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BY:----------

May 8, 2008

Dr. Robert L. Martin
Deputy Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Martin:

The Whole Earth Sweetener Company LLC, a wholly owned subsidiary of Merisant Company, 33 N. Dearborn, Suite 200, Chicago, IL 60602, is submitting in triplicate (plus an electronic copy), the enclosed GRAS Notice for proposed uses of Rebaudioside A (Reb A). We are submitting this document in conformance with the requirements of FDA's proposed regulation 21 CFR 170.36, as outlined in the *Federal Register* of April 17, 1997 (62 FR 18938-18964).

Attached to this GRAS Notice are an Expert Panel Opinion document and a Reb A GRAS Dossier. These two attachments provide the basis for the safety conclusions about the proposed uses of Reb A, and support the general availability and general acceptance of these conclusions.

If you should have any questions about the enclosed materials, please do not hesitate to send an e-mail (sue.andress@merisant.com) or call me at (312) 840-5059. We look forward to receiving your formal response to this Notice. In the meantime, would you please notify me by e-mail that you have received this Notice and that it is currently under review in your Division.

Thank you very much.

Sincerely yours,

Sue Andress
Director of Regulatory Affairs
Whole Earth Sweetener Company LLC

GRAS NOTICE FOR REBAUDIOSIDE A
(REB A)

GRAS NOTICE FOR REBAUDIOSIDE A (REB A)

SUBMITTED BY:

Whole Earth Sweetener Company LLC
A wholly owned subsidiary of Merisant Company
33 N. Dearborn, Suite 200
Chicago, IL 60602

SUBMITTED TO:

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

CONTACT FOR TECHNICAL OR OTHER INFORMATION:

Whole Earth Sweetener Company LLC
A wholly owned subsidiary of Merisant Company
33 N. Dearborn, Suite 200
Chicago, IL 60602

April 28, 2008

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I. GRAS EXEMPTION CLAIM

This section (Section I) of the current GRAS Notice fulfills the requirements of proposed 21 CFR 170.36(c)(1)(i-v). In the remainder of this document, Section II fulfills the requirements of proposed 21 CFR 170.36(c)(2) by providing detailed information about the identity of Reb A, its method of manufacture and its product characteristics. Section III of this GRAS Notice fulfills the requirements of proposed 21 CFR 170.36(c)(3) by providing information on the technical effect of Reb A and any self-limiting levels of use. Together, Sections IV, V and VI of this GRAS Notice fulfill the requirements of proposed 21 CFR 170.36(c)(4)(i) (GRAS determination by scientific procedures) by providing a complete and comprehensive summary of the existing knowledge and information about Reb A that support its status as generally recognized as safe for its proposed uses. Not only do the available data and information about Reb A for its proposed uses satisfy the safety standard of reasonable certainty of no harm, but because this information about Reb A is generally known and generally accepted by a consensus of qualified experts, the proposed uses of Reb A in this notice are generally recognized as safe (GRAS).

A. Name and Address of Notifier

Whole Earth Sweetener Company LLC, a wholly owned subsidiary of Merisant Company, 33 N. Dearborn, Suite 200, Chicago, IL 60602, hereby notifies the Food and Drug Administration that the use of Reb A described below is exempt from the pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act because the Whole Earth Sweetener Company LLC has determined that such use is generally recognized as safe (GRAS) through scientific procedures.

Sue Andress
Director of Regulatory Affairs
Whole Earth Sweetener Company LLC

Date 4/28/08

B. Name of GRAS Substance

Rebaudioside A (common or usual name Reb A) is the subject of this GRAS Notice.

C. Intended Use and Consumer Exposure

Reb A is proposed for use as a sweetener in selected beverages (including sweetened teas, diet soft drinks, energy drinks and flavored waters), cereals (oatmeal, cold cereal, cereal bars) at

levels ranging from 150 to 500 parts per million (150-500 ppm), depending on the product. Reb A also is proposed for tabletop use at a level of 30,000 ppm, which reflects the content in a sachet prior to dilution. Based on these use levels and daily estimates derived from the NHANES 2003-2004 database, the estimated daily intake (EDI) of Reb A is 2 milligrams per kilogram body weight per day (2 mg/kg bw/day) at the mean and 5 mg/kg bw/day at the 90th percentile on a per user basis.

D. Basis for GRAS Determination

Regulatory Framework

The regulatory framework for determining whether a substance can be considered generally recognized as safe (GRAS) in accordance with section 201(s) (21 U.S.C. § 321(s)) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 et. Seq.) (the "Act"), is set forth at 21 CFR 170.30, which states:

> General recognition of safety may be based only on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. 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 based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies which may be corroborated by unpublished studies and other data and information.

GRAS Determination

The basis for the GRAS determination of Reb A is scientific procedures.

Reb A is a steviol glycoside derived from the leaves of *Stevia rebaudiana* (Bertoni). There is an extensive database on structurally-related steviol glycosides including specific studies for which Reb A was the test material. All steviol glycosides, including Reb A,are metabolized by gastrointestinal microflora via the same intermediates and hydrolysis pathways to the aglycone, steviol; therefore, the safety data for all characterized steviol glycosides are relevant to the safety evaluation of Reb A. However, data for other steviol glycosides provide a conservative safety evaluation for Reb A because they all hydrolyze to steviol more rapidly than does Reb A. FDA and other bodies, including JECFA previously reviewed data on the steviol glycosides and identified issues that needed to be resolved. Data are now available that addresses all of those

issues. Thus, there are no remaining toxicology issues to be resolved, and the metabolism and pharmacokinetic data for humans are well understood.

The criteria stated above are applied herein in an analysis of whether the use of Reb A as a sweetener for selected foods (tabletop sweeteners, sweetened ready-to-drink teas, diet carbonated soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal and sweetened cold cereals) is GRAS for the intended conditions of use described herein.

The published literature, supported by unpublished study results, clearly demonstrates the lack of toxicity of Reb A and other steviol glycosides in animals and humans from oral ingestion at levels that are well above those anticipated from Reb A use as a sweetener in tabletop sweeteners, beverages, and cereals.

The members of an Expert Panel (the Panel) were convened to evaluate the safety of Reb A and the Panel's Opinion is provided as Exhibit I of this Notice. All of the available relevant information about Reb A, steviol glycosides and steviol was summarized in a safety assessment document (GRAS Dossier, Exhibit II) entitled, "Safety Evaluation Dossier for Expert Panel Determination of the GRAS Status of Rebaudioside A (Reb A)." The Panel critically evaluated this Dossier and other information they deemed appropriate and relevant. The Panel unanimously concluded that the totality-of-the-evidence satisfies the safety standard of reasonable certainty of no harm for the intended conditions of uses of Reb A. In addition, because the information supporting safety is widely known and accepted by qualified experts, the Panel concluded Reb A is not only safe, but generally recognized as safe (GRAS) for the intended condition of use described herein.

Based on the findings of the Expert Panel and our own knowledge of the information compiled in the Dossier, we affirm that Reb A is GRAS for the intended conditions of use described herein.

E. Newly Implemented Statutory Considerations

The Federal Food, Drug, and Cosmetic Act (FFDCA) recently was amended by the Food and Drug Administration Amendments Act of 2007 (FDAAA; Public Law 110-85). Section 912 of the FDAAA, codified as section 301(ll) of the FFDCA, prohibits, in relevant part, the introduction or delivery for introduction into interstate commerce of any food to which has been added a drug approved under section 505 of the FFDCA or a drug for which substantial clinical investigations have been instituted and for which the existence of such investigations has been made public. To the best of our knowledge, Reb A is neither an approved drug under section 505 of the FFDCA nor a drug for which substantial clinical investigations have been instituted and for which the existence of such investigations has been made public.

F. Availability of Information

The data and information that serve as the basis for the GRAS determination, as well as the information that has become available since the GRAS determination, will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times as arranged by Whole Earth Sweetener Company LLC, 33 N. Dearborn, Suite 200, Chicago, IL 60602 (contact: Sue Andress, telephone (312) 840-5059; email: sue.andress@merisant.com).

II. IDENTITY OF SUBSTANCE AND PRODUCTION

This section of the GRAS notice fulfills the requirements of proposed 21 CFR 170.36(c)(2) by providing information in regard to specific chemical identity information and production and manufacture information for Reb A including product characteristics and specifications.

A. Trade or Common Name

Rebaudioside A (common or usual name Reb A) is the subject of this GRAS Notice. Reb A is a glycoside of steviol.

B. Chemical Name

The chemical name of Reb A is 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.

C. CAS Registry Number

The Chemical Abstract Service (CAS) registry number for Reb A is 58543-16-1.

D. Molecular and Structural Formula

The molecular formula of Reb A is $C_{44}H_{70}O_{23}$ and the molecular weight is 967.03. The structural formula of Reb A is shown in Figure 1 below.

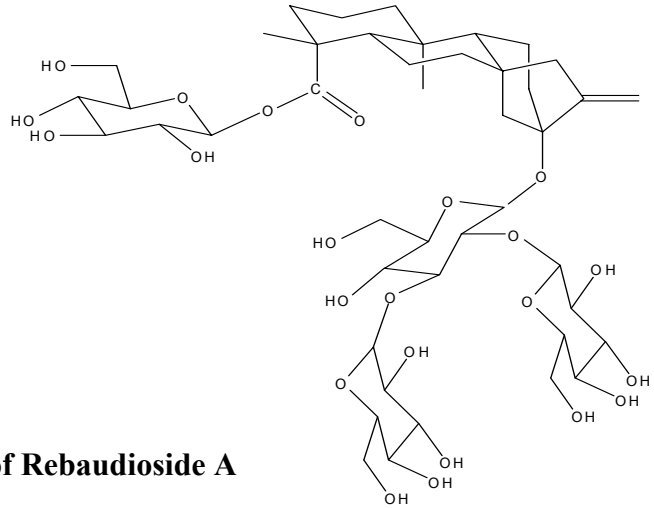
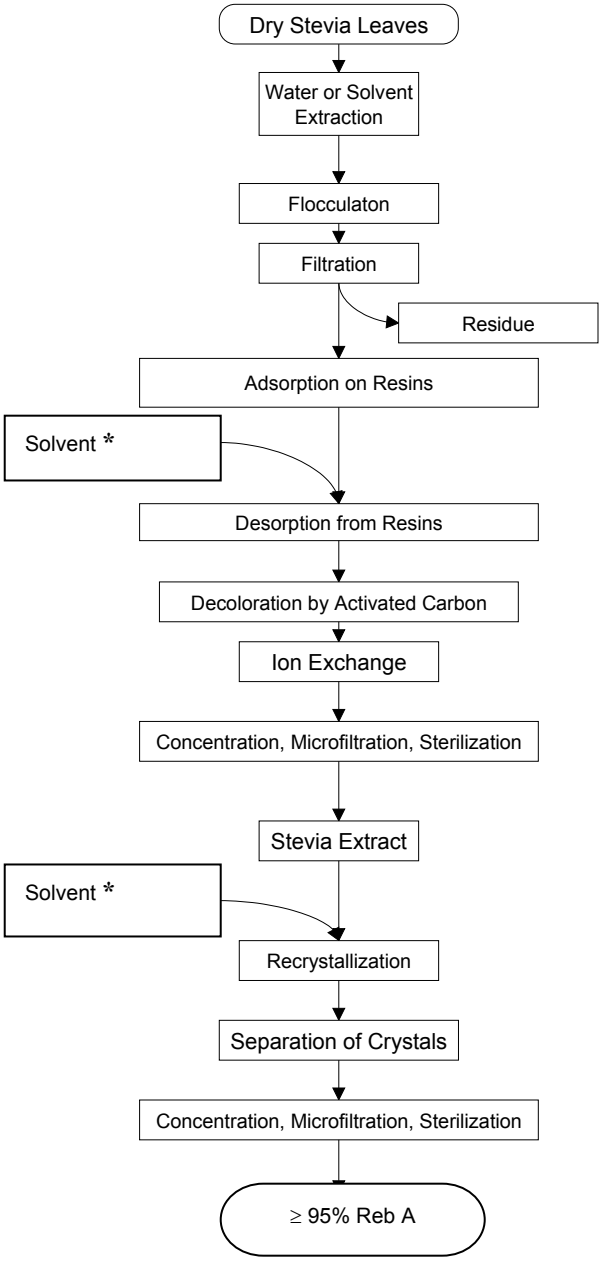


Figure 1. Structure of Rebaudioside A

E. Production Process

The source of Reb A, the leaves of *Stevia rebaudiana* (Bertoni), is a botanical, and as such the steviol glycoside content is slightly influenced by the temperature in the year of cultivation, the latitude, and the time of harvesting. High purity Reb A (≥95%) is obtained by extraction of the stevia leaves with water, ethanol or methanol and the extract is passed through an adsorption resin to trap and concentrate the desired steviol glycosides. The resin is washed with ethanol or methanol to release the glycosides. Through de-coloration and de-salting by ion exchange and microfiltration, consistently higher purity Reb A is obtained than would otherwise be the case. Recrystallization with alcohol/water mixtures results in high purity Reb A (≥95%) as the final product, which may be spray-dried or granulated and residual alcohol removed

Figure 2. Typical Method of Manufacture of Reb A



*Aqueous ethanol or methanol.

