

GRAS Notice (GRN) No. 461

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

ORIGINAL SUBMISSION



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February 7, 2013

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety (HFS-200)
5100 Paint Branch Parkway
College Park, MD 20740-3835

Attention: Dr. Susan Carlson

Re: GRAS Notification – High Purity Rebaudioside A

Dear Dr. Carlson:

On behalf of Almendra Limited of Bangkok, Thailand, we are submitting for FDA review Form 3667 and the enclosed CD containing a GRAS notification for High Purity 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.

We also wish to advise you that the CD provided for agency review is free of viruses.

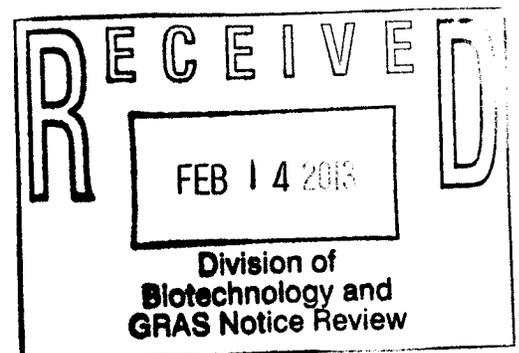
If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via telephone or email.

We look forward to your feedback.

Sincerely,

(b) (6)

Robert S. McQuate, Ph.D.
CEO & Co-Founder
GRAS Associates, LLC
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Enclosure: GRAS Notification for Almendra Limited – High Purity Rebaudioside A



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Enclosure: GRAS Notification for Almendra Limited – High Purity Rebaudioside A

Form Approved: OMB No. ; Expiration Date:
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration
**GENERALLY RECOGNIZED AS SAFE
(GRAS) NOTICE**

Transmit completed form and attachments electronically via the Electronic Submission Gateway (see Instructions); OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety (HFS-200), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission (Check one)

New Amendment to GRN No. _____ Supplement to GRN No. _____

2. All electronic files included in this submission have been checked and found to be virus free. (Check box to verify)

3a. For New Submissions Only: Most recent presubmission meeting (if any) with FDA on the subject substance (yyyy/mm/dd): _____

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? (Check one)
 Yes If yes, enter the date of communication (yyyy/mm/dd): _____
 No

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier	Name of Contact Person Ms. Pnita Chutasmit	Position QA Manager	
	Company (if applicable) Almendra (Thailand) Limited		
	Mailing Address (number and street) All Seasons Places, 23rd Floor, M-Thai Tower, 87 Wireless Road, Lumpini, Phatumwan		
City Bangkok	State or Province <input type="text"/>	Zip Code/Postal Code 10330	Country Thailand
Telephone Number (66) 2-564-7896	Fax Number	E-Mail Address pc@almendra.com.sg	
1b. Agent or Attorney (if applicable)	Name of Contact Person Dr. Robert S. McQuate	Position CEO	
	Company (if applicable) GRAS Associates, LLC		
	Mailing Address (number and street) 20482 Jacklight Lane		
City Bend	State or Province Oregon	Zip Code/Postal Code 97702-3074	Country United States of America
Telephone Number 541-678-5522	Fax Number 541-678-5522 (call first)	E-Mail Address mcquate@gras-associates.com	

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

High Purity Rebaudioside A

2. Submission Format: (Check appropriate box(es))

- Electronic Submission Gateway Electronic files on physical media with paper signature page
 Paper
If applicable give number and type of physical media _____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in FDA's files by reference? (Check one)

- Yes (Proceed to Item 5) No (Proceed to Item 6)

5. The submission incorporates by reference information from a previous submission to FDA as indicated below (Check all that apply)

- a) GRAS Notice No. GRN _____
 b) GRAS Affirmation Petition No. GRP _____
 c) Food Additive Petition No. FAP _____
 d) Food Master File No. FMF _____
 e) Other or Additional (describe or enter information as above) _____

6. Statutory basis for determination of GRAS status (Check one)

- Scientific Procedures (21 CFR 170.30(b)) Experience based on common use in food (21 CFR 170.30(c))

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

- Yes (Proceed to Item 8)
 No (Proceed to Part IV)

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information (Check all that apply)

- Yes, see attached Designation of Confidential Information
 Yes, information is designated at the place where it occurs in the submission
 No

9. Have you attached a redacted copy of some or all of the submission? (Check one)

- Yes, a redacted copy of the complete submission
 Yes, a redacted copy of part(s) of the submission
 No

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance (e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).

Intend to use as table top sweetener and general purpose non-nutritive sweetener for incorporation into foods other than infant formulas and meat and poultry products.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? (Check one)

- Yes No

PART VIII – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Appendix A - Specifications for JT Baker Ethanol	
	Appendix B - Filter Dertification for Sefar Tetex DLW 05-8000-SK 008 Pharma	
	Appencix C - HPLC Analytical Methodology, Methodology Validation & Analytical Report for Rebaudioside A	
	Appendix D - Certificates of Analysis for 5 Production Batches	
	Appendix E - Pesticide Analytical Report	
	Appendix F - Summary of Expert Body Safety Reviews	
	Appendix G - Safety Data on Stevioside & Stevia Extracts That Are Predominantly Stevioside & Rebaudioside A	
	Appendix H - Studies on Principal Metabolite: Steviol	

OMB Statement: Public reporting burden for this collection of information is estimated to average XX hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.) An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.



GRAS ASSESSMENT

HIGH PURITY REBAUDIOSIDE A (≥ 97)

Food Usage Conditions for General Recognition of Safety

for

Almendra (Thailand) Limited
All Seasons Places, 23rd Floor, M-Thai Tower
87 Wireless Road, Lumpini, Phatumwan,
Bangkok, Thailand 10330

Evaluation by

Richard C. Kraska, Ph.D., DABT
Robert S. McQuate, Ph.D.
Robert W. Kapp, Jr., Ph.D., Fellow ATS

February 5, 2013



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I. GRAS EXEMPTION CLAIM

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

Almendra, Ltd. has determined that its high purity rebaudioside A (≥ 97%) product, which is referred to as Reb A ≥97 and which meets the specifications as described in Tables 3-A and 3-B, is Generally Recognized As Safe (GRAS) in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. Almendra, Ltd. made this GRAS determination based on scientific procedures---as described in the following sections---in concert with an appropriately convened panel of experts who are qualified by their scientific training and experience. The GRAS determination accurately reflects the conditions of the stevia-derived sweetener's intended uses in foods.

B. Name Address of Notifier

Almendra (Thailand) Limited
All Seasons Places, 23rd Floor, M-Thai Tower
87 Wireless Road, Lumpini, Phatumwan
Bangkok, Thailand 10330

As the notifier Almendra, Ltd. (Almendra) accepts responsibility for the GRAS determination that has been made for its purified rebaudioside A product² as described in the subject notification; consequently, this rebaudioside A preparation, i.e., having a purity of no less than 97 rebaudioside A, meeting the conditions described herein is exempt from premarket approval requirements for food ingredients.

C. Common Name Identity of Notified Substance

High purity rebaudioside A, commonly abbreviated as reb A or Reb A, is the common name for the notified substance; also see Section III.A.

D. Conditions of Intended Uses in Food

The high purity rebaudioside A 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 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 amount reasonably required to accomplish its intended technical effect.

¹ See 62 FR 18938 (17 April 1997). <http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/ucm083058.htm>.

² Almendra, Ltd. refers to its high purity rebaudioside A product from leaves of *Stevia rebaudiana* Bertoni with the tradename of Reb A ≥97%.

E. Basis for GRAS Determination

Pursuant to 21 CFR 170.30, Almendra's high purity rebaudioside A preparation from the leaves of *Stevia rebaudiana* Bertoni 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.

All communications pertaining to this notification are to be sent to GRAS Associates, LLC who is serving as the authorized agent on behalf of Almendra (Thailand) Limited.

(b) (6)

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

February 5, 2013

II. INTRODUCTION

A. Objective

At the request of Almendra, GRAS Associates, LLC (GA) has undertaken an independent safety evaluation of Almendra's high purity Reb A $\geq 97\%$ preparation. The preparation is composed of high purity rebaudioside A, which is extracted from the leaves of *Stevia rebaudiana* Bertoni and purified to yield rebaudioside A with a purity of $\geq 97\%$. The purpose of the evaluation is to ascertain whether or not the intended food uses of rebaudioside A as a general purpose non-nutritive sweetener as described in Section IV.A are generally recognized as safe, i.e., GRAS.

B. Foreword

Almendra provided GA with background information needed to enable the GRAS assessment to be undertaken. In particular, the information that was 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 its purified rebaudioside A. Almendra 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. Almendra 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 rebaudioside A when consumed as a food ingredient. The composite safety/toxicity studies, 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, Almendra 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 December 27, 2012. A GRAS assessment based 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

Stevia-derived sweeteners have long been 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, Canada and Hong Kong (EFSA, 2010; NutraIngredients, 2010; Health Canada, 2012).

Based on available information from FDA's GRAS Notice Inventory³ website as of January 25, 2013, the agency has written 25 "no questions" letters on GRAS notices on rebaudioside A or steviol glycosides, including those undergoing enzyme treatment. A summary of these filings is presented in Table 1.

³ <http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing>.

Table 1. FDA's GRAS Notice Inventory on Rebaudioside A Steviol Glycosides^{a, b}

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 and 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 and 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. NOW Foods	GRN 337	Enzyme Modified Steviol Glycosides Preparation (EMSGP)	General-purpose sweetener in foods, excluding meat & poultry products, at levels determined by good manufacturing practices
13. GLG Life Tech Ltd	GRN 348	High-Purity Stevioside ≥95%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
14. GLG Life Tech Ltd	GRN 349	High-Purity Steviol Glycosides ≥97%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
15. Guilin Layn Natural Ingredients, Corp.	GRN 354	High-Purity Reb A ≥97%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
16. BrazTek International Inc.	GRN 365	Purified Reb A	General-purpose sweetener, excluding meat & poultry products
17. Sinochem Qingdao Co. Ltd.	GRN 367	High-Purity Steviol Glycosides ≥95%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
18. Shanghai Freeman Americas LLC	GRN 369	Purified Reb A	General-purpose sweetener, excluding meat & poultry products
19. Toyo Sugar Refining Co., Ltd. & Nippon Paper Chemicals Co., Ltd.	GRN 375	Enzyme Modified Steviol Glycosides	General-purpose sweetener in foods, excluding meat and poultry products, at levels determined by good manufacturing practices

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
20. GLG Life Tech Ltd	GRN 380	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
21. Chengdu Wagott Pharmaceutical	GRN 388	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
22. Chengdu Wagott Pharmaceutical	GRN 389	Steviol Glycosides with Stevioside as the Principal Component	General purpose & table top sweetener, excluding meat & poultry products
23. Daepyeong Co., Ltd.	GRN 393	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
24. Daepyeong Co., Ltd.	GRN 395	Steviol Glycosides with Reb A and Stevioside as the Principal Components	General purpose & table top sweetener, excluding meat & poultry products
25. 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). ^b GRN 448, submitted by Daepyeong Co., Ltd regarding glucosylated steviol glycosides, was filed by FDA on November 5, 2012 and is presently under review.

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 of 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 (WHO, 2009), and JECFA subsequently took final action in approving the modified steviol glycosides specifications to include Rebaudioside D and Rebaudioside F (FAO, 2010).

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 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 the 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/kg bw/day, which is similar to JECFA's determination. 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 early 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). 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).

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 is 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).

Lastly, on November 30, 2012, Health Canada published its final clearance for use of steviol glycosides as a sweetener in foods (Health Canada, 2012).

D. FDA Regulatory Framework

In order for their uses in conventional foods, dietary supplements 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

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.

⁴ See 21 CFR 170.3(i)(3).

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

III. CHEMISTRY MANUFACTURE OF ALMENDRA'S HIGH PURITY REBAUDIOSIDE A ($\geq 97\%$)

A. Common or Usual Name

The common or usual name for the product that is the subject of this notification is high purity rebaudioside A, which is derived from the leaves of *Stevia rebaudiana* Bertoni. Rebaudioside A is one of the common steviol glycosides found in nature. The rebaudioside A content of the broadly available commercial products is equal to or higher than 95%. Reb A $\geq 97\%$ is the commercial name used by Almendra 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. Presently, the term, stevia, is used more narrowly to describe the plant or crude extracts of the plant, while reb A---like stevioside---is the common name for another one of the specific glycosides that is extracted from stevia leaves.

B. Description

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 (FCC, 2010). 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).

C. Chemistry of Rebaudioside A

At its 51st meeting, JECFA reviewed the safety related information on steviol glycosides, including the identity and chemistry of these compounds. The following description is taken from the original JECFA monograph (WHO, 2000).

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 and De Oliveira, 1993).

JECFA (FAO, 2007b) identified the sweetener components of stevia and updated the list of common glycosides and their chemical structures (Figure 1) that are slightly different than compounds shown in other older publications (Nanayakkara et al., 1987; Suttajit et al., 1993). The structures of the

components of stevia glycosides were also described in reviews by Kinghorn and Soejarto (1985), Kennelly (2002), and Geuns (2003). Other substances that lack sweetness include the labdane diterpenes, triterpenes, sterols and flavonoid glycosides. Of the eleven different steviol compounds listed in Figure 1, the two principal sweeteners of stevia extracts have been identified as rebaudioside A and stevioside. The chemical identities and key chemical identifiers for these two major components are presented in Table 2.

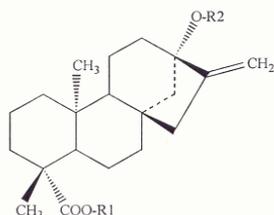
Table 2. Chemical Identity of Rebaudioside A Stevioside

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 Daltons
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 Daltons
CAS Number	57817-89-7

D. Accepted Identity Specifications for Food Grade Steviol Glycosides

In addition to 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). As discussed in Section III.E.1., 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). The component glycosides of particular interest for their sweetening property are rebaudioside A and stevioside.

Figure 1. Chemical Structures of Various Steviol Glycosides^{a, b}



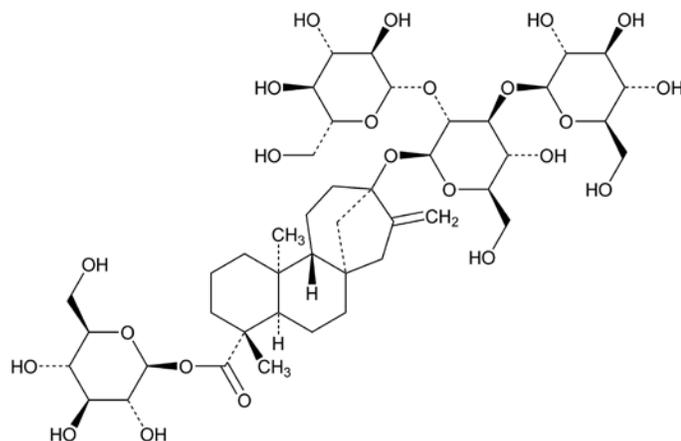
	Compound name	C.A.S. No.	R1	R2
1	Steviol	471-80-7	H	H
2	Steviolbioside	41093-60-1	H	β -Glc- β -Glc(2 \rightarrow 1)
3	Stevioside	57817-89-7	β -Glc	β -Glc- β -Glc(2 \rightarrow 1)
4	Rebaudioside A	58543-16-1	β -Glc	β -Glc- β -Glc(2 \rightarrow 1) β -Glc(3 \rightarrow 1) β -Glc- β -Glc(2 \rightarrow 1)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

The specific chemical structure of rebaudioside A is presented in Figure 2.

Figure 2. Chemical Structure of Rebaudioside A



E. Manufacturing Processes

Based on available scientific and patent literature, several manufacturing processes for steviol glycosides have been reported. These processes are summarized below along with that utilized by Almendra for their high purity Reb A $\geq 97\%$. The manufacturing process for high purity Reb A $\geq 97\%$ is also specifically discussed in Section III.E.2.

1. Scientific Patent Literature

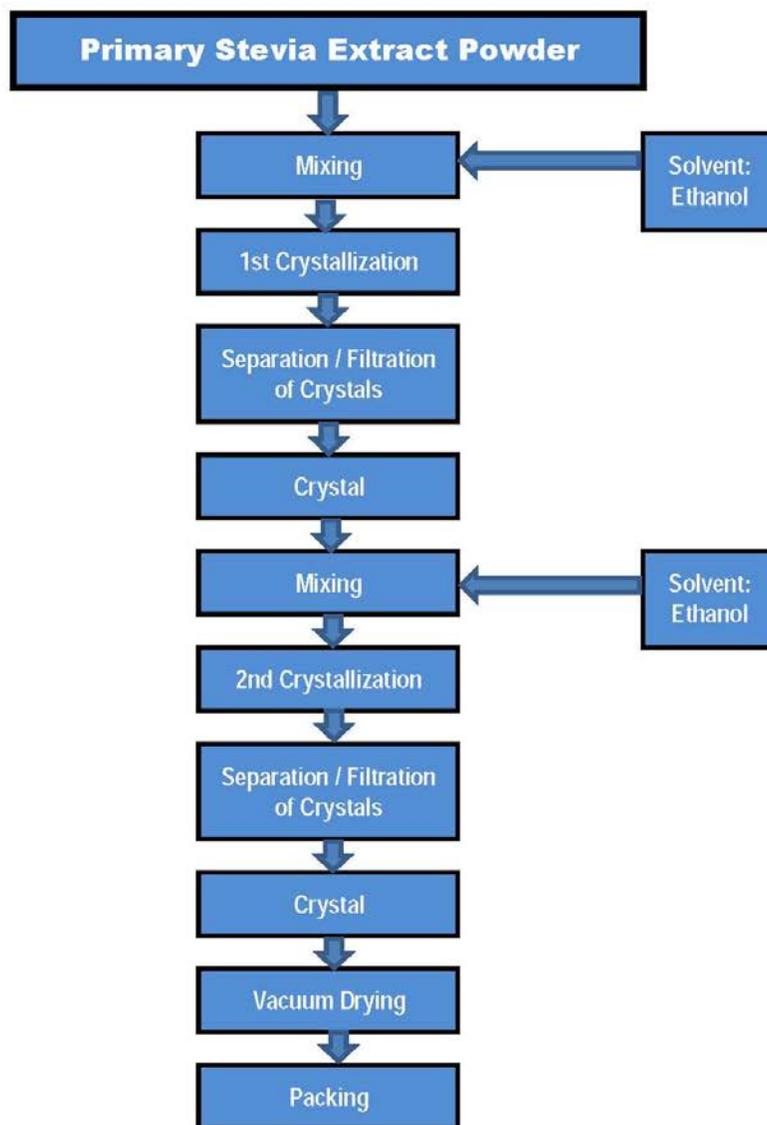
In general, steviol glycosides are obtained by extracting leaves of *Stevia rebaudiana* Bertoni with hot water or alcohols (ethanol or methanol). The extract is a dark particulate solution containing all the key constituents along with leaf pigments, soluble polysaccharides, and other impurities. Some processes remove the "grease" from the leaves with solvents such as chloroform or hexane before extraction occurs (Kinghorn and Soejarto, 1985). There are several extraction patents for the isolation of steviol glycosides. Kinghorn and Soejarto (1985) have 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 the more recent patents, methods using ultrafiltration, metallic ions, supercritical fluid extraction with CO_2 and extract clarification with zeolite are employed.

At the 68th JECFA meeting, steviol glycosides were defined as the products obtained from the leaves of *Stevia rebaudiana* Bertoni. As described by JECFA, the typical manufacture starts with extracting leaves with hot water, and the aqueous extract is passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is washed with 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. Almendra's Manufacturing Process for High Purity Rebaudioside A ($\geq 97\%$)

For the manufacture of high purity Reb A $\geq 97\%$, Almendra employs a fairly typical process that is used in the industry for the production of stevia-derived sweeteners which is prepared in accordance with current Good Manufacturing Practices regulations (cGMP). The source of Almendra's Reb A $\geq 97\%$ preparations is the leaves of the *Stevia rebaudiana* Bertoni plant. In order to extract rebaudioside A from the leaves of stevia, Almendra has developed a state-of-the-art process. High purity Reb A $\geq 97\%$ product is prepared using stevia primary extract which contains an average of 40% rebaudioside A as the starting material. The extract is dissolved in food grade ethanol, mixed and heated. The ethanol used in the purification process meets Ph. Eur. Chemical Specifications (see Appendix A). After cooling the solution is allowed to crystallize. Subsequently, the solution is centrifuged and filtered using a monofilament polypropylene filter - Sefar Tetex DLW 05-8000 series of filters as described in 21 CFR 177 (see Appendix B for the filter specifications). The solid material is re-dissolved in ethanol solution and mixed for re-crystallization. The product undergoes a second crystallization step in ethanol, including centrifugation and filtration. The crystallized precipitate is collected, dried in a vacuum oven and tested for specification compliance. The analytical testing methods, validation and general findings are detailed in Appendices C-1 & C-2. Once the Reb A $\geq 97\%$ product meets specifications, it is packed. The manufacturing process is summarized in a flow chart provided in Figure 3.

