urea nitrogen and creatinine. The authors concluded that the cause of death was acute renal failure (Toskulkao et al., 1997).

Developmental Toxicity Studies

Groups of 20 pregnant golden hamsters were given steviol (purity, 90%) at doses of 0, 250, 500, 750, or 1000 mg/kg bw/day (only 12 animals at the highest dose) by gavage in corn oil on days 6 - 10 of gestation. A significant decrease in body weight gain and increased mortality (1/20, 7/20, and 5/12) were observed at the three highest doses, and the number of live fetuses per litter and mean fetal weight decreased in parallel. Histopathological examination of the maternal kidneys showed a dose-dependent increase in the severity of effects on the convoluted tubules (dilatation, hyaline droplets). However, no dose-dependent teratogenic effects were seen. The NOEL was 250 mg/kg bw/day for both maternal and developmental toxicity (Wasuntarawat et al., 1998).

Mutagenicity □ Genotoxicity Studies

In a number of studies mutagenicity and genotoxicity of steviol has been investigated. These studies reviewed by JECFA are summarized in Table 4.

Table 4. Mutagenicity □ Genotoxicity Studies on Steviol

	<i>IN VIVO/IN VITRO</i>	SYSTEM	TEST SAMPLE PURITY	AUTHOR CONCLUSION	RESULTS AND REMARKS
Sekihashi et al., 2002 ^a	<i>In Vivo/In Vitro</i>	Comet Assay	Not reported	Negative	In <i>in vitro</i> study, steviol at 62.5, 125, 250 and 500 µg/ml did not damage DNA of TK6 and WTK1 cells in presence or absence of S9 mix. In <i>in vivo</i> study, mice sacrificed 3 or 24 hours after one-time oral administration of 250, 500, 1000 or 2000 mg/kg of steviol. Stomach, colon, kidneys, testis and liver DNA not damaged. An identical <i>in vivo</i> experiment with stevia extract performed, which also gave negative results.
Oh et al., 1999 ^b	<i>In Vivo?</i>	Cell Mutation and DNA damage	Not reported	Negative	Steviol gave negative results for cell mutation and DNA damage in cultured cells.
Matsui et al., 1996 ^c	<i>In Vivo?</i>	Mutagenicity and Chromosome aberration (Chinese hamster lung fibroblasts)	Not reported	Positive	Gene mutation and chromosomal aberration found in Chinese hamster lung fibroblasts after metabolic activation of steviol. In hamsters, several metabolites of stevioside found that have not been found in rats or humans. Therefore, experimental relevance should be questioned when hamsters are used.
Terai et al., 2002 ^a	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Positive	Steviol found to be mutagenic in Aroclor induced rat liver S9 fraction. 15-oxo-steviol found to be mutagenic at 10% level of steviol. Specific mutagenicity of lactone derivative in presence of S9 mixture 10x lower than that of derivative without S9 mixture.
Temcharoen et al., 1998 ^c	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Positive	Mutagenic effects of steviol and/or metabolites found in <i>S. typhimurium</i> TM677 by tranversions, transitions, duplications, and deletions at the guanine phosphoribosyltransferase (gpt) gene. Magnitude of increase of these mutations over the control not reported.
Klongpanichpak et al., 1997 ^c	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Negative	Steviol and stevioside inactive in TA strains of <i>S. typhimurium</i> , <i>e. coli</i> WP2, <i>uvrA/PKM101</i> and rec assay using <i>B. subtilis</i> even when microsomal activated fraction present. Magnitude of increase of these mutations over the control not reported.
Matsui et al., 1996 ^a	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Negative	Testing of Southern Blot technique with probe for gpt gene DNA of <i>E. coli</i> . The chromosomal DNA of TM677 and steviol-induced TM677 mutants digested by restriction enzymes and probed. No significant differences found in fragment length between wild-type and mutant DNA.
Matsui et al., 1996 ^a	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Both	Steviol weakly positive in umu test, either with or without metabolic activation. Steviol negative in reverse mutation and other bacterial assays even in presence of S9 activation.
Procinska et al., 1991 ^c	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Negative	The direct mutagenic activity of 15-oxo-steviol was refuted.

	<i>IN VIVO/IN VITRO</i>	SYSTEM	TEST SAMPLE PURITY	AUTHOR CONCLUSION	RESULTS AND REMARKS
Compadre et al., 1988 ^a	<i>In Vitro</i>	Bacterial Mutagenicity, Mass Spec	Not Reported	Positive	Mass spectral analysis of steviol and analogues under conditions known to produce a mutagenic response. 15-oxo-steviol, a product of the metabolite, 15-alpha-hydroxysteviol was found to be direct-acting mutagen. Magnitude of increase over control in assay not discussed.
Pezzuto et al., 1985 ^d	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Positive	Using <i>S. typhimurium</i> TM677 strain, steviol found to be highly mutagenic in presence of 9000 x g supernatant from livers of Aroclor 1254-pretreated rats. This mutagenicity dependent on pretreatment of rats with Aroclor and NADPH addition, as unmetabolized steviol was inactive. None of other metabolites tested was mutagenic. Authors concluded that structural features of requisite importance for the expression of mutagenic activity may include a hydroxy group at position 13 and an unsaturated bond joining the carbon atoms at positions 16 and 17.
Temacharoen et al., 2000 ^c	<i>In Vivo</i>	Micronucleus (rat)	90%	Negative	Very high doses (8 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals.
Temacharoen et al., 2000 ^c	<i>In Vivo</i>	Micronucleus (mouse)	90%	Negative	Very high doses (8 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals.
Matsui et al., 1996 ^a	<i>In Vivo</i>	Micronucleus (mouse)	Not Reported	Negative	Steviol did not increase number of micronuclei observed in this study.
Temacharoen et al., 2000 ^c	<i>In Vivo</i>	Micronucleus (hamster)	90%	Negative	Very high doses (4 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals.

^a Abstract only. ^b As reported in WHO, 2006. ^c As reviewed by Geuns, 2003. ^d Full article.

SUBMISSION END