F. Product Characteristics

Reb A is a natural sweetener. It is a pure diterpene glycoside extracted from the plant *Stevia rebaudiana* (Bertoni), which belongs to the Asteraceae family and is native to Brazil and Paraguay. Reb A is the second most abundant glycoside in stevia leaf and is considered more suitable for use to sweeten foods and beverages than stevioside because of its greater solubility in water and better taste profile. Reb A tastes about 200 to 300 times sweeter than sugar and is non-nutritive. It is structurally similar to other steviol glycosides, including stevioside. In many parts of the world, including Japan, South Korea, Israel, Mexico, Paraguay, Brazil, and Argentina, steviol glycosides, including extracts of the leaves, stevioside and Reb A, are used to sweeten food products and beverages.

Reb A as defined in this GRAS Notice is a single chemically defined substance (≥ 95% purity) and differs from stevia leaves or stevia extracts, which are natural product "mixtures." The food grade specifications for Reb A are summarized in Table 1, below, and are comparable to those in the FAO/WHO Joint Expert Committee on Food Additives (JECFA) Monograph (2007) for Steviol Glycosides. Consistency of Reb A manufacturing has been demonstrated by analyses of three lots of Reb A and certificates of analysis from the vendor (See Exhibit II, Appendix I.-4).

Table 1. Purity and Specifications for Food Grade Reb A

Analytical Parameter	Acceptable Target/Range	Methods of Analysis
Purity Reb A (wt/wt %)	≥ 95% Reb A (on dry basis)	JECFA, 2007 (modified)
Total Steviol Glycosides (wt/wt %)	≥ 95% (on dry basis)	JECFA, 2007 (modified)
Stevioside (wt/wt %)	2% (on dry basis) maximum	JECFA, 2007 (modified)
Steviol (wt/wt %)	< 0.005% (on dry basis)	Bazargan *et al.*, 2007 (LC-MS/MS)
Moisture Content (%) by Loss on Drying	5.0% maximum	FCC, 2003 (p. 855)
Optical Rotation	-29 to -37 degrees	FCC, 2003 (p. 844)
Arsenic (as As)	1.0 mg/kg maximum	FCC, 2003 (p. 861)
Lead	1.0 mg/kg maximum	FCC, 2003 (p. 867)
Cadmium	1.0 mg/kg maximum	FCC, 2003 (p. 863)

Reb A is derived from a botanical product and, therefore, quality specifications have been set to address microbiological contamination. The quality specifications for Reb A are summarized in Table 2, below. There are no detectable pesticide residues in the multi-residue screen.

Table 2. Quality Specifications for Reb A

Analytical Parameter	Acceptable Target/Range	Methods of Analysis
Total Aerobic Plate Count	1000 cfu/g maximum	Downes and Ito, 2001a
Total Aerobic Mold Count	100 mold cfu/g maximum	Downes and Ito, 2001b
Total Aerobic Yeast	100 yeast cfu/g maximum	Downes and Ito, 2001b
Heat Resistant Mold	Non-detectable (< 1 cfu/50 g)	Downes and Ito, 2001c
Coliform	< 10 cfu/g	Downes and Ito, 2001d
E. coli	< 3 MPN/g	Downes and Ito, 2001d
Salmonella ssp	Negative in 25g	Downes and Ito, 2001e
Staphylococcus aureus	Non-detectable (< 1 cfu/g)	Downes and Ito, 2001f
Alicyclobacillus acidoterrestris	Non-detectable (<1 cfu/50 g)	JFJA, 2007
Residual Solvents	Methanol < 300 ppm Ethanol < 1000 ppm	U.S. Pharmacopoeia, 2007
Pesticide Residues	Non-detectable	U.S. FDA, 2004; CDFA, 1988

III. INTENDED TECHNICAL EFFECT

This section of the GRAS Notice fulfills requirements of proposed 21 CFR 170.36(c)(1)(iii) and (c)(3) by providing information about the technical effect of Reb A in food and any self-limiting characteristics of Reb A use.

Reb A is proposed for use as a sweetener in selected beverages (including sweetened teas, diet soft drinks, energy drinks and flavored waters), cereals (oatmeal, cold cereal, cereal bars) and for tabletop use. The perception of sweetness is beverage and concentration dependent. However, qualitative assessments indicate that 20 ppm is the lowest sweetness threshold in a beverage system. Qualitative assessments also indicate that 14 Brix is considered a "too sweet" beverage. In a sweetness equivalency study it was determined that it takes approximately 1200 ppm Reb A in water to match the sweetness of a 14% sugar solution. Most beverages are sweetened to approximately 10 Brix or 10% sugar solution. Experimentation with 500 ppm Reb A indicated that the sweetness potency is 200X; this factor was derived as follows: 10%/0.05% = 200X. The overall sensory stability of Reb A as determined using "bench-produced" beverages stored at 70ºF is significantly better than aspartame over the beverage shelf-life period of 24 weeks. Taste degradation of Reb A was not detected up to 20 weeks.

The functionality of Reb A as a sweetener in foods was demonstrated by sensory panel ratings of coffee sweetened with tabletop powder. Process capability and iso-sweetness data analysis supports a target Reb A level of 26.10 mg/serving. Reb A in a tabletop powder is stable and functional as a sweetener through 26 weeks under accelerated storage conditions, 40 ± 2°C and 75 ± 5% RH.

IV. INTENDED USES AND CONSUMER EXPOSURE

This section of the GRAS Notice fulfills requirements of proposed 21 CFR 170.36(c)(4)(i)(A) in regard to the probable consumption of Reb A as a result of its intended uses and use levels in a variety of foods.

A. Intended Uses and Use Levels

As noted previously, Reb A is proposed for tabletop use and for use as a sweetener in selected beverages (i.e., sweetened ready-to-drink iced teas, fruit juice drinks, diet soft drinks, energy drinks and flavored waters) and cereals (i.e., oatmeal, cold cereal, cereal bars). Specifically, Reb A is proposed for use at the levels in Table 3, below.

Table 3. Proposed Uses and Levels of Reb A (ppm):

Food Group	Reb A (ppm)
Tabletop sweeteners	30,000[1]
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

[1] Reb A content of sachet prior to dilution and not representative of "as consumed"

B. Estimated Daily Intake (EDI) of Reb A

Available Data and Methods

The estimated daily intake (EDI) of Reb A from the proposed uses including tabletop sweeteners, sweetened ready-to-drink iced teas, diet soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal, and sweetened cold cereals, was estimated using the proposed use levels provided in Appendix IV.-1 of the attached Dossier (entitled NHANES 2003-2004 Food Codes and Reb A Use Level Included in EDI Analysis), Exponent Inc.'s Foods and Residue Evaluation Program (FARE™ 8.09) software, and data from the most recent National Health and Nutrition Examination Survey (NHANES 2003-2004).

The NHANES 2003-2004 (NCHS, 2007) is a complex multistage probability sample designed to be representative of the civilian U.S. population. The survey collects two days of food intake data, in addition to nutrition, demographic, and health information. Exponent Inc. used the statistically weighted values from the survey in the analyses. The statistical weights compensate for variable probabilities of selection, adjust for non-response, and provide intake estimates that are representative of the U.S. population and the selected age-gender subgroups.

Exponent Inc. estimated the daily intake on a per "user" basis. In this analysis, a "user" is anyone who reported consuming at least one category of food in which it is proposed to use Reb A (Reb A food category) on either of the survey days, i.e. United States Department of Agriculture's (USDA's) "user" definition. Each individual who reported consuming a Reb A food on either of the survey days was identified, and that individual's responses for both survey days was used. Because Reb A is likely to be consumed over a lifetime it is appropriate to average exposures over a longer period than one day. Therefore, Exponent Inc. used each respondent's food consumption averaged over the two days of the NHANES 2003-2004 survey. A 2-day average typically overestimates lifetime average daily intake especially for foods eaten infrequently; however, only two nonconsecutive days' worth of food consumption data are available in the most recent NHANES 2003-2004 survey database. It is well known that food consumption data collected over longer periods of time, e.g., 14 days as in Market Research Corporation of America (MRCA) consumer surveys, yield estimates of daily intake that may be significantly lower than 2-day averages (Lambe *et al.*, 2000). Therefore, actual consumer exposures are lower than these estimates.

Estimated Daily Intake

The EDI of Reb A was calculated by multiplying each NHANES respondents' 2-day average food intake by the use levels described in Table 3, above. Each individual's intake of Reb A was divided by his/her bodyweight to provide the per capita and per user intakes on a bodyweight basis. Mean and 90th percentile daily intakes on a per user basis, as mg Reb A/kg bw/day, were estimated for the proposed uses of Reb A for the U.S. population (individuals 2+ years old). These results are presented in Table 4, below.

Table 4. Estimated Daily Intake of Reb A from Proposed Uses (U.S. Population, 2+ Age Group)

Food Category		Per User (mg Reb A/ kg bw/day)	
	N (Unweighted)	Mean	90th Percentile
Tabletop sweeteners	665	0.8	1.7
Sweetened ready-to-drink teas	964	1.0	2.4
Diet soft drinks	1182	3.3	7.4
Fruit juice drinks	2801	1.0	2.2
Energy drinks	37	0.4	0.7
Flavored Water	16	1.1	1.6
Cereal bars	336	0.1	0.1
Oatmeal	600	0.4	0.8
Cold cereals	284	0.1	0.2
Total (beverages, cereals, tabletop)	**4959**	**2.0**	**4.7**

Cereal products have the lowest EDI, with mean per user estimates ranging from 0.06 mg/kg bw/day for cereal bars to 0.42 mg/kg bw/day for oatmeal; energy drinks also fall within this low range. Paradoxically, the proposed use in diet soft drinks would appear to result in a higher EDI than the total, with mean and 90th percentile per user estimates at 3.3 mg/kg/day and 7.4 mg/kg/day, respectively. The higher estimate for diet soft drinks is due to the fact that the consumers who consume diet soft drinks are not necessarily the same consumers who consume cereals or tabletop sweeteners, and in many cases will not be the same consumers. Thus, the population of "users" changes for the individual food categories and for the total across all categories.

The soft drink category has fewer consumers than the total for all categories. Also the serving size for diet soft drinks, because they contain a large proportion of water, is larger than the serving size for products such as cereals and tabletop sweeteners. Because the use level of Reb A is on a concentration (ppm) basis, estimated intake of Reb A is directly dependent on the amount of food consumed in a day as well as the levels of Reb A in those foods. When the intake for a consumer of diet soft drinks is combined with the intake for a consumer of cereals, the estimated intake among the population of consumers of all foods containing Reb A is lower than soft drink consumers and higher than cereal consumers due to the averaging effect of the two consumers.

The total EDI from all proposed uses is less than or equal to 2 mg/kg bw/day at the mean and 5 mg/kg bw/day at the 90th percentile of intake among users in the U.S. population (2+ age group).