Figure 3. Almendra (Thailand) Ltd. Production Process for High Purity Rebaudioside A ($\geq 97\%$)



F. Product Specifications Supporting Methods

1. JECFA Specifications for Steviol Glycosides

The composition of extracts of *Stevia rebaudiana* Bertoni depends upon the composition of the harvested leaves, which are, in turn, influenced by soil, climate, and the manufacturing process itself (FAO, 2007b).

In 2007, JECFA recommended that the method of assay should include a minimum requirement of 95% of the total of 7 specific steviol glycosides on a dried weight basis, and JECFA finalized food grade specifications at the 68th JECFA meeting with publication in the FAO JECFA Monograph 4 (FAO, 2007a). Stevioside and rebaudioside A are the major component glycosides of interest because of their sweetening property. The 5 other associated glycosides found in preparations of steviol glycosides accepted by the JECFA specifications with the 95% requirement are rebaudioside C, dulcoside A, rubusoside, steviolbioside, and rebaudioside B. These, however, are typically found at much lower levels than stevioside or rebaudioside A. JECFA updated the specifications for steviol glycosides in 2008 (FAO, 2008), and then again in 2010 when the specifications were expanded to include the original seven specific steviol glycosides plus Reb D and Reb F (FAO, 2010).

Steviol glycosides are described as a white to yellow powder, odorless to having a slight characteristic odor, and exhibiting a sweetness that is 200-300 times greater than sucrose. The ingredient must consist of a minimum of 95% of 9 specific steviol glycosides. The steviol glycosides are freely soluble in water and ethanol, and the 1 in 100 solutions exhibit pH values between 4.5 - 7.0. The product should not have more than 1% ash with no more than a 6% loss on drying at 105°C for 2 hours. Any residual methanol levels should not exceed 200 ppm, and ethanol residues should not exceed 5000 ppm. Arsenic levels should not exceed 1 ppm as determined by the atomic absorption hydride technique. Lead levels should not exceed 1 ppm.

2. Specifications for Almendra's High Purity Rebaudioside A (≥97%) Supporting Methods

Almendra has adopted product specifications for its purified Reb A ≥97% that meets or exceeds JECFA recommendations while also complying with Food Chemicals Codex (FCC, 2010) specifications for rebaudioside A as a consumable human food substances. The compositions of 5 product batches provided by Almendra are compared to the specifications provided by JECFA and FCC and are presented in Table 3-A and 3-B. Results of analyses performed by Almendra quality control laboratories at the manufacturing site demonstrate that five production batches of high purity Reb A ≥97% meet the required specifications. Details of the individual analyses of the rebaudioside A and the chromatograms for these five batches of high purity Reb A ≥97% are provided in Appendix C-3 and in Appendices D-1 through D-5. A test report for analyses of pesticide residues is included in Appendix E. The collection of these reports demonstrates that the substance is well characterized and meets the purity criteria.

Table 3-A. Specifications for Almendra's High Purity Rebaudioside A (≥97%) Product

PARAMETER	JECFA ^a SPECIFICATIONS STEVIOL GLYCOSIDES	FCC ^b SPECIFICATIONS REBAUDIOSIDE A	ALMENDRA SPECIFICATIONS	RESULTS OF BATCH NUMBERS				
				26092011	1092011	28092011	27092011	29032011
Appearance Form	Powder	Crystal, granule or powder	Powder	Powder	Powder	Powder	Powder	Powder
Appearance Color	White to light Yellow	White to off-white	White to light yellow	White	White	White	White	White
Solubility	Freely soluble in water	Freely soluble in water:ethanol (50:50)	Freely soluble in water	Freely soluble in water	Freely soluble in water	Freely soluble in water	Freely soluble in water	Freely soluble in water
Rebaudioside A (HPLC Area %)	NS	≥ 95	≥ 97	98.31	98.61	98.32	98.37	98.49
Residual Ethanol	NMT 5000 mg/kg	NMT 0.5%	500 ppm max	211.0	486.8	384.0	255.0	360.0
Residual Methanol	NMT 200 mg/kg	NMT 0.02%	200 ppm max	ND	ND	ND	ND	ND
Loss on Drying (%)	NMT 6.0 %	NMT 6.0 %	3.0 % max	2.10	1.46	1.44	2.17	1.92
pH	4.5-7.0	4.5-7.0	4.5-7.0	6.4	6.5	6.0	6.2	6.3
Total Ash (%)	NMT 1%	NMT 1%	0.1% max	0.04	0.04	0.04	0.04	0.04
Arsenic	NMT 1 mg/kg	NMT 1 mg/kg	0.02 ppm max	ND	ND	ND	ND	ND
Lead	NMT 1 mg/kg	NMT 1 mg/kg	0.1 ppm max	ND	ND	ND	ND	ND
Mercury (ppm)	NS	NMT 0.01 mg/kg	0.01 ppm max	ND	ND	ND	ND	ND
Cadmium (ppm)	NS	NMT 0.01 mg/kg	0.01 ppm max	ND	ND	ND	ND	ND

^a Prepared at 69th JECFA (WHO, 2008).

^b FCC, 2010. Rebaudioside A monograph. Food Chemicals Codex (7th Ed.)

Abbreviations: NS = not specified; NA = not applicable; NMT = not more than; ND = not detected.

Table 3-B. Additional Specifications for Almendra's High Purity Rebaudioside A (≥97%) Product

MICROBIOLOGICAL PROPERTIES	JECFA ^a SPECIFICATIONS STEVIOL GLYCOSIDES	FCC ^b SPECIFICATIONS REBAUDIOSIDE A	ALMENDRA SPECIFICATIONS	RESULTS OF BATCH NUMBERS				
				26092011	1092011	28092011	27092011	29032011
Total Plate Count (cfu/g, max)	NA	NA	1000 max	260	150	340	220	120
Yeast (cfu/g, max)	NA	NA	100 max	<10	<10	<10	<10	<10
Mold (cfu/g, max)	NA	NA	100 max	<10	<10	<10	<10	<10
Heat Resistant Mold (in 50 g)	NA	NA	Neg	Neg	Neg	Neg	Neg	Neg
<i>Salmonella spp</i>	NA	NA	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g
<i>Staphylococcus aureus</i>	NA	NA	Neg in 10 g	Neg in 10 g	Neg in 10 g	Neg in 10 g	Neg in 10 g	Neg in 10 g
<i>Alicyclobacillus acidoterrestris</i> (in 50 g)	NA	NA	Neg	Neg	Neg	Neg	Neg	Neg
<i>Listeria</i> (in 1 g)	NA	NA	Neg	Neg	Neg	Neg	Neg	Neg
<i>Coliform/E. coli</i> (in 1 g)	NA	NA	<10	<10	<10	<10	<10	<10
Total Coliforms	NA	NA	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g
Fecal Coliforms	NA	NA	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g

^a Prepared at 69th JECFA (WHO, 2008).

^b FCC, 2010. Rebaudioside A monograph. Food Chemicals Codex (7th Ed.)

Abbreviations: NS = not specified; NA = not applicable; NMT = not more than.

G. Stability Data

The stability of the most common steviol glycosides, rebaudioside A and stevioside has been well studied. Stevioside has been reported to be stable over the pH range 3 - 9 and can be heated at 100°C for 1 hour, but at pH levels greater than 9 under these conditions it rapidly decomposes (Kinghorn and Soejarto, 1985). These investigators also speculated that at pH 10 steviolbioside would be the major decomposition product produced from stevioside by alkaline hydrolysis. In another study, Chang and Cook (1983) investigated the stability of pure stevioside and rebaudioside A in carbonated phosphoric and citric acidified beverages. Some degradation of each sweetening component after 2 months of storage at 37°C was noted. However, no significant change at room temperature or below following 5 months of storage of stevioside and 3 months of storage of rebaudioside A was noted. Exposure to 1 week of sunlight did not affect stevioside but resulted in approximately 20% loss of rebaudioside A. Heating at 60°C for 6 days resulted in 0-6% loss of rebaudioside A.

More recently, Merisant (2008) conducted stability testing on rebaudioside A (1) as a powder, (2) as a pure sweetener in solution, and (3) on both cola-type and citrus carbonated beverages. In these investigations no degradation was detected when the powder was stored at 105°C for 96 hours. It was concluded that the powder was stable when stored for 26 weeks at 40±2°C with relative humidity of

75□5 . Both published and unpublished testing results from Merisant revealed that rebaudioside A in carbonated citric acid beverages and phosphoric acid beverages did not significantly degrade during prolonged storage at refrigeration, normal ambient, or elevated ambient temperatures. Minimal loss of rebaudioside A was detected after storage at 60°C, with considerable degradation noted after 13 hours at 100°C for carbonated beverage solutions and pure sweetener solutions (Merisant, 2008).

Cargill (2008) also conducted extensive stability testing on rebaudioside A as a powder under various storage conditions and under a range of pH and temperatures. Additionally, Cargill also investigated rebaudioside A stability in several representative food matrices at room temperature and elevated temperatures. Stability profiles were created for table top sweetener applications, mock beverages including cola, root beer and lemon-lime, thermally processed beverages, yogurt, and white cake. The results of stability testing revealed some degradation products that had not been detected in bulk rebaudioside A. These degradation products were structurally related to the steviol glycosides that are extracted from the leaves of *Stevia rebaudiana* Bertoni. All the degradation products were found to share the same steviol aglycone backbone structure as found in stevioside and rebaudioside A, but they differ by virtue of the glucose moieties present.

The results of stability testing revealed that rebaudioside A is stable in various food matrices following several days or weeks of storage. The extent and rate of degradation is dependent on pH, temperature, and time. When placed in beverages, rebaudioside A is more stable in the pH range 4 to 6 and at temperatures from 5°C to 25°C (Cargill, 2008).

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

In addition to the above described stability reports for purified rebaudioside A, in a GRAS notification by Sunwin and WILD Flavors (2010) on purified steviol glycosides with rebaudioside A and stevioside as the principal components, stability was investigated using a 0.04 solution of Reb A 80 in acidic solutions between pH 2.81 and 4.18. In this study, the solutions were stored at 32□C for 4 weeks, and the Reb A content was determined at 1, 2 and 4 weeks. Reb A 80 was found to be very stable at pH 3.17 and above. At pH 2.81, after 4 weeks of storage under accelerated conditions only a 7 loss of Reb A was noted. Sunwin and WILD Flavors also studied the stability of Reb A 80 in simulated beverages using 0.1 citric acid (pH 3.2). The solutions were pasteurized and stored for 8 weeks at 4□and 32□C, and little difference in sweetness perception was found under these conditions.

The stability data in the scientific literature for stevioside, the JECFA report, and the extensive stability testing presented by Merisant, Cargill and Sunwin and WILD Flavors, along with Almendra's stability testing results support the position that Almendra's high purity Reb A ≥97% preparation is well-suited for the intended food uses as reported by Almendra.

IV. INTENDED DIETARY USES

A. Intended Food Uses

The subject Almendra high purity Reb A ≥97 preparation with rebaudioside A (≥97%) as the principal component is intended to be used as a table top sweetener and as a 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.⁶ 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 high purity Reb A ≥97 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. Food Uses As Addressed by JECFA Previous GRAS Notifications

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 (see Table 1). As part of its safety deliberations, JECFA reviewed various estimates of possible daily intake of steviol glycosides (WHO, 2006). These estimates are presented in Table 4. Merisant also listed intended use levels of rebaudioside A for various food applications in their GRAS Notification (Table 5). Merisant utilized food consumption survey data from 2003-2004 NHANES to determine the estimated daily intake from the proposed uses of rebaudioside A. On a per user basis, the mean and 90th percentile daily consumption of rebaudioside A were estimated as 2.0 and 4.7 mg/kg bw/day, respectively. In its notification, Cargill (2008) utilized a different approach in estimating dietary intake figures for rebaudioside A when incorporated as a general sweetener in a broad cross-section of processed foods. Cargill considered that with a few minor exceptions rebaudioside A uses and use levels would be comparable to those of aspartame uses in the US. Using post-market surveillance consumption data and published data for consumption of aspartame and other high intensity sweeteners (Renwick, 2008), Cargill performed a side-by-side consumption analysis for rebaudioside A versus aspartame. Findings from the above-described different sources along with FSANZ estimates are further discussed in Section IV.C, and the intake estimates are presented in Table 6.

C. Estimated Daily Intake

The very conservative consumer intake estimates provided by JECFA as shown in Table 4 were utilized to gauge the potential human exposures of steviol glycosides and rebaudioside A in foods as reported in the US and in other countries. Since rebaudioside A is about twice as sweet as the mixed glycosides, these levels can be adjusted accordingly. Almendra intends to use its Reb A ≥97% in a number of food categories at levels that comply with GMP uses. The application of Reb A ≥97 to the same foods and at the same levels as those described in earlier FDA notices by Merisant and Cargill is unlikely to affect the dietary intake of rebaudioside A from introduction into the market by another supplier who will have to compete in essentially the same markets and foods. This also negates the need for a cumulative intake analysis.

⁶ Non-nutritive sweeteners: Substances having less than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.

⁷ See 21 CFR 182.1(b)(1).

Table 4. 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.

Table 5. Proposed Uses Levels of Rebaudioside A by Merisant^a

FOOD GROUP	REBAUDIOSIDE A (PPM)
Table top sweeteners	30,000 ^b
Sweetened ready-to-drink teas	90-450
Fruit juice drinks	150-500
Diet soft drinks	150-500
Energy drinks	150
Flavored water	150
Cereals (oatmeal, cold cereal, cereal bars)	150

^a Merisant, 2008.

^b Reb A content of sachet prior to dilution and not representative of "as consumed."

Further consideration was given to anticipated human exposures as projected independently and with different approaches by JECFA (WHO, 2006), Merisant (2008), and Cargill (2008). As described below, the multiple approaches tended to converge to yield estimated daily intakes (EDIs) in the range of 1.3 □ 4.7 mg/kg bw/day that, when compared to the acceptable daily intake (ADI), constitutes an integral component in the subject GRAS evaluation.

**Table 6. Summary of Estimated Daily Intake Assessments for Rebaudioside A
 Calculation of Rebaudioside A Values from JECFA FSANZ
 Estimates of EDI**

SCENARIOS	EDI		
	AS STEVIOL ^a (MG KG BW DAY)	AS REBAUDIOSIDE A ^b (MG KG BW DAY)	TOTAL DAILY INTAKE ^c (MG DAY)
JECFA			
100 Reb A replacement of sugars	5.0	7.5	450
20-30 Reb A replacement of sugars	1.0 - 1.5	1.5 - 2.3	90 - 140
FSANZ			
100 Reb A replacement of sugars	0.3 - 1.0	0.5 - 1.5	30 - 90
MERISANT			
		2.0 - 4.7 ^d	120 - 282
CARGILL			
		1.3 - 3.4 ^d	78 - 204

- ^a Published values for mixed steviol glycosides consumption listed in this column were used for the calculation of Reb A consumption values appearing in next two columns.
- ^b Estimates for Reb A consumption were calculated from JECFA and FSANZ estimates as steviol by multiplying by 3 to correct for the molecular weight of Reb A compared to steviol and by subsequently dividing by 2 because of the increased inherent sweetness of Reb A compared to the mixed steviol glycosides.
- ^c Total daily intake figures were calculated for a 60 kg adult.
- ^d Published values are shown for comparison purposes.

JECFA evaluated information on exposure to steviol glycosides as submitted by Japan and China. Additional information was available from a report on *Stevia rebaudiana* Bertoni plants and leaves that were prepared for the European Commission by the Scientific Committee on Food. JECFA used the GEMS/Food database to prepare international estimates of exposure to steviol glycosides (as steviol). JECFA assumed that steviol glycosides would replace all dietary sugars at the lowest reported relative sweetness ratio for steviol glycosides and sucrose, which is 200:1. The intakes ranged from 1.3 mg/kg bw/day with the African diet to 3.5 mg/kg bw/day with the European diet. Additionally, JECFA also estimated the per capita exposure derived from disappearance (poundage) data supplied by Japan and China. The Committee evaluated exposures to steviol glycosides by assuming full replacement of all dietary sugars in the diets for Japan and the US. The exposures to steviol glycosides (as steviol) as evaluated or derived by the Committee are summarized in Table 7.

Table 7. Summary of Estimates of Exposure to Steviol Glycosides (as Steviol)

ESTIMATE	EXPOSURE (mg kg BW DAY)
GEMS/Food (International) ^a	1.3--3.5 (for a 60 kg person)
Japan, Per Capita	0.04
Japan, Replacement Estimate ^b	3
US, Replacement Estimate ^b	5

^a WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme.

^b These estimates were prepared in parallel to those for the international estimates; it was assumed that all dietary sugars in diets in Japan and the US would be replaced by steviol glycosides on a sweetness equivalent basis, at a ratio of 200:1.

In its assessment, JECFA concluded that the replacement estimates were highly conservative as the calculated dietary exposure overestimates likely consumption and that true dietary intakes of steviol glycosides (as steviol) would probably be 20 □ 30 of these values or 1.0 - 1.5 mg/kg bw/day on a steviol basis, or 3.0 □ 4.5 mg/kg bw/day for rebaudioside A based on the molecular weight adjustment. Furthermore, by adjusting for the 400-fold increased sweetness of rebaudioside A relative to sucrose compared to the mixed steviol glycosides sweetness factor of 200-fold relative to sucrose assumed by JECFA, the estimated dietary intake of rebaudioside A would likely be about 1.5 to □ 2.3 mg/kg bw/day.

Similar to JECFA, FSANZ (2008) also estimated steviol glycosides dietary intake for adult consumers in New Zealand, assuming a full sugar replacement scenario which resulted in estimated exposures of 0.3 - 1.0 mg/kg bw/day on a steviol basis, or 0.5 □ 1.5 mg/kg bw/day for rebaudioside A when making both the molecular weight and sweetness equivalency calculations. Merisant also calculated a dietary estimate for rebaudioside A of 2.0 mg/kg bw/day for the average consumer of the foods listed in Table 5 and 4.7 mg/kg bw/day for a 90th percentile consumer. In another review conducted on behalf of Cargill and included in their GRAS notification, the intake of rebaudioside A when used as a complete sugar replacement was estimated at 1.3 □ 3.4 mg/kg bw/day when calculated as rebaudioside A (Renwick, 2008). The estimated daily intake assessments have been compiled in Table 6. These different assessments suggest that total daily consumption of rebaudioside A for specified food categories and as a general purpose sweetener is unlikely to exceed 5 mg/kg bw/day, for a total daily dietary exposure of up to 300 mg rebaudioside A for an adult weighing 60 kg. EFSA also calculated the daily intake of steviol glycosides (EFSA, 2010) following the JECFA 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 each.

In October 2009, Cargill applied to FSANZ to increase the maximum usage levels of high purity steviol glycosides in the high volume food categories of ice cream and various beverages. Cargill supported its application with increased usage levels by presenting market share analyses which overestimate actual intake while remaining well below the generally accepted ADI. 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 the Cargill application to increase the allowed 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).

On January 13, 2011, EFSA revised its dietary exposure assessment of steviol glycosides. For high consumers, revised European exposure estimates to steviol glycosides remain above the established acceptable daily intake (ADI) of 4 mg/kg bw (steviol equivalent). For European children (aged 1-14), revised intake estimates ranged from 1.7 to 16.3 mg/kg bw/day; and for adults, the range was from 5.6 to 6.8 mg/kg bw/day (EFSA, 2011a). It should be noted that this estimate is based on European consumption patterns. All other estimates of consumption do not suggest that there is a concern of exceeding the ADI.