A further breakout of intakes by age/sex subgroups has similar intake levels, as shown in Table 5.

Table 5. Estimated Daily Intake of Reb A from Proposed Uses (U.S. Population, Children, Teenagers and Adults)

Population	Unweighted Users	Per User (mg Reb A/kg bw/day)	
		Mean	90th Percentile
Children 2-5 years	438	2.4	5.1
Male teenagers	542	1.6	3.4
Female teenagers	567	1.2	2.4
Male >20 years	1207	2.0	4.8
Females >20 years	1416	2.2	5.6

The total EDI from all proposed uses is less than or equal to 2 mg/kg bw/day at the mean and 5 mg/kg bw/day at the 90th percentile of intake among users in the U.S. population (2+ years). The range of exposures among population subgroups is similar, e.g ,1.2 to 2.4 mg/kg bw/day (mean) and 2.4 to 5.6 mg/kg bw/day (90th percentile).

V. REVIEW OF TOXICOLOGICAL AND OTHER SAFETY RELATED DATA

This section of the GRAS Notice also fulfills requirements of proposed 21 CFR 170.36(c)(4)(i)(A) in regard to the generally available and accepted scientific data, information, methods, or principles that are relied on to establish safety. In particular, this section focuses on pharmacokinetic and metabolic data, toxicological data and other safety-related data and information currently available on steviol, and steviol glycosides. These data and this information include clinical studies focusing on blood glucose and blood pressure in human volunteers ingesting steviol glycosides.

The safety of Reb A and steviol glycosides has been evaluated in a wide variety of toxicology and other safety-related studies. The absorption, distribution, metabolism, and excretion (ADME) of Reb A and other steviol glycosides is well understood and has been described in publicly available studies. Understanding of the ADME for the steviol glycosides makes it possible to bring an array of data and information to bear upon the evaluation of the safety of Reb A. Included in the safety related studies on Reb A and other steviol glycosides are acute, subchronic, chronic/carcinogenicity, reproduction and developmental toxicity studies in laboratory animal models as well as genotoxicity studies. In addition, there are a number of studies in human volunteers. These clinical studies evaluate the potential effects of steviol glycosides on blood pressure and on blood glucose.

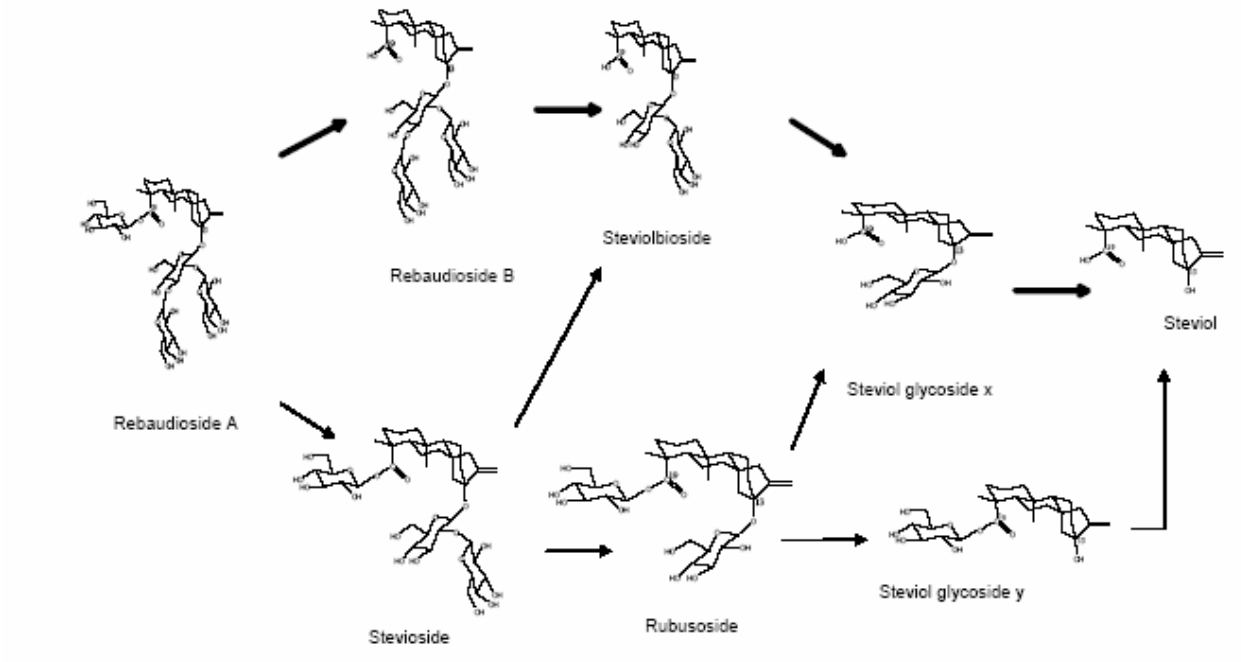
The totality of the evidence, both that which is published and that which is unpublished but corroborative, provides a basis upon which to conclude that the uses of Reb A described in this GRAS Notice satisfy the safety standard of Reasonable Certainty of No Harm. In addition, these data and information are known and accepted by a consensus of qualified experts in the general scientific community. Thus, this information base not only assures that the intended uses of Reb A described in this notice are safe, but also comprises common knowledge that Reb A is also generally recognized as safe under its intended conditions of use.

The following subsections of this Notice provide a general description of the preclinical and other toxicological studies and human safety-related studies on Reb A that support the above conclusions. In addition, the conclusions reached by the Expert Panel are presented below and are considered to be accurate by Whole Earth Sweetener Company LLC.

Absorption, Distribution, Metabolism, and Excretion

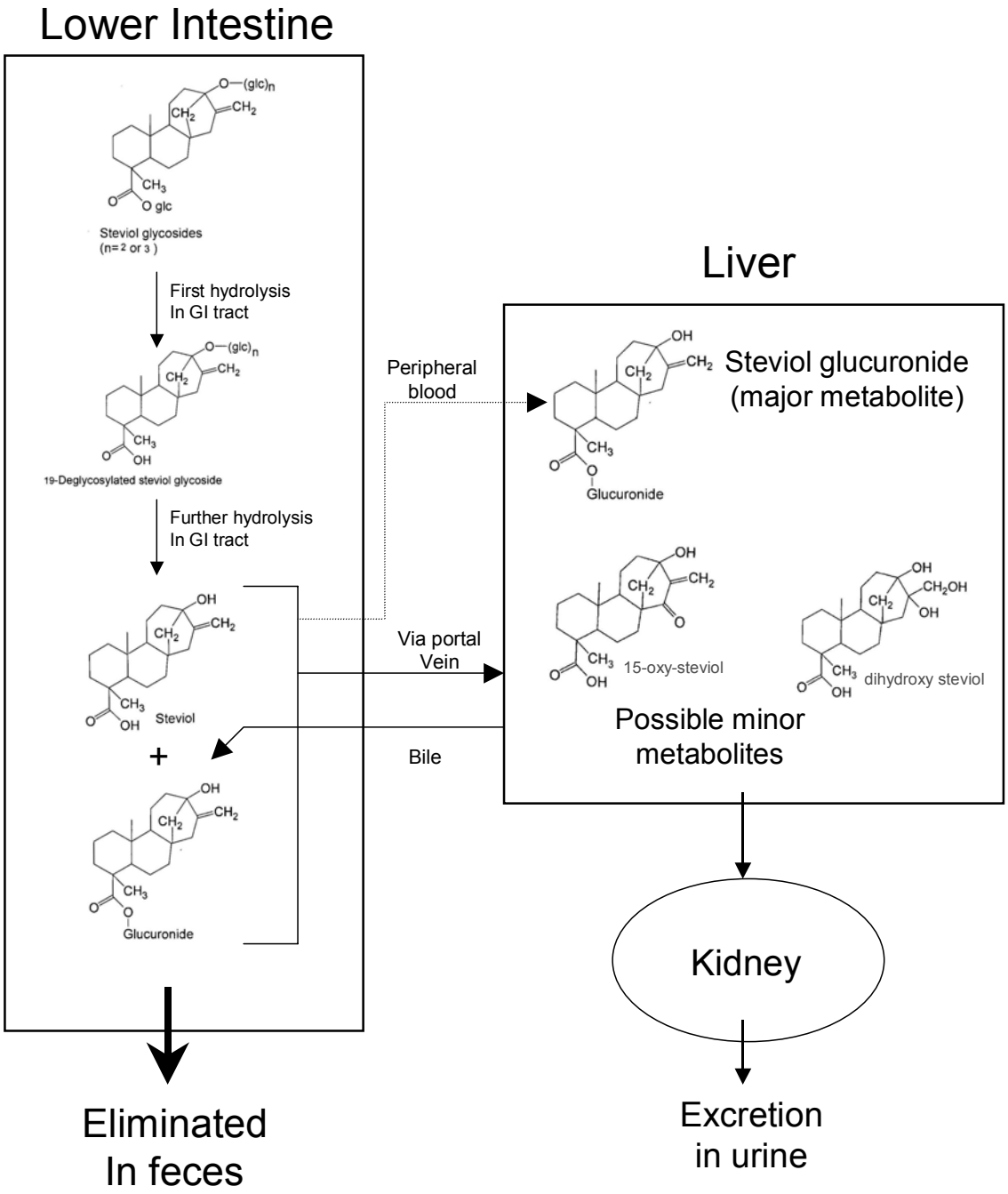
Research has shown Reb A and the other steviol glycosides to be hydrolyzed by common pathways (see Figure 3 below), but at different rates. Reb A is hydrolyzed the slowest of the steviol glycosides.

Figure 3. Common Hydrolysis Pathways of Reb A to the Aglycone, Steviol



The schematic drawing of the metabolic and elimination pathways for steviol glycosides based on the published literature and corroborated by new data for Reb A presented in Figure 4 below demonstrates that Reb A follows the same common pathways as other steviol glycosides. Briefly, the ester and ether linkages are prone to hydrolysis by bacteria in the cecum. The double bond in the aglycone, steviol, may be epoxydated rapidly followed by hydration to form the glycol as shown in Figure 4. Research has shown that all orally administered steviol glycosides, including Reb A, are hydrolyzed in the gut with the amount of degradation dependent on hydrolysis rates and residence time. Because of their large molecular weight and high polarity, the uptake of steviol glycosides by the intestinal tract is extremely low, as expected. This has been confirmed by numerous studies, including experiments with everted gastrointestinal sacs of rats (Koyama *et al.*, 2003a) and Caco-2 cell layers (Geuns *et al.*, 2003). A number of *in vitro* and *in vivo* studies indicated that stevioside is not absorbed across the digestive tract in rats or humans and is not metabolized by any of the normal digestive enzymes or by the acid conditions of the stomach (Wingard *et al.*, 1980; Nakayama *et al.*, 1986; Hutapea *et al.*, 1997; Koyama *et al.*, 2003a, b; Geuns and Pietta, 2004). Based on the results of all of the metabolism studies, ingested steviol glycosides are eliminated primarily in the feces or hydrolyzed to steviol prior to absorption from the gut. Excretion in the urine is a comparatively minor route of elimination.

Figure 4. Summary of possible metabolic reactions and elimination pathways for steviol glycosides



Human fecal microflora were found to completely hydrolyze stevioside and Reb A to their common aglycone, steviol, in 10 and 24 h, respectively, but the microflora did not degrade steviol. After incubation of stevioside or Reb A with human intestinal microflora, steviol epoxide derivatives were not detected. The absence of stevioside in the feces indicates that the bacterial flora hydrolyzed stevioside into steviol, which itself was not further metabolized, as was also shown in pigs *in vivo* (Geuns *et al.*, 2003), and in pig and human feces *in vitro* under anaerobic conditions (Geuns *et al.*, 2003, Koyoma *et al.*, 2003b, Gardana *et al.*, 2003). Geuns *et al.* (2007) reported that between 13 and 40 mg free steviol could be detected in human 24-hr feces sample collected following 3 doses/day of 250 mg stevioside/dose for three days.

Apart from steviol glucuronide and extremely small amounts of test material or free steviol, none of the possible steviol intestinal metabolites has been detected in blood or urine. Geuns *et al.* (2007) did not detect free steviol in the peripheral blood of the study subjects. Free steviol that is absorbed from the intestines is converted into steviol glucuronide by the enterocytes and/or liver (Geuns *et al.*, 2007; Simonetti *et al.*, 2004).

In summary, the major points pertinent to the ADME of Reb A and other steviol glycosides that follow the same common hydrolysis pathways are as follows:

- The half-life of Reb A (if absorbed) in animals and humans is less than 24 hours and there is no bioaccumulation over time even at doses well above those anticipated from the proposed uses.

- Steviol glycosides are not metabolized or transformed in the stomach or intestine by mammalian enzymes.

- The principal steviol glycosides, Reb A and stevioside, are only hydrolyzed in experimental animals and humans by intestinal microflora by sequential hydrolysis of glucose sugar moieties to free steviol.

- Steviol produced by intestinal hydrolysis is excreted primarily into feces (the primary pathway) or to a much lower extent is absorbed into plasma via the portal vein.

- Blood primarily contains the glucuronidated form of steviol; the conjugated form is excreted in urine.

- The metabolic pathways and kinetics of steviol glycosides in animals and humans are similar so the animal test data are appropriate for safety assessment. As a consequence of these similarities, data on the other steviol glycosides, notably stevioside, are also relevant to safety evaluation of Reb A.

In addition to the published data, the ADME of Reb A was investigated in a study in rats receiving Reb A via the diet at dose levels up to 2000 mg/kg bw/day. Besides steviol

glucuronide and extremely small amounts of administered test article or free steviol, the steviol glycosides have not been detected in blood. When Reb A and total steviol were detected in peripheral blood of rats during daily administration of 2000 mg/kg bw/day of Reb A it was at extremely low levels, with mean plasma concentrations of approximately 0.6 and 12 ug/mL, respectively. Estimates of absorbed dose for Reb A and total steviol were approximately 0.02% and 0.06%, respectively, based on the amounts measured in urine collected over 24 hours in comparison to daily administered dietary dose to rats. Mean fecal Reb A and measured hydrolysis products expressed as *Total Reb A Equivalents* compared to daily administered dose results in an estimate of percent of dose recovered ≈ 84% (Sloter, 2008a, unpublished). It has been determined that free steviol that is absorbed from the lower intestines is transported to the liver via the portal vein where it is converted into steviol glucuronide, which is excreted in urine or bile. The findings of the study support the results obtained with other steviol glycosides.

The Panel agreed that studies in animals and humans are relevant in evaluating the safety of Reb A provided that the test material is a characterized steviol glycoside and the route of administration is appropriate. The Panel concluded that studies on unpurified extracts with low concentrations of steviol glycosides may have reported effects that were due to other non-glycosidic substances. The studies in which the test material was characterized as being composed of high levels of steviol glycosides are, thus, more reliable and are used in this evaluation to confirm the safety of Reb A. Studies in which the material was administered orally are the most relevant for the determination of safety of Reb A.

Animal Studies

Acute Toxicity

Stevioside (purity >96%) was not acutely toxic to mice, rats or hamsters at doses as high as 15,000 mg/kg bw/day (Toskulkao *et al.*, 1997, as cited by JECFA 1999; Medon *et al.*, 1982). An earlier study conducted with crude Reb A administered by oral gavage also demonstrated no toxicity in mice up to 2000 mg/kg body weight, the highest dose tested (Medon *et al.*, 1982).

Similarly, steviol (purity 90%) is not acutely toxic to rats and mice at doses as high as 14,000 mg/kg bw (Toskulkao *et al.*, 1997; as cited by JECFA 1999). However, signs of acute toxicity, including death (1/15 male and female for rats and mice) were observed in rats and mice at a dose of 15,000 mg/kg. No morphological or histopathological changes were noted.

Subchronic Toxicity Studies

Subchronic studies are available for Reb A, and stevioside. Dietary administration of Reb A to rats and dogs for 90 days and 6 months, respectively, at doses as high as 2000 mg/kg bw/day resulted in no systemic toxicity.

A rat 90-day dietary toxicity study of Reb A was conducted with treatment doses of 500, 1000 and 2000 mg/kg bw/day. There were no test-article-related effects on clinical observations, food

consumption, functional observational battery or locomotor activity parameters. There were no test article-related macroscopic, organ weight or microscopic findings. Lower body weight gains were noted in the 2000 mg/kg bw/day group in males but not females. The body weight in males was 9.1% lower than the control group at the end of the dosing period (study week 13). The lower body weights were not considered to be adverse due to the small magnitude of difference from the control group value and were most likely due to the large proportion of the diet represented by the test material. (Nikiforov and Eapen, 2008; Eapen, 2007, unpublished). The no-observed-adverse-effect level (NOAEL) was ≥2000 mg/kg bw/day.

Weight loss and/or decreased food efficiency has been reported with other intense sweeteners administered at high dietary concentrations, and it was concluded that body weight gain decreases are not an appropriate basis for determining a NOAEL for this class of ingredients. For example, the reductions in body weight gain that have been reported for neotame, sucralose, and saccharin ranged from 3.7 to >20% in comparison to controls (Flamm *et al.*, 2003). In the absence of toxicity, JECFA has concluded that changes in body weight gain are not appropriate for establishing NOAELs when they are associated with lower food consumption or food efficiency. It is the normal practice of JECFA to recognize when body weight is affected by reduced palatability of food containing high concentrations of test material. For example, JECFA noted that lower body weight gain for sucralose at the high dose in the long-term rat study (1500 mg/kg bw/day) was due to poor palatability of the diet and did not consider this finding adverse. Similarly, the highest dose of acesulfame-potassium, 1500 mg/kg bw/day, was likewise associated with lower body weight gain. These were not considered adverse findings when establishing an ADI for sucralose or acesulfame-potassium of 0-15 mg/kg bw/day (WHO, 1983 as summarized in Flamm *et al.*, 2003).

In a number of studies on Reb A and stevioside, decreases in food efficiency, body weight and/or body weight gain compared to controls are observed at the highest dietary dose levels in rat subchronic toxicity (Nikiforov and Eapen, 2008; Aze *et al.*, 1991), rat chronic toxicity/carcinogenicity (Xili *et al.*, 1992; Toyoda *et al.*, 1997) and rat or hamster reproduction toxicity (Yodyingyuad and Bungawong, 1991; Mori *et al.*, 1981) studies. The Panel agreed with the conclusions of Flamm *et al.* (2003) and in the case of Reb A and stevioside, concluded that decreases in body weight and/or body weight gain in these studies are due to the extremely high levels of Reb A or stevioside administered via the dietary route which provide no caloric value in the diet.

Stevioside administered to rats in the diet at concentrations up to 2500 mg/kg bw/day for 13 weeks resulted in a few sporadic, but statistically significant changes at some doses for some biochemical parameters. A review of the study data tables allows the conclusion that there was no consistent dose-response relationship for these changes, and that the effects were nonspecific and not treatment related (Aze *et al.*, 1991; JECFA, 1999), in agreement with the authors' conclusion.

A 6-month dietary toxicity study in dogs conducted to evaluate the potential toxic effects of Reb A at dosage levels of 0, 500, 1000 or 2000 mg/kg bw/day supports the safety of published findings. There were no test-article-related clinical observations, nor were there any test-article-

related changes in hematology, serum chemistry findings, or urinalysis. No test-article-related gross necropsy observations, alterations in final body weight, alterations in organ weights, or histologic changes were noted at the scheduled necropsy. Based on the results of this study, no toxicity of Reb A was observed at dosage levels up to 2000 mg/kg bw/day and the NOAEL ≥2000 mg/kg bw/day (Eapen, 2008, unpublished).

Chronic Toxicity Studies

While there are no chronic studies of Reb A itself, there are two chronic studies available on stevioside. These studies are relevant to Reb A since Reb A and stevioside are converted via the same metabolic pathways to steviol in the gut prior to absorption. Based on the common ADME of Reb A and stevioside, these studies allowed for evaluation of the chronic toxicity of Reb A. Both studies were 2-year studies that examined the effect of dietary stevioside in rats. In the first study (Xili *et al.*, 1992), stevioside administration in the diet showed no carcinogenic effects in the rat, and a NOAEL of 1.2% (600 mg/kg bw/day) in the diet was reported. In a more recent and more robust study (Toyoda *et al.*, 1997), stevioside administered in the diet was not carcinogenic in rats, and a NOAEL of 2.5% (970 mg/kg bw/day in males) was established. Despite the absence of a study in a second species, the Panel concluded that there is sufficient information to make safety evaluations on the available chronic studies. This conclusion is based on the low systemic exposure levels even at high dietary intakes and the similarities in metabolism among mammalian species (including humans) as well as the lack of effects in the two studies in two different strains of rats.

Genotoxicity Studies

Stevioside was not mutagenic with and without metabolic activation in several experiments using *in vitro* bacterial and mammalian cell mutation, and negative in chromosomal aberration, sister chromatid exchange, and micronucleus assays. Also, stevioside did not increase the micronucleus rate in an *in vivo* mouse micronucleus test in bone marrow (see Exhibit II, Table V.-8).

Unpublished studies with Reb A were corroborative of the stevioside results in that Reb A was not mutagenic with and without metabolic activation when tested *in vitro* in bacterial reverse mutation, and mammalian cell gene mutation assays. In addition, Reb A was negative in an *in vivo* mouse micronucleus assay in bone marrow (see Exhibit II, Table V.-7). Steviol was not mutagenic *in vitro* without metabolic activation in bacterial cell mutation, mammalian gene cell mutation, and chromosomal aberration studies, but with metabolic activation steviol was positive in some of these assays. In three *in vivo* mouse, one hamster and one rat micronucleus assays, chromosomal damage was not observed at dose levels of steviol as high as 8000 mg/kg bw (see Exhibit II, Table V.-9). Therefore, the genotoxicity of steviol *in vitro* is not expressed *in vivo* even at high dose levels.

Reproductive/Developmental Studies

A number of published studies have evaluated the reproduction and developmental toxicity of steviol glycosides and steviol. Studies have been conducted using Reb A, stevioside, and steviol as the test material in hamsters and rats at doses up to 3000 mg/kg bw/day.

Stevioside had no effect on reproduction or development in a 3-generation hamster study with doses up to 2500 mg/kg bw/day (Yodyingyuad & Bunyawong, 1991). No effects were seen in a rat fertility study with doses of stevioside up to 3000 mg/kg bw/day (Mori *et al.*, 1981) or in a developmental study in pregnant rats with doses up to 1000 mg/kg bw/day (Takanaka *et al.*, 1991; Usami *et al.*, 1995).

The results of the published studies are supported by the results of two unpublished studies with Reb A. Reb A was tested by gavage in an embryo/fetal development study in rats (Sloter, 2008b). Intrauterine growth and survival were unaffected by the test article, 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 when Reb A was administered by oral gavage to pregnant rats.

Reb A was also tested in a two-generation dietary reproduction toxicity study in rats (Sloter, 2008a) that confirms and extends the results summarized above for stevioside. There were no effects on reproduction (estrus cycles, mating, fertility, conception or copulation indices, number of days between pairing and coitus, gestation length, and spermatogenic endpoints). A dose level ≥2000 mg/kg bw/day (highest dose administered) was considered to be the NOAEL for parental systemic and reproduction toxicity, and an exposure level ≥2000 mg/kg bw/day was considered to be the NOAEL for neonatal toxicity of Reb A in rats.

In a published study by Wasuntarawat *et al.* (1998) steviol was not teratogenic in hamsters. Decreased weight gain and increased mortality in dams and fetuses (LOAEL = 500 mg/kg bw/day) and a dose dependent effect on kidneys in dams were observed. The effects with steviol but not Reb A and stevioside are likely due to the rapid absorption of steviol leading to higher C_{max} values, whereas Reb A and stevioside must first be hydrolyzed in the gut before absorption of steviol is possible.