There have been many scholarly estimates of potential dietary intake of replacement sweeteners--- including steviol glycosides---that have been published (FSANZ, 2008; Renwick, 2008; WHO, 2003) or submitted to FDA (Merisant, 2008). In GRAS notification 301, a simplified estimate was proposed to and accepted by FDA, based on the estimates of exposure in sucrose equivalents (Renwick, 2008) and the sweetness intensity of any particular sweetener (BioVittoria, 2009). As summarized in GRN 301, the 90th percentile consumer of a sweetener which is 100 times as sweet as sucrose when used as a total sugar replacement would be a maximum of 9.9 mg/kg bw/day for any population subgroup. Using the WHO GEMS/Food database assumption that steviol glycosides in general exhibit a minimum relative sweetness intensity of 200:1 when compared to sucrose, one can assume that high purity rebaudioside A also shows a minimal 200-fold sweetness to that of sucrose (WHO, 2006). This value would apply to Almendra's high purity Reb A ≥97 preparation as well. Therefore, the highest 90th percentile consumption by any population subgroup of Almendra's Reb A ≥97 preparation would be approximately 4.95 mg/kg bw/day. Based on an estimate that Reb A preparations consist of approximately 32.8 steviol equivalents,⁸ the consumption would be less than 1.63 mg/kg bw/day on a steviol equivalents basis for any population group. These calculations are summarized in Table 8.

**Table 8. Daily Intake of Sweeteners (In Sucrose Equivalents)
 Estimated Daily Intakes of Rebaudioside A**

POPULATION GROUP	INTAKES OF SWEETENERS (MG SUCROSE/KG BW/DAY) ^A		INTAKE OF REB A (MG/KG BW/DAY) ^B		INTAKE OF REB A AS STEVIOL EQUIVALENTS ^C	
	LOW	HIGH	LOW	HIGH	LOW	HIGH
HEALTHY POPULATION	255	675	1.28	3.38	0.42	1.11
DIABETIC ADULTS	280	897	1.40	4.49	0.46	1.47
HEALTHY CHILDREN	425	990	2.13	4.95	0.70	1.63
DIABETIC CHILDREN	672	908	3.36	4.54	1.10	1.49

^a Source: Renwick (2008)

^b Calculated by dividing the sucrose intake by the average relative sweetness value of 200 for reb A.

^c Calculated based on the ratio of molecular weights of rebaudioside A and steviol.

The extent that stevia-based sweeteners will penetrate the US food supply and the extent the market will select mixed steviol glycosides products versus reb A products remain uncertain. Furthermore, many competing non-caloric sweeteners are currently available to consumers, which have been successful in the marketplace, most notably aspartame and sucralose.

⁸ Calculated by Expert Panel by as percent of molecular weight of steviol to molecular weight of rebaudioside A.

Based on the totality of dietary intake considerations presented above, the intake estimates are viewed as being conservative. When comparing these EDI assessments for steviol glycosides, we see that total daily consumption of the steviol glycosides and reb A for defined food uses and as a general purpose sweetener is expected to be substantially less than the acceptable daily intake values discussed at length in Section VI.C.

D. Other Information on Human Exposure to Stevia: Use as Food Ingredient Other Uses

For about 20 years, consumers in Japan and Brazil, where stevia has long been approved as a food additive, have been using stevia extracts as non-caloric sweeteners.⁹ It was previously reported that 40% of the artificial sweetener market in Japan is stevia based and that stevia is commonly used in processed foods in Japan (Lester, 1999). Although there are no reported uses of rebaudioside A as a dietary supplement, use of steviol glycosides as a dietary supplement is presently permitted in the US, Australia, and New Zealand and as a natural health product in Canada. It has wide use in China and Japan in food and in dietary supplements. In 2005, it was estimated that sales of stevia in the US reached \$45 million (The Food Institute Report, 2006). More recent reports of consumption figures for stevia reveal pronounced increases in global consumption. Worldwide, Zenith International estimates stevia sales of 3500 metric tons in 2010 which represents a 27% increase over 2009 figures. The market value is estimated to have increased to \$285 million (Zenith, 2011).

Hawke (2003) reported that stevia is commonly used as a treatment for type 2 diabetes in South America. However, for its therapeutic effects, elevated doses in the range of 1 g/person/day or more were reported to be necessary (Gregersen et al., 2004).

V. SAFETY DATA FOR REBAUDIOSIDE A

A. Summary of Safety Data Safety Reviews on Steviol Glycosides

Almendra's high purity Reb A (≥97%) contains rebaudioside A as its major component. Given the structural similarity among rebaudioside A, stevioside and other steviol glycosides, along with metabolic considerations, the scientific data on these other components are relevant to the present safety assessment for Reb A. This is further supported by the fact that EFSA (2010) considers 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.

Stevia and steviol glycosides have been extensively investigated for their biological, toxicological, and clinical effects (Carakostas et al., 2008; Geuns, 2003; Huxtable, 2002). Additionally, the national and international regulatory agencies have thoroughly reviewed the safety of stevia and its glycosides. Most notably, over the years JECFA has evaluated stevia and steviol glycosides multiple times (WHO,

⁹ See Raintree Nutrition Tropical Plant Database. <http://www.rain-tree.com/stevia.htm>.

2000, 2006, 2007, 2008), and this has been summarized in Section II.C. Recently 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.

From the safety perspective, some of the earliest studies on steviol glycosides were of limited value as the actual compositions of materials investigated and their questionable purities undermined drawing firm toxicological conclusions. These early studies reported a decrease in fertility with crude stevia preparations and increased mutagenic activity of the principle metabolite, steviol. Based on these and other questions raised about safety by studies with materials of lesser purity and by studies with unusual protocols in *in vivo* and in *in vitro* systems usually employing high doses or high concentrations of test materials, FDA was reluctant to authorize the use of stevia. These concerns included renal toxicity, effects on glucose metabolism, and inhibition of mitochondrial enzymes. Over the last decade and a half, the safety of steviol glycosides and rebaudioside A in particular have been extensively investigated by employing comprehensive and modern toxicology protocols using scientifically accepted dosing regimens of purified and standardized test substances.

Much of the supporting evidence with respect to the safety of rebaudioside A is derived from safety studies on purified steviol glycosides which were largely composed of mixtures that were predominately stevioside and Reb A. These studies include a complete battery of toxicology and clinical studies on steviol glycosides and evidence of poor GI absorption (Gardana et al., 2003; Geuns and Pietta, 2004; Koyama et al., 2003) of 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 (Koyama et al., 2003b, Renwick and Tarka, 2008). Additional 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. 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 structural similarity, it is reasonable to expect that Reb A is not absorbed in the GI tract but is similarly converted to steviol by the normal flora of the lower GI tract.

Based on the presumption that rebaudioside A is not appreciably absorbed from the GI tract and it is similarly converted to steviol by intestinal flora, the safety review of purified rebaudioside A can be further supported by the large body of published evidence supporting the safety of purified steviol glycosides extracts. 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 thresholds 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 metabolisms 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). 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. JECFA (WHO, 2008), after several years of review, established an ADI of 4 mg/kg bw expressed as steviol equivalents, based on studies on test samples of steviol glycosides with a minimum purity of 95% expressed on a dry weight basis. The critical studies leading to this decision included a chronic study in rats (Toyoda, et al. 1997) which indicated a lack of effects or carcinogenic activity at the highest doses, along with a series of clinical studies conducted at 11 mg/kg bw which ruled out effects on blood glucose and blood pressure.

Given the chemical similarity of rebaudioside A and stevioside, the results of toxicology studies on stevioside and stevia extracts can be used to support the safety assessment of Reb A.

Since the JECFA evaluation (WHO, 2008), more than two dozen GRAS notifications for steviol glycosides or enzyme modified steviol glycosides have been submitted to FDA since 2008, all of which were determined to be GRAS based largely on the ADI established by JECFA, and all have had "no questions" letters of response from FDA (see Table 1).

More detailed reviews on safety of steviol glycosides by expert bodies such as JECFA, FSANZ and EFSA are summarized in Appendix F and more detailed reviews on the safety data on stevioside and stevia extracts and steviol are summarized in Appendices G and H.

B. Safety Studies on Rebaudioside A¹⁰

Since 2008, several well-designed toxicology studies that followed the current regulatory and scientific 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. 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

Studies investigating the ADME of extracts from stevia are available on stevioside, Reb A and other steviol glycosides. Data evaluating the absorption and fate of these extracts from various animal species and humans indicate that one can extrapolate these results from rats to humans. Stevioside is metabolized to steviol *via* intestinal microflora, and the absorption of stevioside after oral administration has been shown to be very low (Koyama et al., 2003a, Geuns et al., 2003).

Studies investigating the hydrolysis of steviol glycosides by intestinal microflora have demonstrated that both stevioside and Reb A are hydrolyzed to steviol following *in vitro* incubation with various cecal microflora (Wingard et al., 1980; Hutapea et al., 1997; Gardana et al., 2003; Geuns et al., 2003). In addition, the *in vitro* hydrolysis of Reb A to steviol was found to be slower than that of stevioside (Koyama et al., 2003a) which is thought to be partly due to the presence of one additional glucose moiety and to differences in structural complexities. Koyama et al (2003a) suggest that the major pathway for Reb A is conversion to stevioside with a minor pathway of conversion to Reb B prior to being ultimately converted to steviol. Stevioside is further converted to steviolbioside, steviolmonosides and finally steviol, with glucose being released with each subsequent hydrolysis.

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

¹⁰ Questions about the safety of rebaudioside A were previously raised by Huxtable (2002) and Kobylewski and Eckhart (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. This matter is discussed by the Expert Panel in Section VI.C.

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 radiolabeled compounds were extensively and rapidly absorbed with plasma concentration-time profiles following similar patterns for stevioside and rebaudioside A.

Roberts and Renwick (2008) identified free steviol (82 to 86 %), steviol, glucuronide (10 to 12 %) and two unidentified metabolites (5-6 %) in rat plasma following treatment with either stevioside or Reb A eight hours post-oral administration. A comparable pharmacokinetic profile was noted following oral treatment of rats with radiolabeled Reb A or stevioside with the time of maximum plasma concentration (T_{max}) for radioactivity ranging between 2 and 8 hours. In comparison, steviol T_{max} for plasma was noted within 30 minutes of oral administration. 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 unidentified metabolites. It is believed that this delay between the occurrence of radioactivity in the plasma and time of administration of steviol glycosides is due to the fact that the Reb A and stevioside are first cleaved to steviol before absorption. Within 72 hours of administration, elimination of radioactivity from plasma was essentially complete. Following elimination in the bile, steviol is available to be released again from its conjugated form by microflora activity and may enter enterohepatic circulation. Consequently, free and conjugated steviol are secreted in the feces along with any unhydrolyzed fraction of the administered glycosides. Following Reb A treatment, significant amounts of unchanged rebaudioside A (29 % in males and 19 % in females) and stevioside (3 % in males and 4 % in females) were excreted in the feces. Following oral stevioside administration, unchanged stevioside was excreted in rat feces. Other unidentified metabolites are also present in fecal samples of rats treated with either glycoside. Rebaudioside A, stevioside, and steviol were metabolized and excreted rapidly, with ≈ 60 % 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 (Roberts and Renwick, 2008).

In summary, Roberts and Renwick (2008) found that steviol was the predominant component found in plasma samples after oral administration of Reb A, stevioside and steviol in rats. Lower amounts of steviol glucuronide(s) and one or two unidentified metabolites were also found. The majority of all samples were found to be excreted rapidly---primarily in the feces---within 48 hours. This is in agreement with the previous *in vitro* hydrolysis data which indicated that both Reb A and stevioside are metabolized to steviol by intestinal microflora. The predominant compound detected in the bile was steviol glucuronide while the prominent material in the intestine is steviol, which the authors suggest indicates that deconjugation occurs 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 with 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. 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 the absence of reporting of adverse events, laboratory assessments of safety, or vital signs (Wheeler et al., 2008).

Another pharmacokinetic investigation was conducted 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 dose 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 doses 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 ≈ 84%.

2. Subchronic Toxicity Studies

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 (9938 and 11,728 mg/kg bw/day for males and females, respectively) for 4 weeks or 50,000 ppm (4161 and 4645 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 4161 and 4645 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 2000 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 ≥2000 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 2000 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 2000 mg/kg bw/day and the assigned NOAEL was ≥ 2000 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 9.

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

Table 9. 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.

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 2048 to 2273 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 [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 estrus 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/person/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/person/day 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 glycosylated hemoglobin levels did not differ significantly between the rebaudioside A (0.11 ± 0.06 , mean \pm standard error) and placebo (0.09 ± 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 C-peptide (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/person/day rebaudioside A does not alter glucose homeostasis or blood pressure in individuals with type 2 diabetes mellitus.

VI. GRAS CRITERIA REVIEWED INFORMATION

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."¹¹

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:

"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."¹²

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:¹³

- 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).

¹¹ See 21 CFR 170.3(i).

¹² See 21 CFR 170.30(a).

¹³ See 62 FR 18938 (17 April 1997) available at <http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/ucm083058.htm>.

As noted below, the safety assessment to ascertain GRAS status for rebaudioside A with the defined food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

B. Expert Safety Reviews of Steviol Glycosides

Because of their sweetness characteristics, steviol glycosides have viable uses as a non-nutritive sweetener in foods.¹⁴ Periodic reviews by JECFA over the years indicate the progress of knowledge on the toxicology of steviol glycosides. Several early safety-related studies on these compounds were performed on crude extracts of stevia. These studies also included multiple investigations with *in vivo* and *in vitro* models which explored the biological activity of stevia extracts at high doses or high concentrations. These early investigations raised several concerns, including impairment of fertility, renal effects, interference with glucose metabolism, and inhibition of mitochondrial enzymes. In recent years as more and more studies were performed on purified glycosides, the toxicology profile of steviol glycosides eventually proved to be rather unremarkable. A number of subchronic, chronic and reproductive studies have been conducted in laboratory animals. These studies were well designed with appropriate dosing regimens and adequate numbers of animals to maximize the probability of detection of important effects. Notably, the initially reported concerns related to the effects of stevia leaves or crude extracts on fertility were refuted by the well-designed reproductive studies with purified steviol glycosides. All other concerns failed to manifest themselves at the doses employed in the long-term rat studies.

As discussed in Section V, at its fifty-first meeting, JECFA determined that there were adequate chronic studies in rats, particularly the study by Toyoda et al. (1997), to establish a temporary ADI of 0 - 2 mg/kg bw/day with an adequate margin of safety. The committee also critically reviewed the lack of carcinogenic response in well-conducted studies. These studies justified the Committee conclusion that the *in vitro* mutagenic activity of steviol did not present a risk of carcinogenic effects *in vivo* and, therefore, all common steviol glycosides which likely share the same basic metabolic and excretory pathway and that the use of high purity preparations of various steviol glycosides are safe to use as a sugar substitute. Subsequently, the additional clinical data reviewed by JECFA allowed the Committee to establish a permanent ADI of 0 - 4 mg/kg bw/day (based on steviol equivalents). The GRAS Expert Panel critically reviewed the JECFA assessment and agrees with the calculation of the ADI for steviol glycosides.

The Panel has reviewed the findings from human clinical studies. The Panel noted that, regarding the clinical effects noted in humans, in order to corroborate the observations in these studies that these effects of steviol glycosides only occur in patients with either elevated blood glucose or blood pressure (or both), JECFA called for studies in individuals that are neither hypertensive nor diabetic (WHO, 2006). The supplemental data presented to JECFA and also published by Barriocanal et al. (2008) demonstrate the lack of pharmacological effects of steviol glycosides at 11 mg/kg bw/day in normal

¹⁴ It has also been reported that steviol glycosides may have pharmacological properties, which can be used to treat certain disease conditions such as hypertension and type 2 diabetes. Chatsudhipong and Muanprasat (2009) published a comprehensive review where they note that such therapeutic applications have not been firmly established as being due to steviol glycosides. The reviewers point out that the effects occur at higher doses than would be used for sweetening purposes. Furthermore, many effects noted in older studies may have been due to impurities in preparations that do not meet the contemporary purity specifications established by JECFA for use as a sweetener. If oral doses of steviol glycosides impart pharmacological effects, such effects would undoubtedly occur due to actions of the principle metabolite, steviol, but the pharmacological effects of steviol have not been comprehensively investigated.

individuals or approximately slightly more than 4 mg/kg bw on the basis of steviol equivalents. It is possible that JECFA may also have reviewed the preliminary results associated with the published clinical studies on rebaudioside A (Maki et al., 2008a, b). The Panel concludes that there will be no effects on blood pressure and glucose metabolism in humans at the doses of rebaudioside A expected from its use in food as a non-nutritive sweetener.

JECFA's review also included anticipated dietary patterns and the use concentrations expected in various foods in order to calculate an estimated daily intake (EDI) (WHO, 2003, 2006). Based on the assumption of 100% substitution of steviol glycosides for sucrose, an EDI of 5 mg/kg bw/day of steviol was calculated for US consumption. JECFA noted that the replacement estimates were highly conservative and that this calculated intake of steviol glycosides (as steviol) would more likely be 20%–30% of these values. Except for the scenario developed by JECFA with 100% replacement of sugars by steviol glycosides, and as discussed in Section IV.C and summarized in Table 6, the highest dietary estimate for use in foods for rebaudioside A is 4.7 mg/kg bw/day. The Panel agrees with the JECFA ADI of 4 mg/kg bw/day based on steviol equivalents which corresponds to 12 mg/kg bw/day for rebaudioside A and notes that the estimates as contained in Table 7 of anticipated dietary intake are below the ADI.

C. Safety of Rebaudioside A

Since July 2008, over ten papers describing the results of a comprehensive research program by different groups on rebaudioside A have been published. These and some other unpublished studies formed the basis of the two initial GRAS notifications to FDA by Cargill (GRN 253) and Merisant (GRN 252). Prior to this, a limited number of toxicology studies specifically on rebaudioside A were conducted. Even before these new studies were completed and as noted in the previous section, JECFA concluded that 7 (which was later expanded to 9) common steviol glycosides are safe for use as sweetener preparations when present in any combination as long as the combined purity of 95% or more was established.

Since a majority of the previous pharmacokinetic research was conducted with steviol glycosides, the presumed strategy adopted for the more recent research on rebaudioside A was to conduct a limited number of well-designed and executed toxicology studies on rebaudioside A itself and to demonstrate in rats and in humans that it is handled pharmacokinetically similarly to stevioside. This approach appears to have been undertaken to justify the JECFA-generated ADI without having to conduct a chronic study in rats with rebaudioside A. Additionally, the Merisant group conducted three mutagenicity assays on rebaudioside A that FDA generally considers to be most predictive for carcinogenicity potential. The Cargill group conducted two clinical studies to assure that rebaudioside A does not have potentially problematic pharmacological effects on blood glucose and blood pressure.

In a review article, Carakostas et al. (2008) summarized the most recent research on rebaudioside A. This review summarized the findings of the Cargill research program as follows:

- 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.
- While studies with Reb A indicated slight GI absorption of the glycoside *per se*, the predominant metabolic pathway is comparable to that of stevioside and the use of the ADI established by JECFA that was determined on studies employing stevioside as the main component can be used 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.

The Expert Panel concurs that the consumption estimates described by JECFA, Renwick (2008) and the GRN 252 and GRN 253 Expert Panels that very conservatively represent a potential high user of rebaudioside A if this non-nutritive sweetener becomes widely available in food.

Regarding the available aggregate safety information, the Panel has concluded that JECFA has critically and extensively evaluated the use of steviol glycosides in foods and agrees that, at the present time, the ADI for steviol glycosides of adequate purity as defined by JECFA specifications has been properly determined to be 4 mg/kg bw/person as steviol equivalents, which corresponds to 12 mg/kg bw/day for rebaudioside A on a dry weight basis. The Panel agrees that unwanted pharmacological effects are not likely to occur at this level and moreover that high consumers of rebaudioside A are not likely to exceed this level. Therefore, the Panel adopts the JECFA-derived ADI as a safe exposure for rebaudioside A and that food uses meeting the specifications within the limits determined by this esteemed international body of food safety experts can be considered to be generally recognized as safe (GRAS).

The Panel recognizes that JECFA is composed of dozens of scientists that are internationally known experts on food ingredient safety that have established ADIs for food ingredients over the last 40 years. Both Merisant and Cargill took rather rigorous scientific approaches to demonstrate the safety of rebaudioside A. The studies were equally well conducted. The safety profiles compiled by Merisant and Cargill differ somewhat, yet the results are complementary and are mutually reinforcing of rebaudioside A safety.

The studies conducted by Cargill provided significant insight into the pharmacokinetics of rebaudioside A while demonstrating clinical safety of rebaudioside A regarding lack of effects on blood pressure and glucose metabolism that could result from doses expected from use in food. The Merisant notification augmented genotoxicity data in three systems recognized by FDA as good predictors of carcinogenic potential. Two of these assays were conducted in mouse systems. Additional mutagenicity and genotoxicity studies have been published on rebaudioside A (Williams and Burdock, 2009). Merisant added a subchronic study in dogs and a teratology study in rats. Both Cargill and Merisant relied on the JECFA ADI for steviol glycosides as determined largely by published chronic studies in rats. Both groups justified the use of the ADI on pharmacokinetic arguments showing the similarity of stevioside and rebaudioside A metabolism and excretion.