Therefore, the totality of evidence demonstrates that Reb A is neither a developmental nor a reproductive toxicant.

<u>Human Studies</u>

Blood Glucose Studies

There are several clinical trials of stevioside and crude stevia extracts with normal and diabetic subjects. Blood glucose levels in these studies were measured at baseline and following oral

administration of the test material for varying time periods. It is clear from these studies that stevioside, at doses up to 30 mg/kg bw/day, does not affect blood glucose levels. A table summarizing these blood glucose studies in humans, while not included in this part of the GRAS Notice for brevity, is, however, included in full in the report of the Reb A GRAS Panel attached to this notice (See Exhibit I, Table 6).

Blood Pressure Studies

Several clinical trials of stevioside and other steviol glycosides, as well as crude stevia extracts, focus on potential effects on blood pressure. These clinical trials include the evaluation of the potential effects of stevioside in normotensive and hypertensive individuals as well as hypertensive individuals with and without antihypertensive therapy. Inter-study comparisons are complicated by the following factors: different product formulations, diverse experimental designs, different subject populations and cohort sizes, and varying durations of treatment.

Human data are available for single dose, short-term (1-3days), intermediate (13-25 weeks) and long-term exposure periods (1-2 years). Doses of stevioside in these studies ranged from 28 mg/dose to 1500 mg/day in clinical studies (up to 30 mg/kg bw/day). This dose expressed in terms of Reb A Equivalents are up to 36 mg/kg bw/day. By comparison, anticipated 90th percentile dietary intakes from the proposed uses of Reb A would be no more than 5 mg/kg bw/day.

A table summarizing these blood pressure studies in humans, while not included in this part of the GRAS Notice for brevity, is, however, included in full in the report of the Reb A GRAS Panel attached to this notice (see Exhibit I, Table 7).

Blood pressure changes in normotensive individuals were small or absent and not meaningful or clinically significant. Barriocanal *et al.* (2008) conducted a 3-month, randomized, double-blind, placebo-controlled, parallel-design study employing type 2 diabetics, type 1 diabetics, and non-diabetics with normal to low normal blood pressure with stevioside at 750 mg/day. No clinically significant differences were observed in systolic or diastolic blood pressure measurements during the treatment period.

Hypertensive subjects were given stevioside for up to 2 years. Antihypertensive medication was discontinued before initiating the studies. Table 7 in the GRAS Panel Opinion summarizes the blood pressure levels in control subjects and stevioside groups (250 mg/3x/day: 15 mg/kg bw/day) for 1 year (Chan, 2000). At the end of one year there was a clinically significant decrease in systolic and diastolic blood pressure. In a second study (500 mg/3x/day for 2 years) reported similar blood pressure decreases (Hsieh *et al.*, 2003). Reductions of the magnitude reported in these studies were only seen in individuals with significant hypertension who were not receiving antihypertensive medications. The Panel concluded that the magnitude of blood pressure reductions reported for these subjects does not present a risk.

The Panel discussed potential concerns about the effects of Reb A or stevioside use on hypertensive individuals who are on antihypertensive medications; the concern primarily relates to reductions in blood pressure and potential for orthostatic hypotension. However, one study using treatment with crude stevia extract did not reveal any changes in carefully measured blood pressure between the supine and upright positions (Haebisch, 1992). Additionally, in other studies there were no significant clinical symptoms that would indicate the occurrence of orthostatic hypotension (Chan, 2000; Hsieh *et al.*, 2003).

It is noteworthy that the largest blood pressure reductions (i.e. ~10-14 mm Hg) are from studies in which subjects received either 750 (15 mg/kg bw/day) or 1500 mg/day (30 mg/kg bw/day) stevioside. These doses expressed in terms of Reb A Equivalents are 18 mg/kg bw/day or 36 mg/kg bw/day. These Reb A Equivalent doses are more than 3.5 - 7 times the anticipated upper 90th percentile of intake from the proposed uses of Reb A. Furthermore, hypertensive individuals who were being treated with antihypertensive medications did not exhibit significant reductions in blood pressure (Barriocanal *et al.*, 2008).

The Panel concluded that although stevioside administration was associated with clinically relevant reductions in blood pressure for individuals with moderately severe hypertension who were not on antihypertensive therapy, clinically relevant reductions in blood pressure were not observed in individuals with normal blood pressure or in individuals with hypertension receiving antihypertensive medication.

VI. SAFETY ASSESSMENT AND GRAS DETERMINATION

A. Introduction

This section of the GRAS Notice fulfills requirements of proposed 21 CFR 170.36(c)(4)(i)(B) and (C) by providing a discussion of the basis for concluding, in light of all generally available and accepted scientific data and information, methods, or principles relevant to establishing safety of the proposed uses of Reb A, that there is a consensus among qualified experts that there is a reasonable certainty that Reb A is not harmful under its intended conditions of use.

This section also includes a reiteration of the available published and unpublished information being relied upon and summarized above (including an assessment of the metabolism, toxicological data and clinical human data available) in light of the probable intake of the substance.

In addition, this section includes a discussion of reports of investigations or other information that may appear to be inconsistent with the GRAS determination. In particular, this section addresses issues that the FDA has raised previously as a result of having received partial, incomplete, or inadequate information in the past from others seeking to establish the GRAS status of stevia and stevia extracts, basing their assertions on ill characterized or incompletely studied aspects of the safety of stevia, its extracts or steviol glycosides in general.

B. Safety Assessment of Reb A for Its Intended Uses

Historical Positions of JECFA and FSANZ

Based on the NOAEL for stevioside of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol) in a 2-year rat study (Toyoda *et al.,* 1997), the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (2006) established a temporary ADI for steviol glycosides of 2 mg/kg bw/day (expressed as steviol). JECFA used a safety factor of 200 (100 for inter- and intra-species differences and additional factor of 2 because JECFA required additional information on pharmacological effects in humans). More recently, Food Standards Australia New Zealand (FSANZ, 2007) established an ADI for steviol glycosides of 4 mg/kg bw/day (expressed as steviol) based on the same 2-year study. FSANZ used a safety factor of 100 for inter- and intra-species differences. FSANZ provided the following rationale for applying a safety factor of 100:

> 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 [applied by JECFA] for uncertainty related to effects in normotensive or diabetic individuals is no longer required.

We believe, based on an evaluation of all the foregoing safety-related information, that the NOAEL used by JECFA and FSANZ (970 mg/kg bw/day) is appropriate to calculate an ADI for Reb A.

Acceptable Daily Intake(ADI) for Reb A

The default safety factor typically used in estimating the acceptable daily intake from toxicological data as outlined in this GRAS Notice is 100-fold. This factor is based on an inter-species factor of 10 and an intra-species factor of 10. As noted above, human data, including a study in which subjects were treated with 30 mg/kg for 2 years, are now available for steviol glycosides, as well as data that demonstrate the similarity of metabolism and kinetics between humans and animals. Therefore, a compound-specific determination of the safety factor is appropriate. The Panel could not use the human clinical trials to derive an ADI for Reb A; however the Panel concluded that because of the similarity in human and animal metabolism a reasonable value for the intra-species factor of 3-4 instead of 10 would be reasonable (WHO, 1994). Thus, for Reb A we believe a reasonable overall safety factor would be 30-40, although ADIs based on the default safety factor are also shown. These estimates for the ADI for Reb A based on the totality of evidence are listed in Table 6 below.

Table 6. Acceptable Daily Intake from Totality of Evidence

Basis of ADI (Safety Factor)	Expressed as Steviol (mg/kg bw/day)	Expressed as Reb A[1] (mg/kg bw/day)
JECFA temporary (200)	2	6
FSANZ (100)	4	12
Corroborating Reb A studies (100)	7	20
Expert Panel ADI (100)	4	12
Expert Panel ADI (40)	10	30
Conclusion for Reb A	**4-10**	**12-30**

1 Calculated using a conversion factor of 3.04 based on the molecular weights of 318.45 and 967.03 for steviol and Reb A, respectively.

Estimated Daily Intake (EDI) for Reb A

As noted above, the EDI of Reb A was calculated using the most recent food consumption survey results from the National Health and Nutrition Examination Survey (NHANES). Reb A intake was determined by multiplying each NHANES respondents' two-day average food intake by the maximum proposed use level for each category. The total EDI from all proposed uses is

approximately 2 mg/kg bw/day at the mean and 5 mg/kg bw/day at the 90th percentile of intake among users. A user is defined as having consumed a food that would contain Reb A during the two days of the NHANES survey. Consideration of only two days of food consumption data results in a conservative upper estimate of potential Reb A intake.

Comparison of ADI and EDI for Reb A

Even using conservative upper estimates of intake (5 mg/kg bw/day) and the lowest potential ADI (12 mg/kg bw/day), consumer intakes of Reb A from the proposed uses would not exceed the ADI.

In summary, the entire body of available information relevant to the safety of Reb A, including identity, specifications, manufacturing process, probable consumer exposure, absorption distribution metabolism and excretion pathways and their pharmacokinetics, toxicological and human clinical data relevant to Reb A and steviol glycosides in general, provides a basis upon which to conclude that there is a reasonable certainty that Reb A is not harmful under its intended conditions of use.

C. Historical issues and concerns about "stevia" and "stevia extracts" and why current information eliminates any such concerns for Reb A

Historically, FDA has received requests to classify botanical stevia and its extracts as GRAS for use as a sweetener or flavoring agent in food. This GRAS Notice represents the first time an assertion of GRAS status for a stevia-related material has focused on a well-defined, single chemical entity of high purity, namely the steviol glycoside, Reb A. To our knowledge, this is the first submission to FDA since additional research has defined the pathways for absorption, distribution, metabolism, and excretion (ADME) of steviol glycosides. Based on that research, several authoritative bodies have concluded that steviol glycosides are safe for use as a sweetener. Specifically the World Health Organization Joint Expert Committee on Food Additives (JECFA) has established a temporary ADI (expressed as steviol) of 0-2 mg/kg bw/day. The Food Standards Australia New Zealand (FSANZ) reviewed the same data plus new research in human volunteers and have proposed an ADI (also expressed as steviol) of 0-4 mg/kg bw/day. Based on molecular weight, these ADIs would be 6 and 12 mg/kg bw/day, respectively, expressed as Reb A.

FDA has received numerous requests, inquiries, and petitions over the years for the use of stevia and stevia-related and derived chemicals for use as sweeteners in food. The FDA record (summarized most completely by Whiteside and Hollingsworth, 1990) dating from at least 1972, contains numerous references to stevia-related FDA actions and positions. In these references, FDA expressed a range of concerns about the use of stevia and related materials as food

ingredients. These concerns are responded to below in the context of the present-day widely known and accepted knowledge and information about the steviol glycosides including, in particular, Reb A.

- **Product Characteristics**

***Issue*:** Available records indicate that FDA requested information about the identity, purity, and specifications of stevia and related products of interest numerous times in the past. FDA reviewers mention the need for identification and characterization of any "residual substances." Source documents request analysis of, and assays and characterization of, the intended ingredient and its impurities; its manufacturing process; its stability in foods; its planned food uses; and adequately documented characterization of any residuals or impurities. Because composition has varied among ingredients of interest and substances tested, FDA has considered this issue to be crucial in establishing the relevance of various safety studies.

***Resolution*:** This notification is for a single chemical Rebaudioside A (Reb A) (≥ 95%) as detailed in Section I. Specifications, manufacturing method, stability in foods, proposed food uses, discussion of potential degradants, analytical methods and analysis of several manufactured lots are included.

- **Stability**

***Issue*:** Hydrolysis conditions, hydrolysis rates and hydrolysis products forming in food from the use of stevia and steviol glycosides have been cited as concerns by FDA in the past. For example, for carbonated beverage use, FDA requested information in the 1990s on stability of stevia-derived food ingredients in aqueous medium down to pH of 2.5 over a period of at least three months at temperatures of up to 40 ºC so as to assess the safety of any degradation products.