The Expert Panel discussed findings from a recently published exploratory subchronic toxicity study in rats by Awney et al. (2011), where a number of toxicological effects of stevioside treatment were reported. This study is summarized in Appendix G. Critical reviews of the publication by Carakostas (2012) and Waddell (2011) revealed a poor study design that included 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. Additionally, the report did not adequately describe mean or individual organ weight data and lacked comparison of study findings against laboratory historical control data. In contrast to the data presented by Awney et al. (2011), several well-designed and well-conducted subchronic toxicity studies did not reveal any adverse effects from rebaudioside A consumption.

The Panel also noted from a study that DNA damage was seen in a variety of organs as assessed by comet assay in rats given drinking water containing 4 mg/mL steviol glycosides for up to 45 days (Nunes et al., 2007a). This study is summarized in Appendix G. 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, along with 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.

In a recent review, (Urban et al., 2013) examined the extensive genotoxicity database on steviol glycosides because some concern has been expressed in 2 recent publications (Brahmachari et al. (2011) and Tandel, 2011) in which they concluded that additional testing is necessary to adequately address the genotoxicity profile. This review aimed to address this matter by evaluating the specific genotoxicity studies that are of concern while evaluating the adequacy of the database that includes more recent genotoxicity data not noted in those publications. The results of this literature review showed that the current database of *in vitro* and *in vivo* studies for steviol glycosides is robust and does not indicate that either stevioside or rebaudioside are genotoxic. This finding, combined with a paucity of evidence for neoplasm development in rat bioassays, establishes the safety of all steviol glycosides with respect to their genotoxic/carcinogenic potential.

In summary, the Expert Panel agrees with the safety conclusions of the 25 GRAS Expert Panels in the notifications previously submitted to FDA that resulted in "no questions" responses from FDA (as summarized in Table 1), JECFA (WHO, 2006; WHO, 2008) and Renwick (2008) 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 sucrose on a sweetness equivalency basis, meets FDA's definition of safe. In addition, the Panel has compared the specifications of Almendra's high purity Reb A ($\geq 97\%$) to the composition of the test materials used in all the published studies. The Panel agrees that the Almendra high purity Reb A product is sufficiently similar to those used in all key studies reviewed by JECFA and those on rebaudioside A subsequently reviewed by FDA, and there is no need for further studies to be conducted on the Almendra product. The Panel also has reviewed the expected levels of dietary intake and agrees that there is sufficient information to conclude that the subject Almendra product can be safely used as a table top sweetener and as a general purpose non-nutritive sweetener in various foods other than infant formulas and meat and poultry products.

D. Common Knowledge Elements of 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 published, peer-reviewed scientific journals. The majority of studies reviewed as part of this safety assessment 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 only reviewed a limited number of studies conducted specifically on rebaudioside A. The collection of supporting data on rebaudioside A has recently been enhanced by a series of studies published during 2008 and cited earlier. The clinical studies that address JECFA's concern on unwanted pharmacological effects with steviol glycosides (Barriocanal et al., 2008) and with rebaudioside A (Maki et al., 2008 a, b) are also published in the peer-reviewed scientific literature.

The Panel recognizes that the safety of steviol glycosides in human foods has been the subject of interest for many years. In addition to the reported substantial history of consumption of stevia, especially in South America and Asia, many scientific studies have been conducted and published. Some of the earlier studies have raised concerns about the safety, and the Expert Panel has given careful attention to such concerns. The overriding evidence has diminished the Panel's concerns based on better study designs, better execution, or simply updated investigations that better reflect state-of-the art toxicological principles and findings.

The remaining common knowledge element for a GRAS determination is that there must be a basis to conclude that there is consensus among qualified scientists about the safety of the substance with its intended use. The JECFA opinion largely meets the common knowledge test on its own. The Panel is cognizant of the scientific rigor and broad base of scientific expertise that resides with the prestigious JECFA. JECFA 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 participated in the JECFA deliberations.

The JECFA conclusion has been reviewed and validated by other respected regulatory agencies including FSANZ, the Switzerland Federal Office of Public Health, France's Agence Francais De Securite Sanitaire Des Alimenta and most recently Health Canada (FSANZ, 2008; Switzerland Federal Office of Public Health, 2008; AFSSA, 2009, Health Canada, 2012). Furthermore, the favorable scientific opinion on the safety of steviol glycosides use as a sweetener in foods as issued by EFSA in 2010 reinforces the safety determinations of many other qualified organizations (EFSA, 2010). In addition, a number of individual 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 common knowledge element has been embellished by the many respected scientists that participated in the Cargill-sponsored research conducted on rebaudioside A, most notably Brusick (2008) and Renwick (2008). An assertion of "general recognition of safety" was made by Carakostas et al. (2008). The authors of a recent review of the genetic toxicology database of steviol glycosides concluded that the available data "establish the safety of all steviol glycosides with respect to their genotoxic/carcinogenic potential" (Urban et al., 2013). We also note that, since December 2008, more than two dozen GRAS notifications have been submitted to FDA for stevia-derived sweetener products, and FDA's detailed reviews have yielded "no questions" letters in each case.

In summary, there are many diverse groups of scientists from all corners of the globe that together provide strong fulfillment of the consensus requirement. Of particular significance from the perspective of establishing consensus for the safety of high purity steviol glycosides are the 25 GRAS notifications with "no questions" determinations by FDA since 2008 (see Table 1). While the scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, the Expert Panel believes

that a wide consensus does exist in the scientific community to support the GRAS conclusion on high purity rebaudioside A as outlined in this notification. The broader scientific community has concluded that past concerns expressed by others over the years (Huxtable, 2002) and earlier safety issues noted by FDA have been resolved by newer data on more purified test materials and the rigid specifications for purity published by JECFA for steviol glycosides, including rebaudioside A. Indeed, scientists from FDA are members of JECFA and have not objected to the safety decision on steviol glycosides. There is also a wider consensus that the body of new research on rebaudioside A is sufficient as opposed to the small group of scientists that argue that more studies need to be done before the sweetener is made available in the US.

VII. CONCLUSIONS¹⁵

Almendra's high purity rebaudioside A (≥ 97%), also referred to as Reb A ≥97%, as expressed on a dry weight basis, is Generally Recognized As Safe when consumed as a general purpose non-nutritive sweetener in foods other than infant formulas and meat and poultry products when: (1) it is produced in accordance with FDA Good Manufacturing Practices requirements; (2) it meets or exceeds the JECFA purity specifications for steviol glycosides as identified in Tables 3-A and 3-B; and (3) it is consumed within the designated JECFA ADI of 12 mg kg bw day on a rebaudioside A basis. 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 shall 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., Fellow ATS

February 5, 2013

¹⁵ The detailed educational and professional credentials for 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 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.

VIII. REFERENCES

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APPENDIX A

Specifications for JT Baker Ethanol



Laboratory chemicals and products - Clinical chemistry and hematology products - Fine & industrial chemicals

Specification Sheet

Product No 2606
ETHANOL, ABSOLUTE, PH EUR
Absolute
'BAKER'
Date November 2010

Meets Ph. Eur. Chemical Specifications.

Test	Specification
Assay (C_2H_5OH)	min. 99.5 % (w/w)
Acidity or Alkalinity (as CH_3COOH)	max. 30 ppm
Appearance	passes test
Identification	passes test
Relative Density	0.790 - 0.793
Residue on Evaporation	max. 25 ppm
<i>Absorbance:</i>	
at 240 nm	max. 0.40
at 250-260 nm	max. 0.30
at 270-340 nm	max. 0.10
<i>Volatile Impurities (by GC):</i>	
Acetaldehyde + Acetal	max. 10 ppm (w/v)
Benzene	max. 2 ppm (w/v)
Methanol (CH_3OH)	max. 200 ppm (w/v)
Total of Other Impurities (based on surface area internal standard 4-Methylpentan-2-ol)	max. 300 ppm

For manufacturing, processing or repacking.

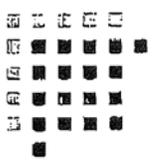
The information in this document is property of Mallinckrodt Baker B.V.
Mallinckrodt Baker B.V.
Teugeweg 20
7418 AM Deventer
The Netherlands
Tel +31 570687500

APPENDIX B

Filter Certification for Sefar Tetex DLW 05-8000-SK 008 Pharma

15/02 '05 DI 10:11 FAX +49 8071 40787 SEFAR GMBH
15.02.2005 09:42 SEFAR AG → SEFAR DE

NR. 999 001
001



SEFAR

KONFORMITÄTSERKLÄRUNG

Registratur Nr: 6796 05 02 518

Kunde : Sefar GmbH / Krauss-Maffei
 Auftragsnummer Kunde : Anfrage
 Auftragsnummer Sefar : Anfrage
 Gewebe aus : E-CTFE
 Verkaufsnummer : SEFR TETEX DLW 08-8000-K 040
 Artikel-Nummer : 3B08-0480-255-00
 Datum : 14.02.2005

Wir bestätigen hiermit, dass das eingangs erwähnte Gewebe aus Materialien gefertigt wurde, die den Anforderungen gemäss

*Directive 90/128/EEC of February 23, 1990 of the European Community
„Directive on plastic materials and articles intended to come into contact with Foodstuffs“*

entsprechen.

Der vorliegende Unbedenklichkeitsnachweis ist nur dann gültig, wenn die sogenannte „Spinnpräparation“ (Substanzen die zur Stabilisierung beim Webprozess benötigt werden), komplett ausgewaschen sind.

Der vorliegende Unbedenklichkeitsnachweis beinhaltet kein Zubehör, das für die Produktion von konfektionierten Filter- und/oder Siebprodukten benötigt wird.

W. Galiart
(b) (6)

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Quality System ISO 9001

/

**SEFAR TETEX DLW
 05-8000-SK 008 PHARMA**

Technical Data Sheet
 Technisches Datenblatt
 Fiche technique

Standard
 Norm
 Norme

Fibre Material Fasermaterial Matière	Polypropylene, monofilament	
Polymer conformity	FDA CFR title 21 part 177 And Directive 90/128/EEC 23.2.1990	
Extractables	< 1000 mg/m ²	Extraction at total reflux Solvent: Ethanol 50%
Weave Pattern Bindung Armure	Double layer weave Doppellagengewebe Tissu double chaîne	
Finish Ausrüstung Traitement	Calendered Kalandriert Calandré	
Thickness [µm] Dicke [µm] Epaisseur [µm]	760	DIN 53 855
Weight [g/m ²] / [oz/sq.yd] Gewicht [g/m ²] / [oz/sq.yd] Poids [g/m ²] / [oz/sq.yd]	420 / 12.39	DIN 53 854
Air Permeability @ 196 Pa Luftdurchlässigkeit @ 196 Pa Perméabilité à l'air @ 196 Pa Air Permeability @ ½ inch H ₂ O	24 40 144 5.2	+/- 6 l/dm ² /min +/- 10 l/m ² /sec +/- 36 m ³ /m ² /h +/- 1.3 cfm DIN 53 887
Nominal Pore Size [µm] Nominale Porengröße [µm] Dimension des pores nominal [µm]	8	Boil over bubble point test method ASTM F-316-86

All stated values are arithmetic means of samples (\bar{x}).
 Alle Angaben sind Stichproben-Mittelwerte (\bar{x})
 Les données indiquées sont des valeurs moyennes.

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01.03.2007/05-8000-SK 008 PHARMA.doc/pl

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SEFAR TETEX DLW
05-8000-SK 012 PHARMA

Technical Data Sheet
 Technisches Datenblatt
 Fiche technique

Standard
 Norm
 Norme

Fibre Material Fasermaterial Matière	Polypropylene, monofilament	
Polymer conformity	FDA CFR title 21 part 177 And Directive 90/128/EEC 23.2.1990	
Extractables	< 1000 mg/m ²	Extraction at total reflux Solvent: Ethanol 50%
Weave Pattern Bindung Armure	Double layer weave Doppellagengewebe Tissu double chaîne	
Finish Ausrüstung Traitement	Calendered Kalandriert Calandré	
Thickness [µm] Dicke [µm] Épaisseur [µm]	850	DIN 53 855
Weight [g/m ²] / [oz/sq.yd] Gewicht [g/m ²] / [oz/sq.yd] Poids [g/m ²] / [oz/sq.yd]	455 / 13.42	DIN 53 854
Air Permeability @ 196 Pa Luftdurchlässigkeit @ 196 Pa Perméabilité à l'air @ 196 Pa Air Permeability @ ½ inch H ₂ O	30 50 180 6.5	+/- 6 l/dm ² /min +/- 10 l/m ² /sec +/- 36 m ³ /m ² /h +/- 1.3 cfm DIN 53 887
Nominal Pore Size [µm] Nominale Porengröße [µm] Dimension des pores nominal [µm]	12	Boil over bubble point test method ASTM F-316-86

All stated values are arithmetic means of samples (\bar{x}).

Alle Angaben sind Stichproben-Mittelwerte (\bar{x})

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19.01.2005/05-8000-SK 012 PHARMA/F

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**SEFAR TETEX DLW
 05-8000-K 020 PHARMA**

Technical Data Sheet Standard
 Technisches Datenblatt Norm
 Fiche technique Norme

Fibre Material Fasermaterial Matière	Polypropylene, monofilament	
Polymer conformity	FDA CFR title 21 part 177 And Directive 90/128/EEC 23.2.1990	
Extractables	< 1000 mg/m ²	Extraction at total reflux Solvent: Ethanol 50%
Weave Pattern Bindung Armure	Double layer weave Doppellagengewebe Tissu double chaîne	
Finish Ausrüstung Traitement	Calendered Kalandriert Calandré	
Thickness [µm] Dicke [µm] Epaisseur [µm]	860	DIN 53 855
Weight [g/m ²] / [oz/sq.yd] Gewicht [g/m ²] / [oz/sq.yd] Poids [g/m ²] / [oz/sq.yd]	425 / 12.54	DIN 53 854
Air Permeability @ 196 Pa Luftdurchlässigkeit @ 196 Pa Perméabilité à l'air @ 196 Pa Air Permeability @ ½ inch H ₂ O	60 +/- 12 l/dm ² /min 100 +/- 20 l/m ² /sec 360 +/- 72 m ³ /m ² /h 13 +/- 2.6 cfm	DIN 53 887
Nominal Pore Size [µm] Nominale Porengröße [µm] Dimension des pores nominal [µm]	20	Boil over bubble point test method ASTM F-316-86

All stated values are arithmetic means of samples (\bar{x}).
 Alle Angaben sind Stichproben-Mittelwerte (\bar{x})
 Les données indiquées sont des valeurs moyennes.

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19.01.2005/05-8000-K 020 PHARMA/F

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**SEFAR TETEX DLW
 05-8000-K 020 PHARMA**

Technical Data Sheet	Standard
Technisches Datenblatt	Norm
Fiche technique	Norme

Fibre Material Fasermaterial Matière	Polypropylene, monofilament	
Polymer conformity	FDA CFR title 21 part 177 And Directive 90/128/EEC 23.2.1990	
Extractables	< 1000 mg/m ²	Extraction at total reflux Solvent: Ethanol 50%
Weave Pattern Bindung Armure	Double layer weave Doppellagengewebe Tissu double chaîne	
Finish Ausrüstung Traitement	Calendered Kalandriert Calandré	
Thickness [µm] Dicke [µm] Epaisseur [µm]	860	DIN 53 855
Weight [g/m ²] / [oz/sq.yd] Gewicht [g/m ²] / [oz/sq.yd] Poids [g/m ²] / [oz/sq.yd]	425 / 12.54	DIN 53 854
Air Permeability @ 196 Pa Luftdurchlässigkeit @ 196 Pa Perméabilité à l'air @ 196 Pa Air Permeability @ ½ inch H ₂ O	60 100 360 13	+/- 12 l/dm ² /min +/- 20 l/m ² /sec +/- 72 m ³ /m ² /h +/- 2.6 cfm DIN 53 887
Nominal Pore Size [µm] Nominale Porengröße [µm] Dimension des pores nominal [µm]	20	Boil over bubble point test method ASTM F-316-86

All stated values are arithmetic means of samples (\bar{x}).
 Alle Angaben sind Stichproben-Mittelwerte (\bar{x})
 Les données indiquées sont des valeurs moyennes.

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19.01.2005/05-8000-K 020 PHARMA/F

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SEFAR TETEX DLW
05-8000-SK 030 PHARMA

Technical Data Sheet
Technisches Datenblatt
Fiche technique

Standard
Norm
Norme

Fibre Material Fasermaterial Matière	Polypropylene, monofilament	
Polymer conformity	FDA CFR title 21 part 177.1520 And Directive 90/128/EEC 23.2.1990	
Extractables	< 1000 mg/m²	Extraction at total reflux Solvent: Ethanol 50%
Weave Pattern Bindung Armure	Double layer weave Doppellagengewebe Tissu double chaîne	
Finish Ausrüstung Traitement	Calendered Kalandriert Calandré	
Thickness [µm] Dicke [µm] Epaisseur [µm]	920	DIN 53 855
Weight [g/m ²] / [oz/sq.yd] Gewicht [g/m ²] / [oz/sq.yd] Poids [g/m ²] / [oz/sq.yd]	440 / 12.98	DIN 53 854
Air Permeability @ 196 Pa Luftdurchlässigkeit @ 196 Pa Perméabilité à l'air @ 196 Pa Air Permeability @ ½ inch H ₂ O	120 +/- 24 l/dm ² /min 200 +/- 40 l/m ² /sec 720 +/- 144 m ³ /m ² /h 26 +/- 5.2 cfm	DIN 53 887
Nominal Pore Size [µm] Nominale Porengröße [µm] Dimension des pores nominal [µm]	30	Boil over bubble point test method ASTM F-316-86

All stated values are arithmetic means of samples (\bar{x}).
 Alle Angaben sind Stichproben-Mittelwerte (\bar{x})
 Les données indiquées sont des valeurs moyennes.

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23.06.2008/05-8000-SK 030 PHARMA |

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SEFAR TETEX DLW
V-05-8000-SK 030
PHARMA

Preliminary Technical Data Sheet	Standard
Provisorisches Technisches	Norm
Datenblatt	
Fiche technique provisoire	Norme

Fibre Material Fasermaterial Matière	Polypropylene, monofilament	
Polymer conformity	FDA CFR title 21 part 177 And Directive 90/128/EEC 23.2.1990	
Extractables	< 1000 mg/m ²	Extraction at total reflux Solvent: Ethanol 50%
Weave Pattern Bindung Armure	Double layer weave Doppellagengewebe Tissu double chaîne	
Finish Ausrüstung Traitement	Calendered Kalandriert Calandré	
Thickness [µm] Dicke [µm] Epaisseur [µm]	920	DIN 53 855
Weight [g/m ²] / [oz/sq.yd] Gewicht [g/m ²] / [oz/sq.yd] Poids [g/m ²] / [oz/sq.yd]	440 / 12.98	DIN 53 854
Air Permeability @ 196 Pa Luftdurchlässigkeit @ 196 Pa Perméabilité à l'air @ 196 Pa Air Permeability @ ½ inch H ₂ O	120 +/- 24 l/dm ² /min 200 +/- 40 l/m ² /sec 720 +/- 144 m ³ /m ² /h 28 +/- 5.2 cfm	DIN 53 887
Nominal Pore Size [µm] Nominale Porengröße [µm] Dimension des pores nominal [µm]	30	Boil over bubble point test method ASTM F-316-86

All stated values are arithmetic means of samples (\bar{x}).
 Alle Angaben sind Stichproben-Mittelwerte (\bar{x})
 Les données indiquées sont des valeurs moyennes.

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18.01 2005/V-05-8000-SK 030 PHARMA/F

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SEFAR TETEX DLW
05-8000-K 050 PHARMA

Technical Data Sheet
 Technisches Datenblatt
 Fiche technique

Standard
 Norm
 Norme

Fibre Material Fasermaterial Matière	Polypropylene, monofilament	
Polymer conformity	FDA CFR title 21 part 177 And Directive 90/128/EEC 23.2.1990	
Extractables	< 1000 mg/m ²	Extraction at total reflux Solvent: Ethanol 50%
Weave Pattern Bindung Armure	Double layer weave Doppellagengewebe Tissu double chaîne	
Finish Ausrüstung Traitement	Calendered Kalandriert Calandré	
Thickness [µm] Dicke [µm] Epaisseur [µm]	920	DIN 53 855
Weight [g/m ²] / [oz/sq.yd] Gewicht [g/m ²] / [oz/sq.yd] Poids [g/m ²] / [oz/sq.yd]	440 / 12.98	DIN 53 854
Air Permeability @ 196 Pa Luftdurchlässigkeit @ 196 Pa Perméabilité à l'air @ 196 Pa Air Permeability @ ½ inch H ₂ O	240 400 1440 52	+/- 60 l/dm ² /min +/- 100 l/m ² /sec +/- 360 m ³ /m ² /h +/- 13 cfm
Nominal Pore Size [µm] Nominale Porengröße [µm] Dimension des pores nominal [µm]	50	Boil over bubble point test method ASTM F-316-86

All stated values are arithmetic means of samples (\bar{x}).
 Alle Angaben sind Stichproben-Mittelwerte (\bar{x})
 Les données indiquées sont des valeurs moyennes.

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APPENDIX C

HPLC Analytical Determinations for Rebaudioside A

C-1 HPLC Analytical Methodology for Rebaudioside A

C-2 HPLC Validation Methodology for Rebaudioside A

C-3 HPLC Analytical Summary Report for Rebaudioside A

C-1 HPLC ANALYTICAL METHODOLOGY FOR REBAUDIOSIDE A

Almendra (Thailand) Ltd.	Date: September 1, 2010
Standard Operating Procedure	Supersedes: September 1, 2010
Purify Analysis by HPLC	Page: 1 of 4

1. Purpose:

To ensure that the HPLC assay of REB A and its impurities in sample are analyzed correctly.

2. Principle:

Rebaudioside A and its major impurities components are separated by using Luna 5 µ C18(2) 100A (Phenomenex) or equivalent, 4.6 mm X 250 mm, 5 µm at 40 oC. The mobile phase consists of 32% acetonitrile and 68% buffer containing 10 mmol/L sodium phosphate buffer (pH2.6). The eluted components are detected by ultraviolet absorbtion (UV) at 210 nm (4 nm bandwidth, reference is 260 nm (100 nm bandwidth)).

3. Scope:

This method is applicable to the quantitation of Rebaudioside A, stevioside and the other impurities, base on stevioside standard.

4. Apparatus:

1. HPLC system, Waters e2695 or equivalent, comprised of a pump, a column thermostat, an autosampler, a UV detector capable of background correction and a data acquisition system.
2. Luna 5 µ C18(2) 100A (Phenomenex) or equivalent, 4.6 mm X 250 mm, 5 µm
3. Analytical balance capable of weighing to 0.0001 g (0.1 mg)
4. Stir plate
5. Small stir bars
6. Volumetric flasks 10 mL and 50 mL
7. Disposable Syringe 5 mL
8. Syringe filter 0.45 µm
9. Screw cap vial 1.5 mL

5. Reagents:

1. Chromadex or Wako reference standard or equivalent.
 - i. Reb A (Rebaudioside A)
 - ii. Reb B
 - iii. Reb C
 - iv. Reb D
 - v. Reb F
 - vi. Stevioside
 - vii. Steviolbioside
 - viii. Rubusoside
 - ix. Dulcoside A
2. Acetonitrile (ACN), HPLC grade or equivalent
3. HPLC grade water or equivalent
4. Phosphoric acid, reagent grade or equivalent
5. Sodium Dihydrogen Phosphate, reagent grade or equivalent

6. PROCEDURE

6.1 Chemical Preparation

6.1.1 10 mmol Sodium Phosphate Buffer Solution (pH 2.6)

The buffer is prepared by dissolving 1.52 g sodium dihydrogen phosphate adjust pH to 2.6 by phosphoric acid in one liter of water. It may be necessary to adjust the ratio of ammonium acetate to acetic acid.

Almendra (Thailand) Ltd.	Date: September 1, 2010
Standard Operating Procedure	Supersedes: September 1, 2010
HPLC Assay for the analysis of REB A	Page: 2 of 4

6.1.2 Mobile Phase (32:68 acetonitrile : buffer)

320 mL Acetonitrile and 640 mL buffer are mixed to achieve a REB A retention time of 8.0 ± 1 min.

6.1.3 Diluent Solution (30% acetonitrile)

Combine 300 mL of acetonitrile and 700 mL of water and mix thoroughly. Be sure that it is at room temperature before use because it cools on mixing.

6.2 Standard Preparation

Steviol Glycoside Standard (Reb A, Reb B, Reb C, Reb D, Reb F, Dulcoside A, Stevioside, Rubusoside, Steviolbioside)

- 1) Place 50.0 ± 0.5 mg of each standard, recorded to the nearest 0.1 mg, of the stevioside standard in a 50 mL volumetric flask and dilute to volume with diluent solution. This will make an approximately 1000 mg/L standard (stock A).
- 2) Stir the solution if necessary until dissolved.
- 3) Dilute standard with diluent to
 - 50 mg/L (Stock B 0.5 mL, dilute to 10 mL)
 - 100 mg/L (Stock B 1.0 mL, dilute to 10 mL)
 - 250 mg/L (Stock B 2.5 mL, dilute to 10 mL)
 - 500 mg/L (Stock A 5 mL, dilute to 10 mL)

For all of the standards, the weights and volumes can be injected once at the beginning and the end of the sequence with no more than 20 injections in between calibration runs.

6.3 Sample Preparation

- 1) Place 50 ± 0.5 mg, recorded to the nearest 0.1 mg, of the sample in a 50 mL volumetric flask and dilute to volume with diluent solution. This will make an approximately 1000 mg/L sample. They are injected in duplicate, 5.0 μ L.
- 2) Measure the moisture content by Karl Fischer analysis every time a sample is prepared.

The weight and volume can be adjusted up proportionally as needed, but not down.

If the sample will not be analyzed immediately, then it is to be stored without headspace, under nitrogen and desiccated.

6.4 Instrumental Conditions

Column	Luna 5 μ C18(2) 100A (Phenomenex) or equivalent, 4.6 mm X 250 mm, 5 μ m
Temperature	40 oC
Mobile Phase	32:68 ; acetonitrile and 10 mmol/L sodium phosphate buffer (pH6.2)
Flow Rate	1.0 mL/min
Injection	5 μ L
Detection	UV at 210 nm (4 nm bandwidth), Reference: 260 nm (100 nm bandwidth)
Run Time	30 min (initially, it will be longer, but should be no more than 60 min)

Almendra (Thailand) Ltd.		Date: September 1, 2010
Standard Operating Procedure		Supersedes: September 1, 2010
Purify Analysis by HPLC		Page: 3 of 4
Autosampler Temp	25 oC	
Sample Concentration	0.1 % or 1000 mg/L in diluent buffer.	

6.5 Analysis Procedure

1) System Installation and Programming:

- 1.1) Install the HPLC system according to the instructions described in the Waters, Perkin Elmer or Agilent HPLC equipment manuals. (See References)
- 1.2) Enter the instrument parameters into the computer software package as describe in instrument condition. Slight variations can be made to optimize an individual HPLC.
- 1.3) Enter the desired processing parameters for integration and for the component table. Choose the desired report format.

2) Preparing the HPLC for Sample Analysis

- 2.1) Before starting the analysis, equilibrate to achieve a stable baseline.

3) Analysis of Steviol Glycoside

3.1) Calibration

The calibration procedure should be performed to monitor daily instrument performance. Standard solutions may be thawed and used as necessary.

- a) Inject 5.0 µl of the standard solution.
- b) Measure the peak areas of the components from the standard solution.
- c) Least squares regression method with R₂>0.999 and a coefficient of variation > 5.0 represents a good linear relationship of the analytical curve.
- d) The average mass weighed and the averages of the areas are used to calculate the results.

3.2) Sample Analysis

- a) Inject 5.0 µl of the sample solution.
- b) Identify the peaks in the sample chromatogram by relating to the corresponding components in the standard curve chromatogram.
- c) Measure the peak areas of the components from the sample solution. Then measure the peak areas of each steviol glycoside from the standard solution.
- d) Calculate the percentage of steviol glycosides by using normalization areas from the following formulas:

$$\% \text{ each steviol glycoside} = [Ws/W] \times [A/As] \times 100 \%$$

where

- Ws = weighed amount (mg) of steviol glycoside in the standard, on dry basis
- W = weighed amount of sample (mg), on a dry basis
- As = Peak area of each steviol glycoside from standard
- A = Peak area of each steviol glycoside from the sample

Almendra (Thailand) Ltd.	Date: September 1, 2010
Standard Operating Procedure	Supersedes: September 1, 2010
HPLC Assay for the analysis of REB A	Page: 4 of 4

e) The purity of the standard used must be deducted from the final result to avoid a quantification error. A moisture correction will convert the final result to a dry basis.

4. System Slow-down and Shut-down

After a run is completed, it is recommended to reduce the flow rate to 0.1 mL/min. between runs and turn off the UV lamp only after the equipment is planned to sit idle between runs to reduce the use of solvent and extend the life of the column and lamp. A run is a comparative set of standards and samples that may be consecutively injected for up to one week.

- 2) The UV detector needs to be turned off every week for recalibration.

7. NOTES

1. Appropriate standard relates to materials that are suitably characterized and purity known with a high degree of certainty. If the purity is not well characterized (or incorrect), then there could be differences in results between labs. It is usually safest to use the same standard to avoid these problems. Otherwise the purities of different standards need to be verified against each other.
2. Acetonitrile is a toxic chemical. Gloves, safety glasses and an apron should be worn when handling. Acetonitrile waste should be collected in an appropriately labeled container, capped tightly when full, and removed for proper disposal.
3. The baseline is important for a consistent integration. There are several factors that could cause the baseline drifting. Column contamination, temperature stability and air in the system are the common problems. Wet prime could eliminate the air in the system, which is added in the procedures. Equilibration can also help to stabilize the baseline drifting. Integration parameters have to be optimized to get better and more consistent integration.

8. REFERENCES

1. Waters Alliance HPLC system Guide (e2695)
2. Waters HPLC Pump Installation and Maintenance Guide
3. Waters 2489 UV Detector Operator's Guide
4. JECFA Tentative Procedure for Steviol Glycosides (2004)
5. JECFA Procedure for Steviol Glycosides (2010)
6. Food Chemicals Codex, current edition Rebaudioside A monograph.
7. Steviol Glycosides Methods Committee validations (Aug/Sept. 2009)

C-2 HPLC Validation Methodology for Rebaudioside A

Validation Method for Determination Reb A and related Steviol Glycoside by HPLC

1. Purpose:

To ensure that analysis method for determination Reb A and related Steviol Glycoside by HPLC is capable of giving reproducible and reliable results.

2. Apparatus:

1. HPLC system, Waters e2695 or equivalent, comprised of a pump, a column thermostat, an autosampler, a UV detector capable of background correction and a data acquisition system.
2. Analytical Column: Luna 5 μ C18(2) 100A (Phenomenex) or equivalent, 4.6 mm X 250 mm, 5 μ m
3. Analytical balance, XS204, Mettler Toledo
4. Karl Fischer, V30, Mettler Toledo
5. Stir plate
6. Small stir bars
7. Volumetric flasks 10 ml and 50 ml, class A
8. Disposable Syringe 5 ml
9. Syringe filter 0.45 μ m
10. Screw cap vial 1.5 ml

3. Reagents:

1. REB A (Rebaudioside A), Chromadex or Wako reference standard or equivalent.
2. Stevioside, Chromadex or Wako reference standard or equivalent.
3. REB B (Rebaudioside B), Chromadex reference standard
4. REB C (Rebaudioside C), Chromadex reference standard
5. REB D (Rebaudioside D), Chromadex reference standard
6. REB F (Rebaudioside F), Chromadex reference standard
7. Dulcoside A, Chromadex reference standard
8. Rubusoside, Chromadex reference standard
9. Steviolbioside, Chromadex reference standard
10. Acetonitrile (ACN), HPLC grade or equivalent
11. HPLC grade water or equivalent
12. Phosphoric acid, reagent grade or equivalent
13. Sodium Dihydrogen Phosphate, reagent grade or equivalent

4. PROCEDURE

4.1 Chemical Preparation:

As JECFA Procedure for Steviol Glycosides (2010)

4.2 Standard Preparation

Steviol Glycoside Standard (Reb A, Reb B, Reb C, Reb D, Reb F, Dulcoside A, Stevioside, Rubusoside, Steviolbioside)

- 1) Place 50.0 ± 0.5 mg of each standard, recorded to the nearest 0.1 mg, of the stevioside standard in a 50 mL volumetric flask and dilute to volume with diluent solution. This will make an approximately 1000 mg/L standard (stock A).
- 2) Stir the solution if necessary until dissolved.
- 3) Dilute standard with diluent to
 - 50 mg/L (Stock B 0.5 mL, dilute to 10 mL)
 - 100 mg/L (Stock B 1.0 mL, dilute to 10 mL)
 - 250 mg/L (Stock B 2.5 mL, dilute to 10 mL)
 - 500 mg/L (Stock A 5 mL, dilute to 10 mL)

4.3 Instrumental Conditions

Column	Luna 5 μ C18(2) 100A (Phenomenex) or equivalent, 4.6 mm X 250 mm, 5 μ m
Temperature	40 $^{\circ}$ C
Mobile Phase	32:68 ; acetonitrile and 10 mmol/L sodium phosphate buffer (pH6.2)
Flow Rate	1.0 mL/min
Injection	5 μ L
Detection	UV at 210 nm (4 nm bandwidth), Reference: 260 nm (100 nm bandwidth)
Run Time	30 min (initially, it will be longer, but should be no more than 60 min)
Autosampler Temp	25 $^{\circ}$ C
Sample Concentration	0.1 % or 1000 mg/L in diluent buffer.

4.4 Analysis Procedure

1) System Installation and Programming:

1.1) Install the HPLC system according to the instructions described in the Waters, Perkin Elmer or Agilent HPLC equipment manuals.

1.2) Enter the instrument parameters into the computer software package as describe in instrument condition. Slight variations can be made to optimize an individual HPLC.

1.3) Enter the desired processing parameters for integration and for the component table. Choose the desired report format.

2) Preparing the HPLC for Sample Analysis

2.1) Before starting the analysis, equilibrate to achieve a stable baseline.

3) Assay Validation for Relative Standard Deviation (Precision)

Inject 5.0 μ l of the each standard solution for 10 replicate injections. Calculate %RSD for retention time, Area and Height.

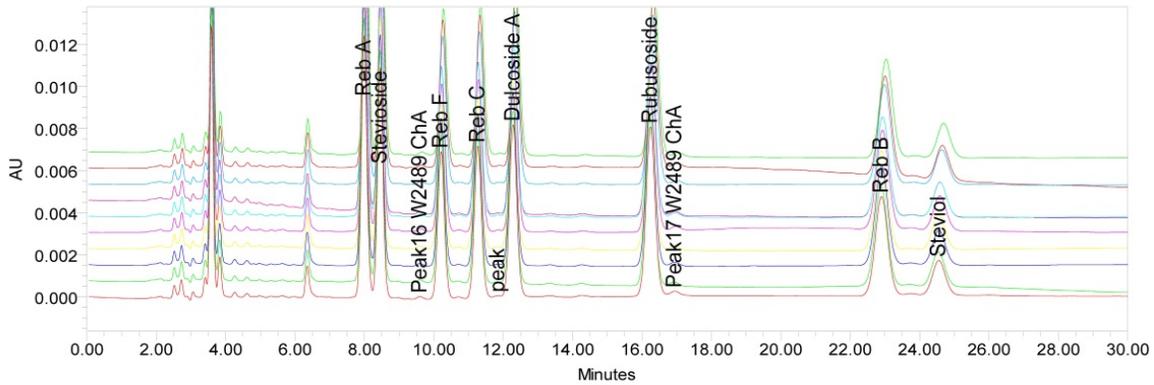
5. RESULTS

Standard	Retention Time			Area			Height		
	Mean	Std. Dev.	%RSD	Mean	Std. Dev.	%RSD	Mean	Std. Dev.	%RSD
Reb A	8.406	0.008	0.100	123059.900	568.400	0.500	10430.900	37.900	0.400
Reb B	24.370	0.026	0.110	129207.400	434.200	0.300	4736.700	14.800	0.300
Reb C	11.918	0.013	0.110	114711.600	351.900	0.300	7430.600	21.900	0.300
Reb F	10.781	0.011	0.100	102797.700	432.900	0.400	7233.900	23.900	0.300
Stevioside	8.885	0.008	0.090	133042.800	573.600	0.400	11161.100	37.600	0.300
Dulcoside A	12.980	0.014	0.110	133247.600	389.400	0.300	8275.100	24.700	0.300
Rubusoside	17.145	0.016	0.090	159896.300	668.900	0.400	8193.600	27.600	0.300
Steviolbioside	26.061	0.026	0.100	42287.800	233.900	0.600	1569.700	5.600	0.400
			0.101			0.400			0.325

C-3 HPLC Analytical Summary Report for Rebaudioside A



Peak Summary Report



- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 1
- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 2
- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 3
- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 4
- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 5
- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 6
- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 7
- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 8
- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 9
- Sample Set Start Date 8/7/2012 2:21:16 PM SGT; Injection 10

Peak Summary with Statistics Name: Dulcoside A

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	1	Dulcoside A	12.283	124923	8174
2	Mixed STD	2	2	Dulcoside A	12.272	125820	8189
3	Mixed STD	2	4	Dulcoside A	12.275	125944	8203
4	Mixed STD	2	3	Dulcoside A	12.279	125298	8176
5	Mixed STD	2	5	Dulcoside A	12.302	125534	8178
6	Mixed STD	2	7	Dulcoside A	12.291	125821	8198
7	Mixed STD	2	8	Dulcoside A	12.335	125626	8176
8	Mixed STD	2	9	Dulcoside A	12.346	125799	8172
9	Mixed STD	2	10	Dulcoside A	12.369	125309	8101
10	Mixed STD	2	6	Dulcoside A	12.298	126420	8248
Mean					12.305	125649.4	8181.5
Std. Dev.					0.034	414.0	36.4
% RSD					0.27	0.3	0.4

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Project Name: Lab Scale
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Peak Summary with Statistics
Name: Peak16 W2489 ChA

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	5	Peak16 W2489 ChA	9.614	1348	124
2	Mixed STD	2	6	Peak16 W2489 ChA	9.611	1356	127
3	Mixed STD	2	10	Peak16 W2489 ChA	9.655	1393	125
4	Mixed STD	2	1	Peak16 W2489 ChA	9.600	1334	123
5	Mixed STD	2	4	Peak16 W2489 ChA	9.599	1475	131
6	Mixed STD	2	3	Peak16 W2489 ChA	9.599	1330	123
7	Mixed STD	2	8	Peak16 W2489 ChA	9.637	1394	127
8	Mixed STD	2	2	Peak16 W2489 ChA	9.598	1429	129
9	Mixed STD	2	7	Peak16 W2489 ChA	9.605	1410	127
10	Mixed STD	2	9	Peak16 W2489 ChA	9.644	1406	128
Mean					9.616	1387.4	126.2
Std. Dev.					0.021	45.8	2.5
% RSD					0.22	3.3	2.0

Peak Summary with Statistics
Name: Peak17 W2489 ChA

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	10	Peak17 W2489 ChA	17.048	2842	170
2	Mixed STD	2	9	Peak17 W2489 ChA	17.022	2762	169
3	Mixed STD	2	8	Peak17 W2489 ChA	17.007	3049	180
4	Mixed STD	2	7	Peak17 W2489 ChA	16.958	3192	185
5	Mixed STD	2	6	Peak17 W2489 ChA	16.963	2927	175
6	Mixed STD	2	3	Peak17 W2489 ChA	16.940	2717	168
7	Mixed STD	2	4	Peak17 W2489 ChA	16.934	2832	171
8	Mixed STD	2	2	Peak17 W2489 ChA	16.927	3016	179
9	Mixed STD	2	5	Peak17 W2489 ChA	16.972	2636	162
10	Mixed STD	2	1	Peak17 W2489 ChA	16.945	3076	181
Mean					16.972	2904.9	174.0
Std. Dev.					0.041	177.5	7.2
% RSD					0.24	6.1	4.1

Peak Summary with Statistics
Name: Reb A

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	10	Reb A	8.041	142143	12279
2	Mixed STD	2	7	Reb A	7.995	143697	12512
3	Mixed STD	2	3	Reb A	7.987	142960	12449
4	Mixed STD	2	1	Reb A	7.988	143084	12436
5	Mixed STD	2	9	Reb A	8.028	143139	12410

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Peak Summary with Statistics
Name: Reb A