***Resolution*:** Comprehensive stability studies have been conducted to confirm stability under normal use conditions. In addition, studies have been conducted for long-term storage under extreme temperature (up to 110ºF) and pH (2.0 to 7.0) conditions.

- **Adequacy of Toxicological Data**

***Issue*:** Among the publicly available records on stevia and its glycosides, FDA has expressed concern about the lack of toxicity data and/or the lack of quality in the existing data at that time to support safe use of stevia-derived ingredients in food. For example:

- Existing studies were not consistent with FDA's guidelines or standards outlined for toxicological testing.
- Toxicological studies at the time lacked complete characterization of the substance fed and the identity of the substances fed varied among the various studies.
- There was an inadequate characterization of the ADME of stevia and related compounds in animals or humans. In particular, FDA wanted evidence whether stevia or its glycosides were metabolized to steviol in the GI tract by human gut microflora, as appeared to be the case in rats.
- FDA expressed concern that the carcinogenic potential of stevia-derived ingredients was not well determined in animals or humans.
- FDA has commented that steviol is "highly mutagenic" in salmonella mutation assays in the presence of metabolic activation, increasing the importance of good carcinogenicity and ADME studies.
- Most studies were of short duration (3 months or less).
- FDA cites inadequate reproduction studies.

***Resolution*:** The toxicology database has been expanded and strengthened by the completion of numerous studies conducted according to FDA GLPs and test guidelines with Reb A as the test material (rat and dog subchronic, *in vitro* and *in vivo* genotoxicity, toxicokinetics and rat development/reproductive studies). New clinical studies have been conducted using human volunteers to evaluate potential effects of steviol glycosides on blood pressure and blood glucose. The results of these new toxicology studies conducted with Reb A are corroborative of published studies with stevioside, and together with new clinical studies uniformly support the GRAS status of Reb A.

- **Anti-fertility Effects or Other Reproductive Effects in Animals and Humans**

Issue: FDA expressed concern about the presence of some historical data pointing to the possibility that stevia-derived products can induce reproductive effects, including a pronounced antifertility effect when administered in the drinking water of test animals and a no-effect level had not been determined. FDA was also concerned about a report that steviol induced androgenic and anti-androgenic effects in chickens and its potential relevance to humans. Cited often was [Mazzei] Planas and Kuc (1968), apparently confirmed by the Nunes *et al.* (1988), where it was reported that an aqueous extract of stevia decreased fertility in female rats. Also cited are anecdotal reports of Guarani of Paraguay using stevia and its aqueous extracts as an oral contraceptive.

***Resolution*:** Published studies reported no reproductive or developmental effects of stevioside in rats or hamsters (Yodyingyuad and Bunyawong, 1991; Mori *et al.,* 1981; Takanaka *et al.,* 1991; Usami *et al.,* 1995). These studies are corroborated by new FDA GLP and test guideline studies with Reb A that demonstrate a lack of reproductive or developmental effects even at the extremely high doses tested (2000 mg/kg bw/day).

- **Hypoglycemia**

***Issue*:** FDA has cited the potential for stevia and its extracts to produce hypoglycemia in animals and humans.

Resolution: Sufficient data now available, including several new studies in which steviol glycosides were administered to human volunteers, demonstrate that there will not be an adverse effect on blood glucose by use of Reb A under conditions of intended use.

- **Blood Pressure Effects**

***Issue*:** FDA has expressed concern about the potential of stevia and its extracts to produce adverse effects on the cardiovascular system in animals and, potentially, in humans.

***Resolution*:** This notification specifically evaluated potential effects on blood pressure in animals and humans (see Exhibit II, Sections V.A.3 and V.B.). The data demonstrate that in some studies there are effects (albeit small) on blood pressure in animals, particularly with stevia extracts. Although stevioside administration was associated with clinically relevant reductions in blood pressure for individuals with moderately severe hypertension who were not on antihypertensive therapy, clinically relevant reductions in blood pressure were not observed in individuals with normal blood pressure or in individuals with hypertension receiving antihypertensive medication.

D. General Recognition of the Safety of Reb A

Safe for Intended Use

The intended uses of Reb A have been determined, as outlined above, to be safe through scientific procedures as set forth in 21 CFR§170.30(b).

Common Knowledge – Generally Available

Because this safety evaluation was based on information in the peer reviewed published scientific literature, it satisfies the first component of the "common knowledge element" of a GRAS determination, namely, that the data and information being relied upon is generally available. This Notice, and the deliberations of the Panel, did take notice of some other supporting information that is not published. The role of this type of information, however, is merely corroborative. The safety evaluation relies on a host of published studies, most of which have been publicly available for an extended period of time. Among the many published studies evaluated, two are included whose appearance in the public domain are relatively recent. These are the Nikiforov and Eapen (2008) paper and the Barriocanal *et al.* (2008) paper.

We are aware of FDA's historical guidance that for information to be "generally available" in the GRAS context it normally would require a published paper to be in the public domain for a period of approximately six months prior to making a GRAS claim in order to assure that it has been thoroughly disseminated throughout the interested scientific community, including subscribers to journals and to relevant libraries. We believe that in today's electronically based information environment, such a "six-month" guideline is no longer necessary or appropriate. Rather we believe that adequate distribution and dissemination of published scientific results depends more on other factors. These may include the following: a) whether the results already have been the subject of discussions of authoritative bodies (such as JECFA or other national or international food authorities such as FSANZ (such as was the case for the data of Barriocanal *et al*,(2008)); b) on whether the paper and/or its abstract have been picked up and disseminated by the electronic media and are therefore available through internet keyword searches such as Google (as has been the case for both the Nikiforov and Eapen (2008) and the Barriocanal *et al.* (2008) papers); and c) whether the types of journals selected for publication are of high quality and wide circulation so that the largest possible body of experts can quickly become familiar with the new information. The second and third three criteria have been satisfied by both recently published studies cited above. Accordingly, we believe the information relied upon to establish GRAS has been "generally available" for purposes of reaching that conclusion.

Common Knowledge – Generally Accepted by Qualified Experts

In order to assure that the common knowledge about the safety of Reb A is not only generally available, but that it is generally accepted by a consensus of qualified experts, the notifier has assembled a distinguished panel of experts (the Reb A GRAS Panel) comprising five prominent experts in the field of food and food ingredient safety. The individuals comprising the Panel are qualified by scientific training and experience to evaluate the safety of substances intended to be added to food. They have critically reviewed and evaluated the available information summarized in this document and have individually and collectively unanimously concluded that Reb A, produced consistent with Good Manufacturing Practice and meeting the specifications described herein, is safe under its intended conditions of use. The Panel further unanimously concluded that these uses of Reb A satisfy the safety standard of reasonable certainty of no harm. The Panel's GRAS opinion is included as an attachment to this document (See Exhibit I).

The Whole Earth Sweetener Company LLC is not aware of information that would be inconsistent with a finding that the proposed uses of Reb A, meeting appropriate specifications and used according to Good Manufacturing Practice, are GRAS.

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EXHIBIT I.

EXPERT PANEL OPINION

EXPERT PANEL OPINION

Introduction

The undersigned, an independent Panel of Experts, qualified by scientific training and experience to evaluate the safety of food and food ingredients, were requested by The Whole Earth Sweetener Company, to determine the safety and generally recognized as safe (GRAS) status of Rebaudioside A (common name: Reb A) for use as a natural sweetener in selected beverages (including sweetened teas, diet soft drinks, energy drinks and flavored waters), cereals (oatmeal, cold cereal, cereal bars) and for tabletop use. Reb A would be used at proposed levels presented in Table 1.

Table 1. Proposed Uses and Levels of Reb A (ppm):

Food Categories	Reb A (ppm)
Tabletop sweeteners	30,000[1]
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

[1]Reb A content of sachet prior to dilution and not representative of "as consumed"

A comprehensive survey of all of the relevant scientific literature concerning safety related information on Reb A, steviol glycosides, and steviol, was conducted by Exponent, Inc. and Toxicological Regulatory Services (TRS). The data searched include identity, structure, purity and specifications; toxicological data including acute, subchronic, and chronic toxicity, genotoxicity, reproductive/developmental toxicity, and carcinogenicity studies in animals; human clinical studies (including short term and long term studies in normotensive and hypertensive subjects and in diabetics and nondiabetics) and data and information about the absorption, distribution, metabolism and excretion (ADME) of Reb A, steviol glycosides and steviol in animals and humans. The literature search was concluded on April 10, 2008.

All of the available relevant information about Reb A, steviol glycosides and steviol was summarized in a safety assessment document (GRAS Dossier) entitled, "Safety Evaluation Dossier for Expert Panel Determination of the GRAS Status of Rebaudioside A (Reb A)," prepared by Exponent and TRS. The Expert Panel members critically evaluated this document and other information they deemed appropriate and relevant, and unanimously agreed to the opinion described herein.

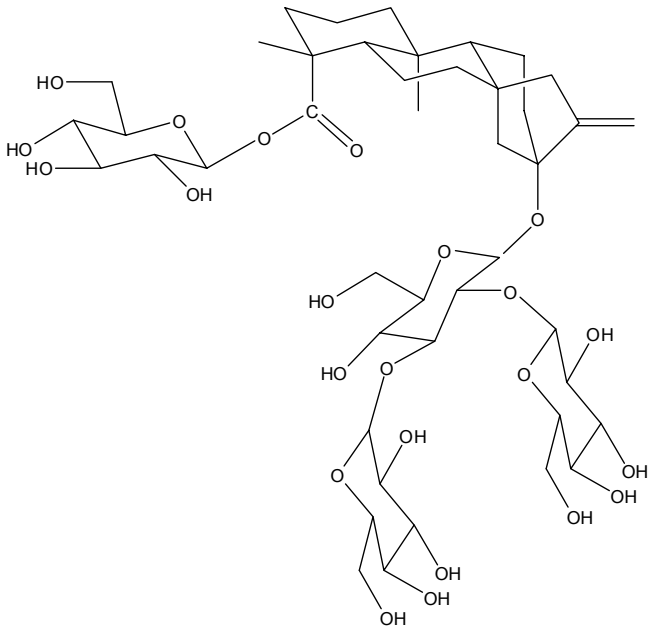
Recent unpublished data were reviewed by the Panel for comparison to published literature, including Clarke, 2006; Eapen, 2007; Eapen, 2008; Eisenberg, 2007; Krsmanovic and Huston, 2006; Sloter, 2008a,b; Stetson, 2008a-c; and Wagner and Van Dyke, 2006.

Description

The subject matter of this review is Rebaudioside A (common name: Reb A) a glycoside of steviol. The chemical name of Reb A is: 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.

The CAS Number of Reb A is: 58543-16-1, and its chemical formula is: $C_{44}H_{70}O_{23}$ (Formula Weight: 967.03).