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
6	Mixed STD	2	2	Reb A	7.986	143430	12494
7	Mixed STD	2	8	Reb A	8.022	143119	12416
8	Mixed STD	2	6	Reb A	7.999	144743	12562
9	Mixed STD	2	5	Reb A	8.002	143395	12460
10	Mixed STD	2	4	Reb A	7.987	143353	12494
Mean					8.003	143306.3	12451.1
Std. Dev.					0.020	652.3	76.4
% RSD					0.25	0.5	0.6

Peak Summary with Statistics
Name: Reb B

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	7	Reb B	22.940	121800	4704
2	Mixed STD	2	10	Reb B	23.038	120574	4648
3	Mixed STD	2	5	Reb B	22.936	120683	4674
4	Mixed STD	2	2	Reb B	22.894	121361	4682
5	Mixed STD	2	8	Reb B	22.989	121315	4700
6	Mixed STD	2	4	Reb B	22.904	121566	4700
7	Mixed STD	2	9	Reb B	23.005	121378	4692
8	Mixed STD	2	1	Reb B	22.906	120678	4675
9	Mixed STD	2	6	Reb B	22.938	121932	4722
10	Mixed STD	2	3	Reb B	22.908	120847	4682
Mean					22.946	121213.5	4687.9
Std. Dev.					0.049	489.1	20.3
% RSD					0.21	0.4	0.4

Peak Summary with Statistics
Name: Reb C

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	2	Reb C	11.260	106373	7233
2	Mixed STD	2	5	Reb C	11.287	106391	7229
3	Mixed STD	2	6	Reb C	11.283	107105	7285
4	Mixed STD	2	8	Reb C	11.318	106525	7220
5	Mixed STD	2	4	Reb C	11.263	106550	7248
6	Mixed STD	2	9	Reb C	11.328	106677	7216
7	Mixed STD	2	10	Reb C	11.348	106028	7140
8	Mixed STD	2	3	Reb C	11.265	106111	7224
9	Mixed STD	2	1	Reb C	11.268	106421	7223
10	Mixed STD	2	7	Reb C	11.277	107215	7264

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Peak Summary with Statistics
Name: Reb C

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
Mean					11.290	106539.6	7228.2
Std. Dev.					0.031	380.8	38.1
% RSD					0.27	0.4	0.5

Peak Summary with Statistics
Name: Reb F

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	9	Reb F	10.258	95400	6995
2	Mixed STD	2	8	Reb F	10.250	95217	6996
3	Mixed STD	2	7	Reb F	10.214	95526	7031
4	Mixed STD	2	6	Reb F	10.220	96136	7070
5	Mixed STD	2	3	Reb F	10.205	95080	7005
6	Mixed STD	2	4	Reb F	10.204	95278	7025
7	Mixed STD	2	2	Reb F	10.202	95345	7018
8	Mixed STD	2	1	Reb F	10.207	95224	7000
9	Mixed STD	2	5	Reb F	10.223	95321	7009
10	Mixed STD	2	10	Reb F	10.275	94593	6920
Mean					10.226	95311.9	7007.1
Std. Dev.					0.026	383.2	37.7
% RSD					0.25	0.4	0.5

Peak Summary with Statistics
Name: Rubusoside

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	6	Rubusoside	16.265	150569	8093
2	Mixed STD	2	1	Rubusoside	16.248	149469	8016
3	Mixed STD	2	3	Rubusoside	16.244	148976	8019
4	Mixed STD	2	10	Rubusoside	16.346	148906	7957
5	Mixed STD	2	8	Rubusoside	16.307	149614	8032
6	Mixed STD	2	2	Rubusoside	16.235	149166	8013
7	Mixed STD	2	5	Rubusoside	16.268	148736	8003
8	Mixed STD	2	7	Rubusoside	16.262	150448	8060
9	Mixed STD	2	9	Rubusoside	16.320	149751	8027
10	Mixed STD	2	4	Rubusoside	16.239	149326	8036
Mean					16.273	149496.2	8025.7
Std. Dev.					0.038	621.2	35.3
% RSD					0.23	0.4	0.4

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Peak Summary with Statistics

Name: Steviol

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	1	Steviol	24.558	42106	1621
2	Mixed STD	2	10	Steviol	24.694	41683	1597
3	Mixed STD	2	9	Steviol	24.658	41211	1595
4	Mixed STD	2	8	Steviol	24.643	41831	1615
5	Mixed STD	2	7	Steviol	24.592	42269	1624
6	Mixed STD	2	6	Steviol	24.589	42044	1628
7	Mixed STD	2	3	Steviol	24.558	41847	1614
8	Mixed STD	2	4	Steviol	24.553	41715	1614
9	Mixed STD	2	2	Steviol	24.544	41632	1609
10	Mixed STD	2	5	Steviol	24.586	41710	1611
Mean					24.597	41804.9	1612.8
Std. Dev.					0.051	294.3	10.7
% RSD					0.21	0.7	0.7

Peak Summary with Statistics

Name: Stevioside

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	9	Stevioside	8.493	126106	10910
2	Mixed STD	2	6	Stevioside	8.463	126711	11021
3	Mixed STD	2	10	Stevioside	8.507	125105	10807
4	Mixed STD	2	3	Stevioside	8.451	125627	10929
5	Mixed STD	2	1	Stevioside	8.452	125683	10914
6	Mixed STD	2	4	Stevioside	8.450	125923	10965
7	Mixed STD	2	5	Stevioside	8.466	125943	10947
8	Mixed STD	2	2	Stevioside	8.449	125840	10956
9	Mixed STD	2	7	Stevioside	8.458	126389	10981
10	Mixed STD	2	8	Stevioside	8.486	125822	10916
Mean					8.467	125914.7	10934.7
Std. Dev.					0.021	435.0	56.5
% RSD					0.25	0.3	0.5

Peak Summary with Statistics

Name: Unk1

	Sample Name	Vial	Inj	Name	Retention Time (min)
1	Mixed STD	2	2	Unk1	10.818
2	Mixed STD	2	6	Unk1	10.818
3	Mixed STD	2	7	Unk1	10.818
4	Mixed STD	2	10	Unk1	10.818
5	Mixed STD	2	3	Unk1	10.818
6	Mixed STD	2	9	Unk1	10.818
7	Mixed STD	2	4	Unk1	10.818
8	Mixed STD	2	5	Unk1	10.818
9	Mixed STD	2	8	Unk1	10.818
10	Mixed STD	2	1	Unk1	10.818

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Peak Summary with Statistics
Name: Unk1

	Sample Name	Vial	Inj	Name	Retention Time (min)
Mean					10.818
Std. Dev.					0.000
% RSD					0.00

Peak Summary with Statistics
Name: Unk2

	Sample Name	Vial	Inj	Name	Retention Time (min)
1	Mixed STD	2	6	Unk2	13.076
2	Mixed STD	2	3	Unk2	13.076
3	Mixed STD	2	4	Unk2	13.076
4	Mixed STD	2	2	Unk2	13.076
5	Mixed STD	2	1	Unk2	13.076
6	Mixed STD	2	5	Unk2	13.076
7	Mixed STD	2	10	Unk2	13.076
8	Mixed STD	2	9	Unk2	13.076
9	Mixed STD	2	8	Unk2	13.076
10	Mixed STD	2	7	Unk2	13.076
Mean					13.076
Std. Dev.					0.000
% RSD					0.00

Peak Summary with Statistics
Name: Unk3

	Sample Name	Vial	Inj	Name	Retention Time (min)
1	Mixed STD	2	8	Unk3	14.413
2	Mixed STD	2	2	Unk3	14.413
3	Mixed STD	2	10	Unk3	14.413
4	Mixed STD	2	9	Unk3	14.413
5	Mixed STD	2	6	Unk3	14.413
6	Mixed STD	2	4	Unk3	14.413
7	Mixed STD	2	7	Unk3	14.413
8	Mixed STD	2	1	Unk3	14.413
9	Mixed STD	2	3	Unk3	14.413
10	Mixed STD	2	5	Unk3	14.413
Mean					14.413
Std. Dev.					0.000
% RSD					0.00

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Peak Summary with Statistics
Name: Unk4

	Sample Name	Vial	Inj	Name	Retention Time (min)
1	Mixed STD	2	1	Unk4	26.974
2	Mixed STD	2	2	Unk4	26.974
3	Mixed STD	2	4	Unk4	26.974
4	Mixed STD	2	3	Unk4	26.974
5	Mixed STD	2	6	Unk4	26.974
6	Mixed STD	2	7	Unk4	26.974
7	Mixed STD	2	8	Unk4	26.974
8	Mixed STD	2	9	Unk4	26.974
9	Mixed STD	2	10	Unk4	26.974
10	Mixed STD	2	5	Unk4	26.974
Mean					26.974
Std. Dev.					0.000
% RSD					0.00

Peak Summary with Statistics
Name: Unknown

	Sample Name	Vial	Inj	Name	Retention Time (min)
1	Mixed STD	2	5	Unknown	8.748
2	Mixed STD	2	8	Unknown	8.748
3	Mixed STD	2	1	Unknown	8.748
4	Mixed STD	2	7	Unknown	8.748
5	Mixed STD	2	2	Unknown	8.748
6	Mixed STD	2	6	Unknown	8.748
7	Mixed STD	2	3	Unknown	8.748
8	Mixed STD	2	9	Unknown	8.748
9	Mixed STD	2	4	Unknown	8.748
10	Mixed STD	2	10	Unknown	8.748
Mean					8.748
Std. Dev.					0.000
% RSD					0.00

Peak Summary with Statistics
Name: peak

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
1	Mixed STD	2	7	peak	11.842		
2	Mixed STD	2	8	peak	11.842		
3	Mixed STD	2	6	peak	11.842		
4	Mixed STD	2	3	peak	11.842		
5	Mixed STD	2	4	peak	11.842		

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Peak Summary with Statistics
Name: peak

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	Height
6	Mixed STD	2	1	peak	11.842	915	80
7	Mixed STD	2	5	peak	11.842		
8	Mixed STD	2	10	peak	11.842		
9	Mixed STD	2	2	peak	11.842		
10	Mixed STD	2	9	peak	11.842		
Mean					11.842	915.4	80.3
Std. Dev.					0.000		
% RSD					0.00		

Peak Summary with Statistics
Name: unk6

	Sample Name	Vial	Inj	Name	Retention Time (min)
1	Mixed STD	2	5	unk6	11.587
2	Mixed STD	2	10	unk6	11.587
3	Mixed STD	2	9	unk6	11.587
4	Mixed STD	2	8	unk6	11.587
5	Mixed STD	2	7	unk6	11.587
6	Mixed STD	2	6	unk6	11.587
7	Mixed STD	2	3	unk6	11.587
8	Mixed STD	2	4	unk6	11.587
9	Mixed STD	2	2	unk6	11.587
10	Mixed STD	2	1	unk6	11.587
Mean					11.587
Std. Dev.					0.000
% RSD					0.00

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Project Name: Lab Scale
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APPENDIX D

Certifications of Analyses for Batches

- D-1 Certificate of Analysis for Batch 01092011
- D-2 Certificate of Analysis for Batch 26092011
- D-3 Certificate of Analysis for Batch 27092011
- D-4 Certificate of Analysis for Batch 28092011
- D-5 Certificate of Analysis for Batch 29032011

D-1 Certificate of Analysis for Batch 01092011



Certificate Of Analysis

Customer Name: _____
Product Name: Rebaudioside A, 97%
Batch Number: 01092011
Amount: _____
Manufacturing Date: September 01, 2011
Expired Date: August 31, 2013
COA No: 010/2011
Date: December 22, 2011

ANALYTICAL RESULTS

Analytical Parameters	Specification	Result
Appearance Form	Powder	Powder
Appearance Color	White	White
Solubility (in water)	Freely Soluble	Freely Soluble
Purity (HPLC Area,%)	≥ 97	98.61
Residual Ethanol (ppm)	≤ 500	486.8
Residual Methanol (ppm)	≤ 200	Not Detected
Loss on Drying (%)	≤ 3.0	1.46
pH	4.5 – 7.0	6.5
Total Ash (%)	≤ 0.1	0.04
Arsenic (ppm)	≤ 0.02	Not Detected
Lead (ppm)	≤ 0.1	Not Detected
Mercury (ppm)	≤ 0.01	Not Detected
Cadmium (ppm)	≤ 0.01	Not Detected



Microbiological Properties	Specification	Result
Total Plate Count (cfu/g, max)	1,000	150
Yeast (cfu/g, max)	100	< 10
Mold (cfu/g, max)	100	< 10
Heat Resistant Mold (in 50g)	Negative	Negative
Salmonella spp. (in 25g)	Negative	Negative
Staphylococcus aureus (in 10g)	Negative	Negative
Alicyclobacillus acidoterrestris (in 50g)	Negative	Negative
Listeria (in 1g)	Negative	Negative
Coliform/E.coli (in 1g)	Negative	Negative
Total Coliforms (in 25g)	Negative	Negative
Fecal Coliforms (in 25g)	Negative	Negative

(b) (6)

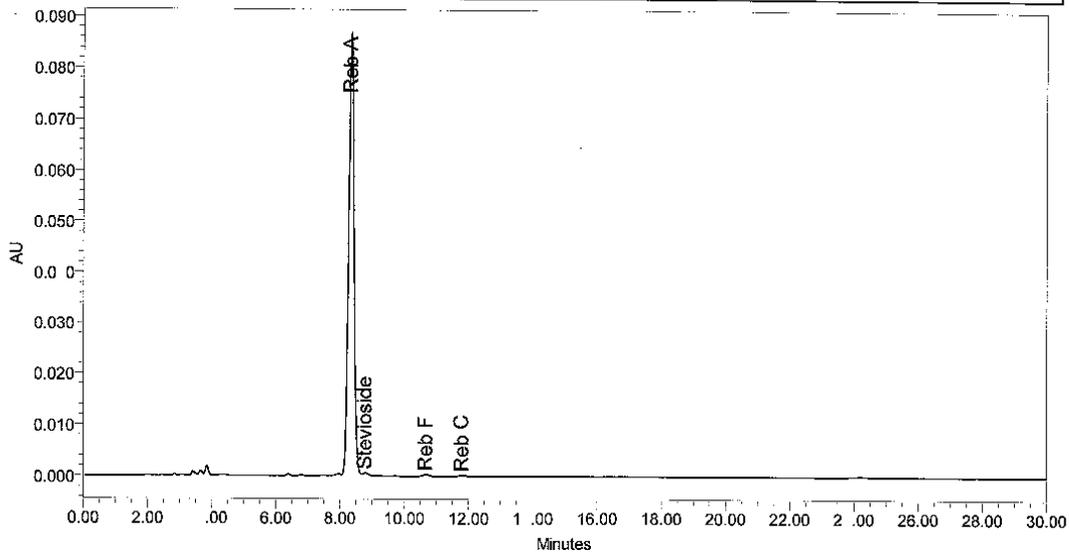


Ms. Pnita Chutasmit
Quality Assurance Manager



Almendra (Thailand) Ltd.

SAMPLE INFORMATION			
Sample Name:	01092011	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	Sep7_11
Vial:	13	Acq. Method Set:	Jecfa2010
Injection #:	1	Processing Method:	calibration8_3
Injection Volume:	5.00 ul	Channel Name:	W2 89 ChA
Run Time:	30.0 Minutes	Proc. Chnl. Descr.:	W2 89 ChA 210nm
Date Acquired:	9/8/2011 11:12:25 AM SGT		
Date Processed:	12/13/2011 3:53: 7 PM SGT		



	Name	Retention Time (min)	Area (?V*sec)	% Area
1	Reb A	8.3	1029982	98.61
2	Stevioside	8.797	5901	0.56
3	Reb F	10.678	52.1	0.50
	Reb C	11.805	3325	0.32

D-2 Certificate of Analysis for Batch 26092011



Certificate Of Analysis

Customer Name: _____
Product Name: Rebaudioside A, 97%
Batch Number: 26092011
Amount: _____
Manufacturing Date: September 26, 2011
Expired Date: September 25, 2013
COA No: 015/2011
Date: December 22, 2011

ANALYTICAL RESULTS

Analytical Parameters	Specification	Result
Appearance Form	Powder	Powder
Appearance Color	White	White
Solubility (in water)	Freely Soluble	Freely Soluble
Purity (HPLC Area,%)	≥ 97	98.31
Residual Ethanol (ppm)	≤ 500	211
Residual Methanol (ppm)	≤ 200	Not Detected
Loss on Drying (%)	≤ 3.0	2.10
pH	4.5 – 7.0	6.4
Total Ash (%)	≤ 0.1	0.04
Arsenic (ppm)	≤ 0.02	Not Detected
Lead (ppm)	≤ 0.1	Not Detected
Mercury (ppm)	≤ 0.01	Not Detected
Cadmium (ppm)	≤ 0.01	Not Detected



Microbiological Properties	Specification	Result
Total Plate Count (cfu/g, max)	1,000	260
Yeast (cfu/g, max)	100	< 10
Mold (cfu/g, max)	100	< 10
Heat Resistant Mold (in 50g)	Negative	Negative
Salmonella spp. (in 25g)	Negative	Negative
Staphylococcus aureus (in 10g)	Negative	Negative
Alicyclobacillus acidoterrestris (in 50g)	Negative	Negative
Listeria (in 1g)	Negative	Negative
Coliform/E.coli (in 1g)	Negative	Negative
Total Coliforms (in 25g)	Negative	Negative
Fecal Coliforms (in 25g)	Negative	Negative

(b) (6)



Ms. Pnita Chutasmit
Quality Assurance Manager



Microbiological Properties	Specification	Result
Total Plate Count (cfu/g, max)	1,000	260
Yeast (cfu/g, max)	100	< 10
Mold (cfu/g, max)	100	< 10
Heat Resistant Mold (in 50g)	Negative	Negative
Salmonella spp. (in 25g)	Negative	Negative
Staphylococcus aureus (in 10g)	Negative	Negative
Alicyclobacillus acidoterrestris (in 50g)	Negative	Negative
Listeria (in 1g)	Negative	Negative
Coliform/E.coli (in 1g)	Negative	Negative
Total Coliforms (in 25g)	Negative	Negative
Fecal Coliforms (in 25g)	Negative	Negative

(b) (6)

Ms. Pnita Chutasmit
Quality Assurance Manager

D-3 Certificate of Analysis for Batch 27092011



Certificate Of Analysis

Customer Name: _____
Product Name: Rebaudioside A, 97%
Batch Number: 27092011
Amount: _____
Manufacturing Date: September 27, 2011
Expired Date: September 26, 2013
COA No: 016/2011
Date: December 22, 2011

ANALYTICAL RESULTS

Analytical Parameters	Specification	Result
Appearance Form	Powder	Powder
Appearance Color	White	White
Solubility (in water)	Freely Soluble	Freely Soluble
Purity (HPLC Area,%)	≥ 97	98.37
Residual Ethanol (ppm)	≤ 500	255
Residual Methanol (ppm)	≤ 200	Not Detected
Loss on Drying (%)	≤ 3.0	2.17
pH	4.5 – 7.0	6.2
Total Ash (%)	≤ 0.1	0.04
Arsenic (ppm)	≤ 0.02	Not Detected
Lead (ppm)	≤ 0.1	Not Detected
Mercury (ppm)	≤ 0.01	Not Detected
Cadmium (ppm)	≤ 0.01	Not Detected



Microbiological Properties	Specification	Result
Total Plate Count (cfu/g, max)	1,000	220
Yeast (cfu/g, max)	100	< 10
Mold (cfu/g, max)	100	< 10
Heat Resistant Mold (in 50g)	Negative	Negative
Salmonella spp. (in 25g)	Negative	Negative
Staphylococcus aureus (in 10g)	Negative	Negative
Alicyclobacillus acidoterrestris (in 50g)	Negative	Negative
Listeria (in 1g)	Negative	Negative
Coliform/E.coli (in 1g)	Negative	Negative
Total Coliforms (in 25g)	Negative	Negative
Fecal Coliforms (in 25g)	Negative	Negative

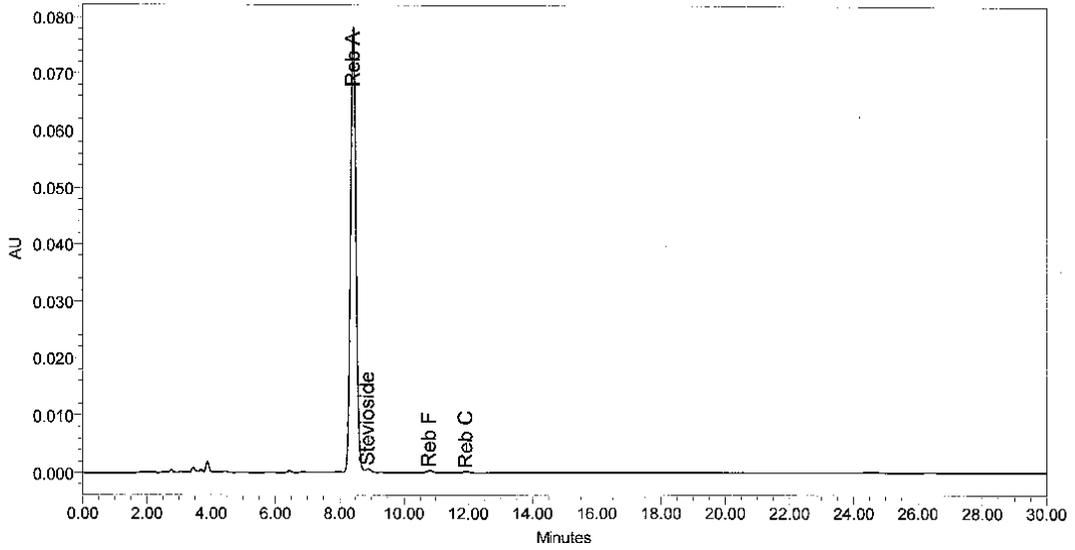
(b) (6)

Ms. Pnita Chutasmit
Quality Assurance Manager



Almendra (Thailand) Ltd.

SAMPLE INFORMATION			
Sample Name:	27092011	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	Oct3_2011
Vial:	6	Acq. Method Set:	Jecfa2010
Injection #:	1	Processing Method:	calibration8_3
Injection Volume:	5.00 ul	Channel Name:	2489 ChA
Run Time:	30.0 Minutes	Proc. Chnl. Descr.:	2489 ChA 210nm
Date Acquired:	10/4/2011 2:47:32 AM SGT		
Date Processed:	12/13/2011 3:29:21 PM SGT		



	Name	Retention Time (min)	Area (?V*sec)	% Area
1	Reb A	8.436	933368	98.37
2	Stevioside	8.893	7291	0.77
3	Reb F	10.807	5046	0.53
4	Reb C	11.948	3176	0.33

D-4 Certificate of Analysis for Batch 28092011



Certificate Of Analysis

Customer Name: _____
Product Name: Rebaudioside A, 97%
Batch Number: 28092011
Amount: _____
Manufacturing Date: September 28, 2011
Expired Date: September 27, 2013
COA No: 017/2011
Date: December 22, 2011

ANALYTICAL RESULTS

Analytical Parameters	Specification	Result
Appearance Form	Powder	Powder
Appearance Color	White	White
Solubility (in water)	Freely Soluble	Freely Soluble
Purity (HPLC Area,%)	≥ 97	98.32
Residual Ethanol (ppm)	≤ 500	384
Residual Methanol (ppm)	≤ 200	Not Detected
Loss on Drying (%)	≤ 3.0	1.44
pH	4.5 – 7.0	6.0
Total Ash (%)	≤ 0.1	0.04
Arsenic (ppm)	≤ 0.02	Not Detected
Lead (ppm)	≤ 0.1	Not Detected
Mercury (ppm)	≤ 0.01	Not Detected
Cadmium (ppm)	≤ 0.01	Not Detected



Certificate Of Analysis

Customer Name: _____
Product Name: Rebaudioside A, 97%
Batch Number: 28092011
Amount: _____
Manufacturing Date: September 28, 2011
Expired Date: September 27, 2013
COA No: 017/2011
Date: December 22, 2011

ANALYTICAL RESULTS

Analytical Parameters	Specification	Result
Appearance Form	Powder	Powder
Appearance Color	White	White
Solubility (in water)	Freely Soluble	Freely Soluble
Purity (HPLC Area,%)	≥ 97	98.32
Residual Ethanol (ppm)	≤ 500	384
Residual Methanol (ppm)	≤ 200	Not Detected
Loss on Drying (%)	≤ 3.0	1.44
pH	4.5 – 7.0	6.0
Total Ash (%)	≤ 0.1	0.04
Arsenic (ppm)	≤ 0.02	Not Detected
Lead (ppm)	≤ 0.1	Not Detected
Mercury (ppm)	≤ 0.01	Not Detected
Cadmium (ppm)	≤ 0.01	Not Detected



Microbiological Properties	Specification	Result
Total Plate Count (cfu/g, max)	1,000	340
Yeast (cfu/g, max)	100	< 10
Mold (cfu/g, max)	100	< 10
Heat Resistant Mold (in 50g)	Negative	Negative
Salmonella spp. (in 25g)	Negative	Negative
Staphylococcus aureus (in 10g)	Negative	Negative
Alicyclobacillus acidoterrestris (in 50g)	Negative	Negative
Listeria (in 1g)	Negative	Negative
Coliform/E.coli (in 1g)	Negative	Negative
Total Coliforms (in 25g)	Negative	Negative
Fecal Coliforms (in 25g)	Negative	Negative

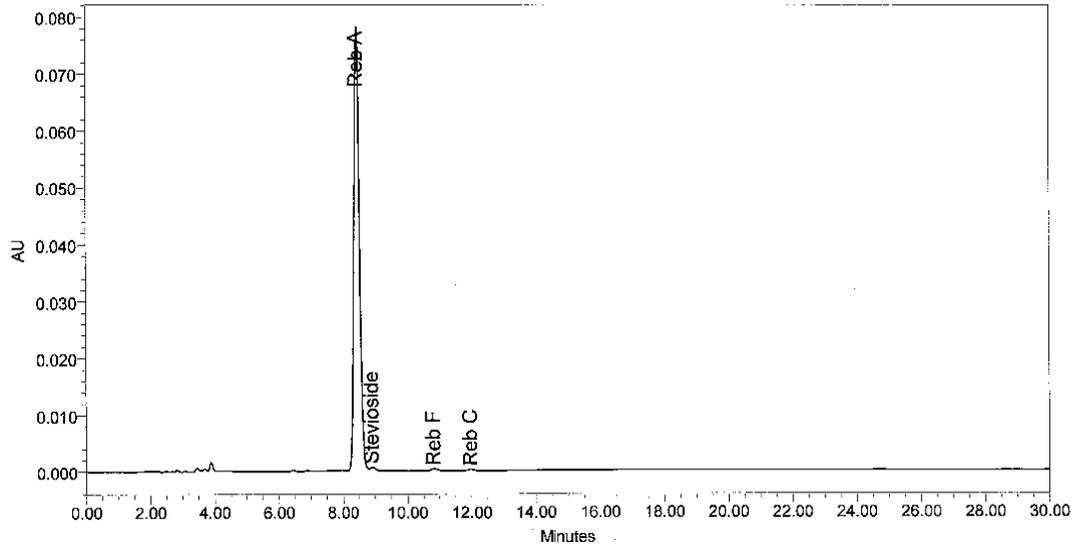
(b) (6) 

Ms. Pnita Chutasmit
Quality Assurance Manager



Almendra (Thailand) Ltd.

SAMPLE INFORMATION			
Sample Name:	28092011	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	Oct3_2011
Vial:	8	Acq. Method Set:	Jecfa2010
Injection #:	1	Processing Method:	calibration8_3
Injection Volume:	5.00 ul	Channel Name:	W2489 ChA
Run Time:	30.0 Minutes	Proc. Chnl. descr.:	W2489 ChA 210nm
ate Acquired:	10/4/2011 6:55:58 PM SGT		
ate Processed:	12/13/2011 3:33:06 PM SGT		



	Name	Retention Time (min)	Area (?V*sec)	% Area
1	Reb A	8.457	937668	98.32
2	Stevioside	8.914	7376	0.77
3	Reb F	10.838	5102	0.53
4	Reb C	11.985	3574	0.37

D-5 Certificate of Analysis for Batch 29032011



Certificate Of Analysis

Customer Name: _____
Product Name: Rebaudioside A, 97%
Batch Number: 29032011
Amount: _____
Manufacturing Date: March 29, 2011
Expired Date: March 28, 2013
COA No: 003/2011
Date: December 22, 2011

ANALYTICAL RESULTS

Analytical Parameters	Specification	Result
Appearance Form	Powder	Powder
Appearance Color	White	White
Solubility (in water)	Freely Soluble	Freely Soluble
Purity (HPLC Area,%)	≥ 97	98.49
Residual Ethanol (ppm)	≤ 500	360
Residual Methanol (ppm)	≤ 200	Not Detected
Loss on Drying (%)	≤ 3.0	1.92
pH	4.5 – 7.0	6.3
Total Ash (%)	≤ 0.1	0.04
Arsenic (ppm)	≤ 0.02	Not Detected
Lead (ppm)	≤ 0.1	Not Detected
Mercury (ppm)	≤ 0.01	Not Detected
Cadmium (ppm)	≤ 0.01	Not Detected



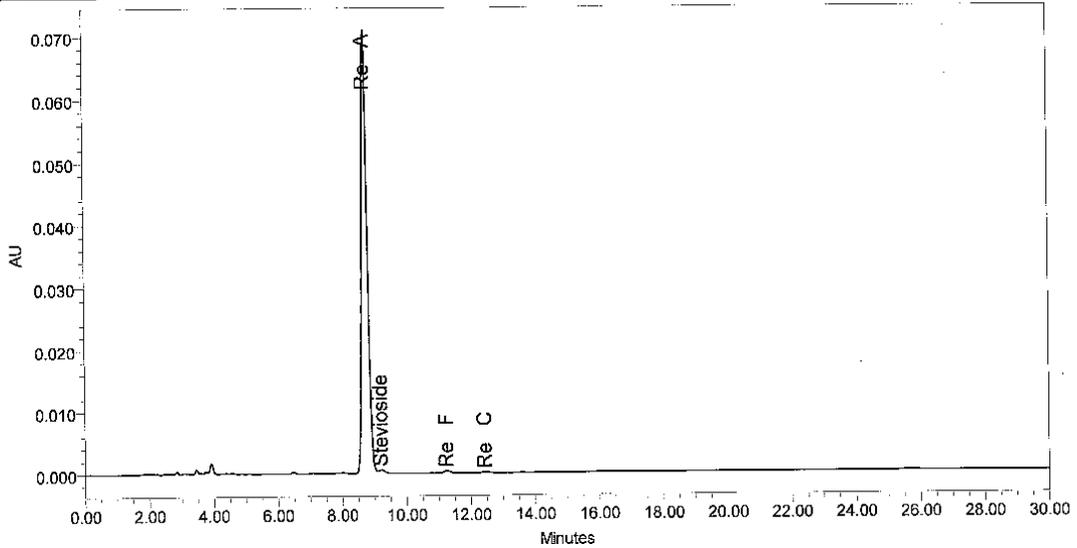
Microbiological Properties	Specification	Result
Total Plate Count (cfu/g, max)	1,000	120
Yeast (cfu/g, max)	100	< 10
Mold (cfu/g, max)	100	< 10
Heat Resistant Mold (in 50g)	Negative	Negative
Salmonella spp. (in 25g)	Negative	Negative
Staphylococcus aureus (in 10g)	Negative	Negative
Alicyclobacillus acidoterrestris (in 50g)	Negative	Negative
Listeria (in 1g)	Negative	Negative
Coliform/E.coli (in 1g)	Negative	Negative
Total Coliforms (in 25g)	Negative	Negative
Fecal Coliforms (in 25g)	Negative	Negative

(b) (6)

Ms. Pnita Chutasmit
Quality Assurance Manager



SAMPLE INFORMATION			
Sample Name:	29032011	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	Aug01_11_10ml_50ml
Vial:	54	Acq. Method Set:	Jecfa2010
Injection #:	1	Processing Method:	R100 RT 8_7
Injection Volume:	5.00 ul	Channel Name:	W2489 ChA
Run Time:	30.0 Minutes	Proc. Chnl. Descr.:	W2489 ChA 210nm
Date Acquired:	8/3/2011 3:09:47 AM SGT		
Date Processed:	12/13/2011 4:02:08 PM SGT		



	Name	Retention Time (min)	Area (?V*sec)	% Area
1	Re A	8.760	874290	98.46
2	Stevioside	9.227	6437	0.72
3	Re F	11.257	4612	0.52
4	Re C	12.465	2583	0.29

APPENDIX E

Pesticide Analytical Report from Overseas Merchandize Inspection Co. Ltd.



Overseas Merchandise Inspection Co., Ltd. 海外貨物検査株式会社
 No. 12 - 14 Yen Akas Soi 3 Chongnonsri, Yannawa, Bangkok 10120 Thailand
 Tel No. : +66-2-286-4120-3 Fax No. : +66-2-287-2570-1 URL : http://www.omicnet.com Page 1 of 5

TEST REPORT

ORIGINAL

Report No. : CBL58-13684(R)
 Sample ID : 58-13684
 Batch No. : JBL58/06383
 Report Date : December 6,2011

Customer : Almendra (Thailand) Ltd.
 All Seasons Places, 23 rd Fl., M-Thai Tower,
 87, Wireless Road, Lumpini, Phatumwan, Bangkok

Sample Description : FG P01
 Customer's Reference : BATCH NO. 26092011
 Received Date : November 11,2011
 Sample Condition : Sample is contained in Plastic bag, sealed.
 Analysis Commenced Date : November 11 ,2011
 Analysis Completed Date : November 15 ,2011

Test Item	Method	MDL	Result	Unit
Carbamate Group	In-house method CH-101-TM based on Journal of AOAC International. Vol. 86, No.2, 2003,p. 412-431.			
• 1-Naphthol		0.005	Not Detected	mg/kg
• Aldicarb		0.005	Not Detected	mg/kg
• Aldicarb sulfoxide		0.005	Not Detected	mg/kg
• Aldoxycarb		0.005	Not Detected	mg/kg
• Bendiocarb		0.005	Not Detected	mg/kg
• Bufencarb		0.005	Not Detected	mg/kg
• Carbaryl		0.005	Not Detected	mg/kg
• Carbofuran		0.005	Not Detected	mg/kg
• Etofol		0.005	Not Detected	mg/kg
• Fenobucarb		0.005	Not Detected	mg/kg
• Isoprocarb		0.006	Not Detected	mg/kg
• Methiocarb		0.005	Not Detected	mg/kg
• Methomyl		0.005	Not Detected	mg/kg
• Metolcarb		0.005	Not Detected	mg/kg
• Oxamyl		0.001	Not Detected	mg/kg
• Promecarb		0.005	Not Detected	mg/kg
• Propoxur		0.001	Not Detected	mg/kg
• Thiodicarb		0.001	Not Detected	mg/kg
• XMC		0.003	Not Detected	mg/kg
• Xyllycarb (MPMC)		0.005	Not Detected	mg/kg
Organochlorine Group	In-house method CH-094-TM based on Journal of AOAC International Vol. 86, No.2, 2003.			
• 1,1-Dichloro-2,2-Bis (4-Ethylphenyl) Ethane (Perthane)		0.2	Not Detected	mg/kg
• Acetochlor		0.06	Not Detected	mg/kg
• Alachlor		0.04	Not Detected	mg/kg
• Aldrin		0.004	Not Detected	mg/kg
• Allethrin		0.01	Not Detected	mg/kg
• Alpha-BHC		0.004	Not Detected	mg/kg
• Azaconazole		0.02	Not Detected	mg/kg
• Benfluralin		0.008	Not Detected	mg/kg

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Overseas Merchandise Inspection Co., Ltd. 海外貨物検査株式会社

No. 12 - 14 Yen Akas Soi 3 Chongnonsri, Yannawa, Bangkok 10120 Thailand
 Tel No. : +66-2-266-4120-3 Fax No. : +66-2-287-2570-1 URL : http://www.omicnet.com Page 2 of 5

ORIGINAL

Report No. : CBL58-13684(R)
 Sample ID : 58-13684
 Batch No. : JBL58/06383

Test Item	Method	MDL	Result	Unit
• Benoxacor		0.016	Not Detected	mg/kg
• Beta-BHC		0.008	Not Detected	mg/kg
• Bifenox		0.016	Not Detected	mg/kg
• Boscalid		0.001	Not Detected	mg/kg
• Bromophos		0.003	Not Detected	mg/kg
• Bromophos-ethyl		0.014	Not Detected	mg/kg
• Bromopropylate		0.012	Not Detected	mg/kg
• Bupirimate		0.04	Not Detected	mg/kg
• Butachlor		0.06	Not Detected	mg/kg
• Captafol		0.04	Not Detected	mg/kg
• Captan		0.006	Not Detected	mg/kg
• Carfentrazone-ethyl		0.001	Not Detected	mg/kg
• Chlorbenside		0.005	Not Detected	mg/kg
• Chlordane		0.004	Not Detected	mg/kg
• Chlorfenapyr		0.006	Not Detected	mg/kg
• Chlorfenson		0.006	Not Detected	mg/kg
• Chloroneb		0.04	Not Detected	mg/kg
• Chlorothalonil		0.01	Not Detected	mg/kg
• Chlorthal-dimethyl		0.006	Not Detected	mg/kg
• Chlozolinat		0.004	Not Detected	mg/kg
• Cinidon-ethyl		0.002	Not Detected	mg/kg
• Clomeprop		0.005	Not Detected	mg/kg
• Coumafos/Coumaphos		0.01	Not Detected	mg/kg
• DDD		0.006	Not Detected	mg/kg
• DDE		0.004	Not Detected	mg/kg
• DDT		0.006	Not Detected	mg/kg
• Delta-BHC		0.006	Not Detected	mg/kg
• Di-allate		0.01	Not Detected	mg/kg
• Dichlofluanid		0.02	Not Detected	mg/kg
• Dichloran		0.001	Not Detected	mg/kg
• Dichlormid		0.012	Not Detected	mg/kg
• Diclocymet		0.001	Not Detected	mg/kg
• Dicofol		0.04	Not Detected	mg/kg
• Dieldrin		0.005	Not Detected	mg/kg
• Dimethenamid		0.01	Not Detected	mg/kg
• Dimethipin		0.001	Not Detected	mg/kg
• Endosulfan Sulphate		0.006	Not Detected	mg/kg
• Endosulfan-Alpha		0.006	Not Detected	mg/kg
• Endosulfan-Beta		0.006	Not Detected	mg/kg
• Endrin		0.01	Not Detected	mg/kg
• Endrin aldehyde		0.02	Not Detected	mg/kg
• Endrin ketone		0.008	Not Detected	mg/kg
• Ethalfuralin		0.008	Not Detected	mg/kg
• Etridiazole		0.008	Not Detected	mg/kg
• Fenamidone		0.1	Not Detected	mg/kg
• Fipronil		0.008	Not Detected	mg/kg
• Flufenacet		0.02	Not Detected	mg/kg
• Fluthiacet-methyl		0.001	Not Detected	mg/kg
• Folpet		0.02	Not Detected	mg/kg
• Fthalide		0.004	Not Detected	mg/kg

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Overseas Merchandise Inspection Co., Ltd. 海外貨物検査株式会社

No. 12 - 14 Yen Akas Soi 3 Chongnonsri, Yannawa, Bangkok 10120 Thailand

Tel No. : +66-2-286-4120-3 Fax No. : +66-2-287-2570-1 URL : http://www.omicnet.com

Page 3 of 5

ORIGINAL

Report No. : CBL58-13684(R)