Its structural formula is shown below:

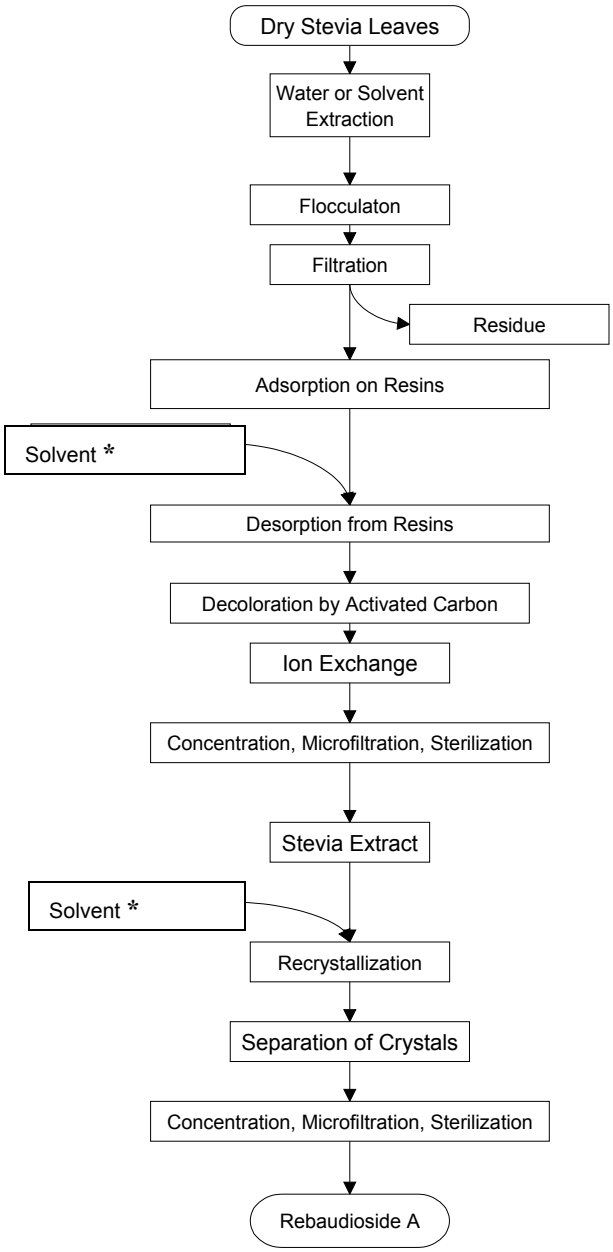


Reb A is a natural sweetener. It is a pure diterpene glycoside extracted from the plant *Stevia rebaudiana* (Bertoni), which belongs to the Asteraceae family and is native to Brazil and Paraguay. Reb A is the second most abundant glycoside in stevia and is considered more suitable for use to sweeten foods and beverages than stevioside because of its greater solubility in water and better taste profile. Reb A tastes about 200 to 300 times sweeter than sugar and is non-nutritive. It is structurally similar to other steviol glycosides, including stevioside. In many parts of the world, including Japan, South Korea, Israel, Mexico, Paraguay, Brazil, and Argentina, stevia glycosides, including extracts of the leaves, stevioside and Reb A, are used to sweeten food products and beverages.

Manufacturing Process

The source of Reb A, the leaves of *Stevia rebaudiana* (Bertoni), is a botanical and as such the steviol glycoside content is slightly influenced by the temperature in the year of cultivation, the latitude, and the time of harvesting. Highly purified Reb A (≥95%) is obtained by extraction of the stevia leaves with water, ethanol or methanol and the extract is passed through an adsorption resin to trap and concentrate the desired steviol glycosides. The resin is washed with ethanol or methanol to release the glycosides. Through de-coloration and de-salting by ion exchange and microfiltration, consistently higher purity Reb A is obtained. Recrystallization with alcohol/water mixtures results in very high purity Reb A (≥95%) which may be spray-dried or granulated and residual alcohol removed.

Typical Method of Manufacture of Reb A



*Aqueous ethanol or methanol.

Specifications for Food Grade Reb A

Reb A is a single chemically defined substance (≥ 95% purity) and differs from stevia leaves or stevia extracts which are crude natural product "mixtures." The food grade specifications for Reb A summarized in Table 2 are comparable to those in the JECFA Monograph (2007) for Steviol Glycosides. Consistency of Reb A manufacturing has been demonstrated by analyses of three lots of Reb A and certificates of analysis from the vendor.

Table 2. Purity and Specifications for Food Grade Reb A

Analytical Parameter	Acceptable Target/Range	Methods of Analysis
Purity Reb A (wt/wt %)	≥ 95% Reb A (on dry basis)	JECFA, 2007 (modified)
Total Steviol Glycosides (wt/wt %)	≥ 95% (on dry basis)	JECFA, 2007 (modified)
Stevioside (wt/wt %)	2% (on dry basis) maximum	JECFA, 2007 (modified)
Steviol (wt/wt %)	< 0.005% (on dry basis)	Bazargan *et al*., 2007 (LC-MS/MS)
Moisture Content (%) by Loss on Drying	5.0% maximum	FCC, 2003 (p. 855)
Optical Rotation	-29 to -37 degrees	FCC, 2003 (p. 844)
Arsenic (as As)	1.0 mg/kg maximum	FCC, 2003 (p. 861)
Lead	1.0 mg/kg maximum	FCC, 2003 (p. 867)
Cadmium	1.0 mg/kg maximum	FCC, 2003 (p. 863)

Quality Specifications for Reb A

Reb A is derived from a botanical product and, therefore, quality specifications have been set to address microbiological contamination. There are no detectable pesticide residues in the multi-residue screen.

Table 3. Quality Specifications for Reb A

Analytical Parameter	Acceptable Target/Range	Methods of Analysis
Total Aerobic Plate Count	1000 cfu/g maximum	Downes and Ito, 2001a
Total Aerobic Mold Count	100 mold cfu/g maximum	Downes and Ito, 2001b
Total Aerobic Yeast	100 yeast cfu/g maximum	Downes and Ito, 2001b
Heat Resistant Mold	Non detectable (< 1 cfu/50 g)	Downes and Ito, 2001c
Coliform	< 10 cfu/g	Downes and Ito, 2001d
E. coli	< 3 MPN/g	Downes and Ito, 2001d
Salmonella ssp	Negative in 25g	Downes and Ito, 2001e
Staphylococcus aureus	Non detectable (< 1 cfu/g)	Downes and Ito, 2001f
Alicyclobacillus acidoterrestris	Non detectable (<1 cfu/50 g)	JFJA, 2007
Residual Solvents	Methanol < 300 ppm Ethanol < 1000 ppm	U.S. Pharmacopoeia, 2007
Pesticide Residues	None Detectable	U.S. FDA, 2004; CDFA, 1988

Functionality

Reb A, the subject of this GRAS determination, is proposed for use as a sweetener in selected beverages (including sweetened teas, diet soft drinks, energy drinks and flavored waters), cereals (oatmeal, cold cereal, cereal bars) and for tabletop use.

The perception of sweetness is beverage and concentration dependent. However, qualitative assessments indicate that 20 ppm is the lowest sweetness threshold in a beverage system. Qualitative assessments also indicate that 14 Brix is considered a "too sweet" beverage. In a sweetness equivalency study it was determined that it takes approximately 1200 ppm Reb A in water to match the sweetness of a 14% sugar solution. Most beverages are sweetened to approximately 10 Brix or 10% sugar solution. Experimentation with 500 ppm Reb A indicated that the sweetness potency is 200X, therefore the potency is presented as: 10%/0.05% = 200X. The overall sensory stability of Reb A is significantly better than aspartame over the beverage shelf-life period of 24 weeks. Taste degradation of Reb A was not detected up to 20 weeks.

Functionality of Reb A as a sweetener in foods was demonstrated by sensory panel ratings of coffee sweetened with tabletop powder. Process capability and isosweetness data analysis supports a target Reb A level of 26.10 mg/serving. Reb A in a tabletop powder is stable and functional as a sweetener through 26 weeks under accelerated storage conditions, 40 ± 2°C, 75 ± 5% RH.

Exposure

Available Data and Methods

The estimated daily intake (EDI) of Reb A from the proposed uses including tabletop sweeteners, sweetened ready-to-drink iced teas, diet soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal, and sweetened cold cereals, was estimated using the proposed use levels provided in Appendix 1 of the Dossier (entitled NHANES 2003-2004 Food Codes and Reb A Use Level Included in EDI Analysis), Exponent®'s Foods and Residue Evaluation Program (FARE™ 8.09) software, and data from the most recent National Health and Nutrition Examination Survey (NHANES 2003-2004).

The NHANES 2003-2004 (NCHS, 2007) is a complex multistage probability sample designed to be representative of the civilian U.S. population. The survey collects two days of food intake data, in addition to nutrition, demographic, and health information. The NHANES survey over-samples minorities, low-income groups, adolescents (12-19 years), and adults 60 years of age and older and statistical weights are provided by the National Center for Health Statistics (NCHS) to adjust for the differential probabilities of selection. Participants included 10,122 subjects in 2003-2004. Only individuals with complete and reliable 2-day dietary records were included in the analysis; therefore, N=8,354. Exponent® used the statistically weighted values from the survey in the analyses. The statistical weights compensate for variable probabilities of selection, adjust for non-response, and provide intake estimates that are representative of the U.S. population and the selected age-gender subgroups.

Exponent® estimated the daily intake on a per "user" basis. In this analysis, a "user" is anyone who reported consuming at least one category of food in which it is proposed to use Reb A (Reb A food category) on either of the survey days, i.e. United States Department of Agriculture's (USDA's) "user" definition. Each individual who reported consuming a Reb A food on either of the survey days was identified, and that individual's responses for both survey days was used. Because Reb A is likely to be consumed over a lifetime it is appropriate to average exposures over a longer period than one day. Therefore, Exponent® used each respondent's food consumption averaged over the two days of the NHANES 2003-2004 survey. A 2-day average typically overestimates long-term, chronic daily intake; however, only two nonconsecutive days' worth of food consumption data are available in the most recent NHANES 2003-2004 survey database. The 2-day average is typically a poor estimator of lifetime average daily intake, especially for foods eaten infrequently. It is well known that food consumption data collected over longer periods of time, e.g., 14 days as in Market Research Corporation of America (MRCA) consumer surveys, yield estimates of daily intake that may be significantly lower than 2-day averages (Lambe *et al*., 2000).

The EDI of Reb A was calculated by multiplying each NHANES respondents' 2-day average food intake by the use levels described in Table 1. Results are presented in Table 4 as mg Reb A/kg bw/day. Each individual's intake of Reb A was divided by his/her bodyweight to provide the per capita and per user intakes on a bodyweight basis. Analyses were conducted using Exponent®'s proprietary software program FARE™

version 8.09. Mean and 90th percentile daily intakes on a per user basis, mg/kg bw/day, were estimated for the proposed uses of Reb A for the U.S. population (individuals 2+ years old).

Proposed Use Level of Reb A
Reb A that is the subject of this GRAS notification is proposed for use as a natural sweetener in tabletop sweeteners, sweetened ready-to-drink iced teas, diet soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal, and sweetened cold cereals. As noted above, Reb A would be used at or lower than the levels proposed in Table 1.

Estimated Daily Intake
The per user intake estimates of Reb A for the U.S. population from the proposed uses are presented in Table 4.

Table 4. Estimated Daily Intake of Reb A from Proposed Uses (U.S. Population: 2+ Age Group)

Food Category		Per User (mg Reb A/kg bw/day)	
	N (Unweighted)	Mean	90th Percentile
Tabletop sweeteners	665	0.8	1.7
Sweetened ready-to-drink teas	964	1.0	2.4
Diet soft drinks	1182	3.3	7.4
Fruit juice drinks	2801	1.0	2.2
Energy drinks	37	0.4	0.7
Flavored Water	16	1.1	1.6
Cereal bars	336	0.1	0.1
Oatmeal	600	0.4	0.8
Cold cereals	284	0.1	0.2
Total (beverages, cereals, tabletop)	**4959**	**2.0**	**4.7**

The total EDI from all proposed uses is 2 mg/kg bw/day at the mean and 5 mg/kg bw/day at the 90th percentile of intake among users. Intakes by age/sex subgroups are similar (Table 5).

Table 5. Estimated Daily Intake of Reb A from Proposed Uses (U.S. Population: Children, Teenagers and Adults)

Population	Unweighted Users	Per User (mg/kg bw/day)	
		Mean	90th Percentile
Children 2-5 years	438	2.4	5.1
Male teenagers	542	1.6	3.4
Female teenagers	567	1.2	2.4
Male >20 years	1207	2.0	4.8
Females >20 years	1416	2.2	5.6

Safety Data

Absorption, Distribution, Metabolism and Excretion (ADME)

The Panel concluded the following regarding the ADME of steviol glycosides including Reb A. The data show that the half-life in animals and humans is less than 24 hours and that there is no bioaccumulation over time even at doses well above those anticipated from the proposed uses.