Sample ID : 58-13684

Batch No. : JBL58/06383

Test Item	Method	MDL	Result	Unit
• Heptachlor		0.006	Not Detected	mg/kg
• Heptachlor epoxide		0.004	Not Detected	mg/kg
• Hexachlorobenzene		0.002	Not Detected	mg/kg
• Isoxaflutole		0.05	Not Detected	mg/kg
• Lindane (gamma-BHC)		0.003	Not Detected	mg/kg
• Mefenpyr-diethyl		0.016	Not Detected	mg/kg
• Methoxychlor		0.01	Not Detected	mg/kg
• Myclobutanil		0.09	Not Detected	mg/kg
• Nitrapyrin		0.003	Not Detected	mg/kg
• Norflurazon		0.02	Not Detected	mg/kg
• Oxabetrinil		0.003	Not Detected	mg/kg
• Pentoxazone		0.016	Not Detected	mg/kg
• Pyridaben		0.003	Not Detected	mg/kg
• Pyridalyl		0.002	Not Detected	mg/kg
• Pyrifenoxy		0.002	Not Detected	mg/kg
• Quintozene		0.004	Not Detected	mg/kg
• Tecnazene		0.004	Not Detected	mg/kg
• Tetradifon		0.008	Not Detected	mg/kg
• Thiazopyr		0.02	Not Detected	mg/kg
• Thifluzamide		0.008	Not Detected	mg/kg
• Tolyfluanid		0.01	Not Detected	mg/kg
• Tri-Allate		0.008	Not Detected	mg/kg
• Trichlamide		0.001	Not Detected	mg/kg
• Triflumizole		0.002	Not Detected	mg/kg
• Trifluralin		0.008	Not Detected	mg/kg
• Vinclozolin		0.008	Not Detected	mg/kg
Pyrethroid Group	In-house method CH-094-TM based on Journal of AOAC International Vol. 86, No.2, 2003.			
• Bifenthrin		0.02	Not Detected	mg/kg
• Cyfluthrin		0.04	Not Detected	mg/kg
• Cyhalothrin		0.02	Not Detected	mg/kg
• Cypermethrin		0.04	Not Detected	mg/kg
• Deltamethrin, Tralomethrin		0.02	Not Detected	mg/kg
• Fenpropathrin		0.02	Not Detected	mg/kg
• Fenvalerate		0.02	Not Detected	mg/kg
• Flucythrinate		0.004	Not Detected	mg/kg
• Fluvalinate		0.01	Not Detected	mg/kg
• Permethrin		0.04	Not Detected	mg/kg
• Pyrethrin		0.03	Not Detected	mg/kg
Organophosphorus Group	In-house method CH-093-TM based on Anastassiades et al, Journal of AOAC International Vol. 86, No. 2, 2003.			
• Acephate		0.01	Not Detected	mg/kg
• Anilofos		0.03	Not Detected	mg/kg
• Azinphos-methyl		0.05	Not Detected	mg/kg
• Butamifos		0.005	Not Detected	mg/kg
• Cadusaphos		0.007	Not Detected	mg/kg
• Carbophenothion		0.01	Not Detected	mg/kg
• Chlorethoxyphos		0.005	Not Detected	mg/kg

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ORIGINAL

Report No. : CBL58-13684(R)
 Sample ID : 58-13684
 Batch No. : JBL58/06383

Test Item	Method	MDL	Result	Unit
• Chlorfenvinphos		0.01	Not Detected	mg/kg
• Chlorpyrifos		0.01	Not Detected	mg/kg
• Chlorpyrifos-methyl		0.01	Not Detected	mg/kg
• Cyanofenphos		0.01	Not Detected	mg/kg
• Cyanophos		0.005	Not Detected	mg/kg
• Demeton-S-methyl		0.005	Not Detected	mg/kg
• Diazinon		0.005	Not Detected	mg/kg
• Dichlofenthion		0.01	Not Detected	mg/kg
• Dichlorvos, Naled		0.003	Not Detected	mg/kg
• Dicrotophos		0.015	Not Detected	mg/kg
• Dimethoate		0.01	Not Detected	mg/kg
• Dimethylvinphos		0.01	Not Detected	mg/kg
• Dioxathion		0.02	Not Detected	mg/kg
• Disulfoton		0.005	Not Detected	mg/kg
• Edifenphos		0.01	Not Detected	mg/kg
• EPN		0.01	Not Detected	mg/kg
• Ethion		0.002	Not Detected	mg/kg
• Ethoprophos		0.005	Not Detected	mg/kg
• Etrimfos		0.006	Not Detected	mg/kg
• Famphur		0.03	Not Detected	mg/kg
• Fenamiphos		0.005	Not Detected	mg/kg
• Fenchlorphos		0.01	Not Detected	mg/kg
• Fenitrothion		0.01	Not Detected	mg/kg
• Fensulfothion		0.01	Not Detected	mg/kg
• Fenthion		0.01	Not Detected	mg/kg
• Fonofos		0.005	Not Detected	mg/kg
• Formothion		0.005	Not Detected	mg/kg
• Fosthiazate		0.02	Not Detected	mg/kg
• Iprobenfos		0.01	Not Detected	mg/kg
• Isazophos		0.01	Not Detected	mg/kg
• Isocarbofos		0.01	Not Detected	mg/kg
• Isofenphos		0.01	Not Detected	mg/kg
• Isofenphos Methyl		0.01	Not Detected	mg/kg
• Isoxathion		0.01	Not Detected	mg/kg
• Malathion		0.02	Not Detected	mg/kg
• Mecarbam		0.01	Not Detected	mg/kg
• Mephospholan		0.005	Not Detected	mg/kg
• Methacrifos		0.003	Not Detected	mg/kg
• Methamidophos		0.003	Not Detected	mg/kg
• Methidathion		0.01	Not Detected	mg/kg
• Mevinphos		0.005	Not Detected	mg/kg
• Monocrotophos		0.01	Not Detected	mg/kg
• Omethoate		0.02	Not Detected	mg/kg
• Parathion		0.01	Not Detected	mg/kg
• Parathion-Methyl		0.01	Not Detected	mg/kg
• Phenthoate		0.01	Not Detected	mg/kg
• Phorate		0.005	Not Detected	mg/kg
• Phosalone		0.03	Not Detected	mg/kg
• Phosmet		0.05	Not Detected	mg/kg
• Phosphamidon		0.02	Not Detected	mg/kg

No person or entity should rely upon this Certificate/Report or any information contained therein without having express knowledge of the terms and conditions of the OMIC General Conditions of Business as printed on the reverse side of this certificate subject to which this Certificate/Report



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ORIGINAL

Report No. : CBL58-13684(R)

Sample ID : 58-13684

Batch No. : JBL58/06383

Test Item	Method	MDL	Result	Unit
• Piperophos		0.01	Not Detected	mg/kg
• Pirimioxyphos		0.02	Not Detected	mg/kg
• Pirimiphos-Ethyl		0.01	Not Detected	mg/kg
• Pirimiphos-Methyl		0.01	Not Detected	mg/kg
• Profenofos		0.01	Not Detected	mg/kg
• Propaphos		0.01	Not Detected	mg/kg
• Propetamphos		0.005	Not Detected	mg/kg
• Prothiophos		0.01	Not Detected	mg/kg
• Pyrazophos		0.03	Not Detected	mg/kg
• Pyridafenthion		0.01	Not Detected	mg/kg
• Quinalphos		0.01	Not Detected	mg/kg
• Salithion		0.005	Not Detected	mg/kg
• Sulprofos		0.005	Not Detected	mg/kg
• Terbufos		0.005	Not Detected	mg/kg
• Tetrachlorvinphos		0.01	Not Detected	mg/kg
• Thiometon		0.005	Not Detected	mg/kg
• Tolclofos-Methyl		0.005	Not Detected	mg/kg
• Triazophos		0.01	Not Detected	mg/kg
• Tribufos		0.01	Not Detected	mg/kg
• Trichlorfon		0.005	Not Detected	mg/kg
• Vamidothion		0.03	Not Detected	mg/kg

The sample(s) will be held for thirty days from the date of the report.

Reported result refers to submitted sample(s) only.

This report shall not be reproduced except in full, without written approval of the laboratory.

Overseas Merchandise Inspection Co., Ltd.
 Bangkok Laboratory

(b) (6)

(KUNAPORN SANGUANKAEW)
 Supervisor of Chromatography Section

APPENDIX F

Summary of Regulatory and Expert Body Safety Reviews

1. Summary of JECFA Reviews

At an early review at its 51st meeting, JECFA (WHO, 2000) expressed the following reservations about the safety data available at that time for steviol glycosides:

The Committee noted several shortcomings in the information available on stevioside. In some studies, the material tested (stevioside or steviol) was poorly specified or of variable quality, and no information was available on other constituents or contaminants. Furthermore, no studies of human metabolism of stevioside and steviol were available. In addition, data on long-term toxicity and carcinogenicity were available for stevioside in only one species. The mutagenic potential of steviol has been tested sufficiently only *in vitro*.

In view of the absence of information for the elaboration of specifications for stevioside and since the evaluation of the available toxicological data revealed several limitations, the Committee was unable to relate the results of the toxicological investigations to the commercial product and could not allocate an ADI to stevioside.

Before reviewing stevioside again, the Committee considered that it would be necessary to develop specifications to ensure that the material tested was representative of the commercial product. Further information on the nature of the substance that was tested, data on the metabolism of stevioside in humans and the results of suitable *in vivo* genotoxicity studies with steviol would also be necessary.

Subsequently, additional data were generated on the metabolism of steviol glycosides and submitted to JECFA. This information suggested that the common steviol glycosides are converted to steviol by intestinal bacteria and then rapidly converted to glucuronides that are excreted. The committee now had a molecular basis to become comfortable with new toxicology studies on test materials which consisted of variable composition but were relatively high purity mixtures of the common steviol glycosides. The new information also revealed that in *in vitro* studies steviol is mutagenic, while *in vivo* condition it is not mutagenic. The committee became convinced that purified steviol glycosides did not impair reproductive performance as did crude preparations of stevia and that there were sufficient chronic studies in rats with adequate no observed effect levels (NOEL) that could support a reasonable acceptable daily intake (ADI) in the range of doses that would be encountered by the use of steviol glycosides as a sugar substitute. However, JECFA wanted more clinical data to rule out pharmacological effects at the expected doses. The following excerpt was taken from the report of the 63rd meeting (WHO, 2006):

The Committee noted that most of the data requested at its fifty-first meeting, e.g., data on the metabolism of stevioside in humans, and on the activity of steviol in suitable studies of genotoxicity *in vivo*, had been made available. The Committee concluded that stevioside and rebaudioside A are not genotoxic *in vitro* or *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives *in vitro* is not expressed *in vivo*.

The NOEL for stevioside was 970 mg/kg bw/day in a long-term study (Toyoda et al., 1997) evaluated by the Committee at its fifty-first meeting. The Committee noted that stevioside has shown some evidence of pharmacological effects in patients with hypertension or with type-2 diabetes at doses corresponding to about 12.5–25 mg/kg bw/day (equivalent to 5–10 mg/kg bw/day expressed as steviol). The evidence available at present was inadequate to assess whether these pharmacological effects would also occur at lower levels of dietary exposure, which could lead to adverse effects in some individuals (e.g., those with hypotension or diabetes).

The Committee therefore decided to allocate a temporary ADI, pending submission of further data on the pharmacological effects of steviol glycosides in humans. A temporary ADI of 0–2 mg/kg bw was established for steviol glycosides, expressed as steviol, on the basis of the NOEL for stevioside of 970 mg/kg bw/day (or 383 mg/kg bw/day,

expressed as steviol) in the 2-year study in rats and a safety factor of 200. This safety factor incorporates a factor of 100 for inter- and intra-species differences and an additional factor of 2 because of the need for further information. The Committee noted that this temporary ADI only applies to products complying with the specifications.

The Committee required additional information, to be provided by 2007, on the pharmacological effects of steviol glycosides in humans. These studies should involve repeated exposure to dietary and therapeutic doses, in normotensive and hypotensive individuals and in insulin-dependent and insulin-independent diabetics.

In 2007, at its 68th meeting, JECFA (WHO, 2007) concluded that sufficient progress had been made on the clinical studies and extended the temporary ADI until 2008. Subsequently, sufficient data had been received by JECFA to revise and finalize food additive specifications for steviol glycosides (FAO, 2007a). The Chemical and Technical Assessment report written after the 2007 meeting, explained the Committee's thinking which resulted in flexibility in the identity specifications (FAO, 2007b).

In response to the call for data on stevioside for the 63rd meeting of the Committee, submissions from several countries showed that the main components of the commercially available extracts of stevia are stevioside and rebaudioside A, in various amounts ranging from about 10-70% stevioside and 20-70% rebaudioside A. The information indicated that most commercial products contained more than 90% steviol glycosides with the two main steviol glycosides comprising about 80% of the material. The 63rd JECFA required that the summed content of stevioside and rebaudioside A was not less than 70% and established a minimum purity of 95% total steviol glycosides. Analytical data showed that most of the remaining 5% could be accounted for by saccharides other than those associated with the individual steviol glycosides.

Noting that the additive could be produced with high purity (at least 95%) and that all the steviol glycosides hydrolyze upon ingestion to steviol, on which the temporary ADI is based, the 68th JECFA decided it was unnecessary to maintain a limit for the sum of stevioside and rebaudioside content. The Committee recognized that the newly revised specifications would cover a range of compositions that could include, on the dried basis, product that was at least 95% stevioside or at least 95% rebaudioside A.

In 2008, based on additional clinical studies, at its 69th meeting, 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 in the final toxicology monograph addendum (WHO, 2009) were stated as follows:

From a long-term study with stevioside, which had already been discussed by the Committee at its fifty-first meeting, a NOEL of 970 mg/kg bw per day was identified. At its sixty-third meeting, the Committee set a temporary ADI of 0-2 mg/kg bw for steviol glycosides, expressed as steviol, on the basis of this NOEL for stevioside of 970 mg/kg bw per day (383 mg/kg bw per day expressed as steviol) and a safety factor of 200, pending further information. The further information was required because the Committee had noted that stevioside had shown some evidence of pharmacological effects in patients with hypertension or with type 2 diabetes at doses corresponding to about 12.5-25.0 mg/kg bw per day (5-10 mg/kg bw per day expressed as steviol).

The results of the new studies presented to the Committee at its present meeting have shown no adverse effects of steviol glycosides when taken at doses of about 4 mg/kg bw per day, expressed as steviol, for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal or low-normal blood pressure for 4 weeks. The Committee concluded that the new data were sufficient to allow the additional safety factor of 2 and the temporary designation to be removed and established an ADI for steviol glycosides of 0-4 mg/kg bw expressed as steviol.

The Committee noted that some estimates of high-percentile dietary exposure to steviol glycosides exceeded the ADI, particularly when assuming complete replacement of caloric sweeteners with steviol glycosides, but recognized that these estimates were highly conservative and that actual intakes were likely to be within the ADI range.

2. Summary of FSANZ Review of Steviol Glycosides

In 2008, FSANZ completed a review of the safety of steviol glycosides for use as a sweetener in foods. FSANZ concluded that steviol glycosides are well tolerated and unlikely to have adverse effects on blood pressure, blood glucose or other parameters in normal, hypotensive or diabetic subjects at doses up to 11 mg/kg bw/day. FSANZ agreed with JECFA in setting an ADI of 4 mg steviol equivalents/kg bw/day, which was derived by applying a 100-fold safety factor to the NOEL of 970 mg/kg bw/day established by a 2-year rat study (Toyoda et al., 1997). The FSANZ review discussed the adequacy of the existing database and several new studies, including the clinical studies reviewed by JECFA in the summer of 2007, most notably the work of Barriocanal et al. (2008), which was later published in 2008.

In their draft document, FSANZ also indicated that the new data in humans provides a basis for revising the uncertainty factors that were used by JECFA to derive the temporary ADI for steviol glycosides in 2005. In particular, the evidence surrounding the pharmacological effects of steviol glycosides on blood pressure and blood glucose has been strengthened so that the additional 2-fold safety factor for uncertainty related to effects in normotensive or diabetic individuals is no longer required. Therefore, FSANZ established an ADI of 4 mg/kg bw/day for steviol glycosides as steviol equivalents, 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 (FSANZ, 2008). In December 2010, FSANZ recommended accepting the increased usage levels 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).

3. Summary of EFSA Review of Steviol Glycosides

On March 10, 2010, EFSA adopted a scientific opinion on the safety of steviol glycosides (mixtures that comprise not less than 95% of stevioside and/or rebaudioside A) as a food additive. Earlier---in 1984, 1989 and 1999---the Scientific Committee for Food (SCF) evaluated stevioside as a sweetener. At the time, the SCF concluded that the use of stevioside was "toxicologically not acceptable" due to insufficient available data to assess its safety. However, in light of JECFA's 2008 findings and in response to a June 2008 request by the European Commission, EFSA reevaluated the safety of steviol glycosides as a sweetener. As both rebaudioside A and stevioside are metabolized and excreted by similar pathways, with steviol being the common metabolite for both glycosides, the EFSA Panel agreed that the results of toxicology studies on either stevioside or rebaudioside A are applicable for the safety assessment of steviol glycosides. Considering the available safety data (*in vitro* and *in vivo* animal studies and some human tolerance studies), the EFSA Panel concluded that steviol glycosides, complying with JECFA specifications, are not carcinogenic, genotoxic, or associated with any reproductive/developmental toxicity. The EFSA Panel established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg/kg bw/day based on the application of a 100-fold uncertainty factor to the NOAEL in the 2-year carcinogenicity study in the rat when administering 2.5% stevioside in the diet. This is equal to 967 mg stevioside/kg bw/day (corresponding to approximately 388 mg steviol equivalents/kg bw/day). Conservative estimates of steviol glycosides exposures both in adults and in children suggest that the ADI could possibly be exceeded by European consumers of certain ages and geographies at the maximum proposed use levels.

Recently, EFSA (2011a) revised its exposure assessment of steviol glycosides from its uses as a food additive for children and adults and published the reduced usage levels in 16 foods by a factor of 1.5 to

3, with no changes for 12 food groups. Additionally, 15 other foods were removed, mainly within the category of desserts and other products, while 3 new food uses were added. The mean estimated exposure to steviol glycosides (equivalents) in European children (aged 1-14 years) ranged from 0.4 to 6.4 mg/kg bw/day and from 1.7 to 16.3 mg/kg bw/day at the 95th percentile. A correction was considered to be necessary for the consumption of non-alcoholic flavored drinks (soft drinks) by children, and the corrected exposure estimate at the 95th percentile for children ranged from 1.0 to 12.7 mg/kg bw/day. For adults, the mean and 97.5th percentile intakes were estimated to range from 1.9 to 2.3 and 5.6 to 6.8 mg/kg bw/day, respectively. Non-alcoholic flavored drinks (soft drinks) are the main contributors to the total anticipated exposure to steviol glycosides for both consumer categories. For high consumers, EFSA noted that revised exposure estimates to steviol glycosides remain above the established ADI of 4 mg/kg bw (steviol equivalent).

In addition, EFSA (2011b) recently accepted rebaudioside A as a flavoring agent in a variety of foods. EFSA reviewed the available safety data on rebaudioside A and agreed that the ADI of 4mg/kg bw/day established for steviol glycosides applied also to rebaudioside A in a purified form. The dietary intake for use as a flavoring agent was calculated by two different methods, and EFSA determined that the worst case exposure would be 10,888 microgram/person/day, which is equivalent to 181 microgram rebaudioside A/kg bw/day, for a person weighing 60 kg. This corresponds to a daily intake of 60 microgram steviol/kg bw/day, using a conversion factor of 0.33 for converting the amount of rebaudioside A into steviol equivalents.

APPENDIX G

Safety Data on Stevioside Stevia Extracts That Are Predominantly Stevioside Rebaudioside A

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).

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.

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 ($\geq 97\%$) 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 G-1. 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 G-1. 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 G-2. 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 G-2. 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 G-3). 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 4, 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).

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).

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 G-4.

Table G-3. 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.

^a Only abstract available.

Table G-4. 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 Oommen, 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.
Yodyingyud 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, 1999.

Mutagenicity Genotoxicity Studies

In a series of studies mutagenic and genotoxic effects of stevia and stevioside were investigated. These studies are summarized in Table G-5. 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). Recently, the genotoxicity data on steviol glycosides was reviewed and considered adequate to support the safety of its use as a sweetener in foods (Urban et al., 2013).

Table G-5. 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)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Stevioside	NS	50 mg/plate	Negative	Klongpanichpak et al (1997)
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.

Clinical Studies

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 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%), rebaudioside A (24%) 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).

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 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 (Barriocanal et al., 2006).

In a follow-up study, Barriocanal et al. (2008) evaluated the effects of steviol glycosides on blood glucose and blood pressure (BP) for three months in subjects with type 1 diabetes, subjects with type 2 diabetes, and subjects without diabetes and with normal/low-normal BP levels. Patients in each group received either 250 mg t.d.s. (total dissolved solids) steviol glycoside stevioside or placebo treatment. The purity of the steviol glycosides was $\geq 92\%$. Three months of follow up revealed no changes in systolic BP, diastolic BP, glucose and glycated hemoglobin from baseline. In placebo type 1 diabetics there was a significant difference in systolic BP and glucose. There were no adverse effects observed in either treatment group, and the authors concluded that oral steviol glycosides are well-tolerated and have no pharmacological effect.

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).

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).

APPENDIX H

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 \geq 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 H-1.

Table H-1. 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.
Compadre et al., 1988 ^a	<i>In Vitro</i>	Bacterial Mutagenicity,	Not Reported	Positive	Mass spectral analysis of steviol and analogues under conditions known to produce a mutagenic response.

	<i>IN VIVO/IN VITRO</i>	SYSTEM	TEST SAMPLE PURITY	AUTHOR CONCLUSION	RESULTS AND REMARKS
		Mass Spec			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