- Steviol glycosides are not metabolized or transformed in the stomach or intestine by mammalian enzymes.
- Intestinal metabolism of Reb A is primarily hydrolysis by the gut microflora, and the pathways are similar in rats and in humans. The principal steviol glycosides, Reb A and stevioside, are only metabolized in experimental animals and humans by intestinal microflora by sequential hydrolysis of glucose sugar moieties to free steviol.
- Steviol produced by intestinal hydrolysis is excreted primarily into feces (the primary pathway) or to a much lower extent is absorbed into plasma via the portal vein.
- The metabolic pathways and kinetics of steviol glycosides in animals and humans are similar so the animal test data are appropriate for safety assessment. As a consequence of these similarities, data on the other steviol glycosides, notably stevioside, are also relevant to safety evaluation of Reb A.

Human fecal microflora were found to completely hydrolyze stevioside and Reb A to their common aglycone, steviol, in 10 and 24 h, respectively, but the microflora did not degrade steviol. After incubation of stevioside or Reb A with human intestinal microflora, steviol epoxide derivatives were not detected. The absence of stevioside in the feces indicates that the bacterial flora hydrolyzed stevioside into steviol, which itself was not further metabolized, as was also shown in pigs *in vivo* (Geuns *et al.,* 2003a), and in pig and human feces *in vitro* under anaerobic conditions (Geuns *et al*., 2003a, Koyoma *et al.,* 2003b, Gardana *et al.,* 2003).

Geuns *et al.* (2007) reported that between 13 and 40 mg free steviol could be detected in human 24-hr feces sample collected following 3 doses/day of 250 mg stevioside/dose for three days.

- Blood primarily contains the glucuronidated form of steviol; the conjugated form is excreted in urine.

Apart from steviol glucuronide and extremely small amounts of test material or free steviol, none of the possible steviol intestinal metabolites have been detected in blood or urine. Geuns *et al.* (2007) did not detect free steviol in the peripheral blood of the study subjects. Free steviol that is absorbed from the intestines is converted into steviol glucuronide by the enterocytes and/or liver (Geuns *et al.,* 2007; Simonetti *et al.,* 2004).

In addition to the published data, the ADME of Reb A was investigated in a study in rats receiving Reb A via the diet at dose levels up to 2000 mg/kg bw/day, Reb A, free steviol and steviol glucuronide were detected in peripheral blood of rats, but at extremely low levels representing less than 0.08% of the administered dose (Sloter, 2008a, unpublished). The findings of the study support the results obtained with other steviol glycosides.

The Panel concluded that studies in animals and humans are relevant in evaluating the safety of Reb A provided that the test material is a characterized steviol glycoside and the route of administration is appropriate. The Panel also concluded that studies on unpurified extracts with low concentrations of steviol glycosides may have reported effects that were due to other non-glycosidic substances. The studies in which the test material was characterized are thus more reliable, and are used in this evaluation to confirm the safety of Reb A. Studies in which the material was administered orally are the most relevant for the determination of safety of Reb A.

Acute Toxicity

Stevioside (purity >96%) was not acutely toxic to mice, rats or hamsters at doses as high as 15,000 mg/kg bw/d (Toskulkao *et al*. 1997, as cited by JECFA 1999; Medon *et al*. 1982). An earlier study conducted with crude Reb A administered by oral gavage also demonstrated no toxicity in mice up to 2000 mg/kg body weight (Medon *et al.*, 1982).

Similarly, steviol (purity 90%) is not acutely toxic to rats and mice at doses as high as 14,000 mg/kg bw (Toskulkao *et al*. 1997; as cited by JECFA 1999). However, signs of acute toxicity, including death (1/15 male and female for rats and mice) were observed in rats and mice at a dose of 15,000 mg/kg. No morphological or histopathological changes were noted.

Subchronic Toxicity

Subchronic studies are available for Reb A, and stevioside. Dietary administration of Reb A to rats and dogs for 90 days and 6 months, respectively, at doses as high as 2000 mg/kg bw/day resulted in no systemic toxicity.

A 90-day dietary toxicity study was conducted with treatment doses of 500, 1000 and 2000 mg/kg bw/day. There were no test-article-related effects on clinical observations, food consumption, functional observational battery or locomotor activity parameters. There were no test article-related macroscopic, organ weight or microscopic findings. Lower body weight gains were noted in the 2000 mg/kg bw/day group in males but not females. The body weight in males was 9.1% lower than the control group at the end of the dosing period (study week 13). The lower body weights were not considered to be adverse due to the small magnitude of difference from the control group value and were most likely due to the large proportion of the diet represented by the test material. (Nikiforov and Eapen, 2008; Eapen, 2007, unpublished). The no-observed-adverse-effect level (NOAEL) was ≥2000 mg/kg bw/day.

Weight loss and/or decreased food efficiency has been reported with other intense sweeteners administered at high dietary concentrations, and it was concluded that body weight gain decreases are not an appropriate basis for determining a NOAEL for this class of ingredients. For example, the reductions in body weight gain that have been reported for neotame, sucralose, and saccharin ranged from 3.7 to >20% in comparison to controls (Flamm *et al*., 2003). In the absence of toxicity, JECFA has concluded that changes in body weight gain are not appropriate for establishing NOAELs when they are associated with lower food consumption or food efficiency. It is the normal practice of JECFA to recognize when body weight is affected by reduced palatability of food containing high concentrations of test material. For example, JECFA noted that lower body weight gain for sucralose at the high dose in the long-term rat study (1500 mg/kg bw/day) was due to poor palatability of the diet and did not consider this finding adverse. Similarly, the highest dose of acesulfame-potassium, 1500 mg/kg bw/day, was likewise associated with lower body weight gain. These were not considered adverse findings when establishing an ADI for sucralose or acesulfame-potassium of 0-15 mg/kg bw/day (WHO, 1983 as summarized in Flamm, *et al*., 2003).

In a number of studies reviewed by the Panel, decreases in food efficiency, body weight and/or body weight gain compared to controls are observed at the highest dietary dose levels in rat subchronic toxicity (Nikiforov and Eapen, 2008; Aze *et al*., 1991), rat chronic toxicity/carcinogenicity (Xili *et al*., 1992; Toyoda *et al*., 1997) and rat or hamster reproduction toxicity (Yodyingyuad and Bungawong, 1991; Mori *et al*., 1981) studies. The Panel concluded that decreases in body weight and/or body weight gain in these studies are due to the extremely high levels of Reb A or stevioside administered via the dietary route which provide no caloric value in the diet.

Stevioside administered to rats in the diet at concentrations up to 2500 mg/kg bw/day for 13 weeks resulted in a few sporadic, but statistically significant changes at some doses

for some biochemical parameters. The Panel reviewed the study data tables and concluded that there was no consistent dose-response relationship for these changes and agreed with the authors' conclusion that the effects were nonspecific and not treatment related (Aze *et al.,* 1991; JECFA, 1999).

A 6-month dietary toxicity study in dogs conducted to evaluate the potential toxic effects of Reb A at dosage levels of 0, 500, 1000 or 2000 mg/kg bw/day supports the safety of published findings. There were no test-article-related clinical observations, nor were there any test-article-related changes in hematology, serum chemistry findings, or urinalysis. No test-article-related gross necropsy observations, alterations in final body weight, alterations in organ weights, or histologic changes were noted at the scheduled necropsy. Based on the results of this study, no toxicity of Reb A was observed at dosage levels up to 2000 mg/kg bw/day and the NOAEL ≥2000 mg/kg bw/day (Eapen, 2008, unpublished).

Chronic Toxicity

While there are no chronic studies of Reb A itself, there are two chronic studies available on stevioside. These studies are relevant to Reb A since Reb A and stevioside are converted via the same metabolic pathways to steviol in the gut prior to absorption. Based on the ADME, these studies allowed the Panel to evaluate the chronic toxicity of Reb A. Both studies were 2-year studies that examined the effect of dietary stevioside in rats. In the first study (Xili *et al*., 1992), stevioside administration in the diet showed no carcinogenic effects in the rat, and a NOAEL of 1.2% (600 mg/kg bw/day) in the diet was reported. In a more recent and more robust study (Toyoda *et al*., 1997), stevioside administered in the diet was not carcinogenic in rats, and a NOAEL of 2.5% (969 mg/kg bw/day in males) was established.

Despite the absence of a study in a second species, the Panel concluded that there was sufficient information to make safety evaluations on the available chronic studies. This conclusion is based on the low systemic exposure levels and the similarities in metabolism among mammalian species (including humans) as well as the lack of effects in the 2 studies in two different strains of rats.

Genotoxicity Studies

Reb A was not mutagenic with and without metabolic activation when tested *in vitro* in bacterial reverse mutation, bacterial forward mutation, and mammalian cell gene mutation assays. In addition, Reb A was negative in an *in vivo* mouse micronucleus assay in bone marrow.

Stevioside was not mutagenic with and without metabolic activation in several experiments using *in vitro* bacterial and mammalian cell mutation, and negative in chromosomal aberration, sister chromatid exchange, and micronucleus assays. Also, stevioside did not increase the micronucleus rate in an *in vivo* mouse micronucleus test in bone marrow.

Steviol was not mutagenic *in vitro* without metabolic activation in bacterial cell mutation, mammalian gene cell mutation, and chromosomal aberration studies, but with metabolic activation steviol was positive in some of these assays. In three *in vivo* mouse, one hamster and one rat micronucleus assays, chromosomal damage was not observed at dose levels of steviol as high as 8000 mg/kg bw. Therefore, the Panel concluded that the genotoxicity of steviol *in vitro* is not expressed *in vivo* even at high dose levels.

Reproductive/Developmental Toxicity Studies

The Panel reviewed a number of published studies that have evaluated the reproductive and developmental toxicity of steviol glycosides and steviol. Studies have been conducted using Reb A, stevioside, and steviol as the test material in hamsters and rats at doses up to 3000 mg/kg bw/day.

Stevioside had no effect on reproduction or development in a 3-generation hamster study with doses up to 2500 mg/kg bw/day (Yodyingyuad & Bunyawong, 1991). No effects were seen in a rat fertility study with doses of stevioside up to 3000 mg/kg bw/day (Mori *et al.*, 1981) or in a developmental study in pregnant rats with doses up to 1000 mg/kg bw/day (Takanaka *et al.*, 1991; Usami *et al.*, 1995).

The results of the published studies are supported by the results of two unpublished studies. Reb A was tested by gavage in an embryo/fetal development study in rats (Sloter, 2008b). Intrauterine growth and survival were unaffected by the test article, 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 when Reb A was administered by oral gavage to pregnant rats.

Reb A was also tested in a two-generation dietary reproduction toxicity study in rats (Sloter, 2008a) that confirms the results summarized above. There were no effects on reproduction (estrus cycles, mating, fertility, conception or copulation indices, number of days between pairing and coitus, gestation length, and spermatogenic endpoints). A dose level ≥2000 mg/kg bw/day (highest dose administered) was considered to be the NOAEL for parental systemic and reproductive toxicity, and an exposure level ≥2000 mg/kg bw/day was considered to be the NOAEL for neonatal toxicity of Reb A in rats.

In a published study by Wasuntarawat *et al*. (1998) steviol was not teratogenic in hamsters. Decreased weight gain and increased mortality in dams and fetuses (LOAEL = 500 mg/kg bw/day) and a dose dependent effect on kidneys in dams were observed. The effects with steviol but not Reb A and stevioside are likely due to the rapid absorption of steviol leading to higher C_{max} values, whereas Reb A and stevioside must first be hydrolyzed in the gut before absorption of steviol is possible.

The Panel concluded that the totality of evidence demonstrates that Reb A is neither a developmental nor a reproductive toxicant.

Human Studies

Blood Glucose Studies

The Panel reviewed clinical trials of stevioside and crude stevia extracts with normal and diabetic subjects. Blood glucose levels were measured at baseline and following oral administration of the test material for varying time periods (Table 6). The Panel concluded that stevioside, at doses up to 30 mg/kg bw/day, does not affect blood glucose levels.

Table 6: Summary of Blood Glucose Levels in Human Volunteers taking Steviol Glycosides for periods from 1 day to 2 years

Study	Substance tested	Doses	Duration	Subjects	Glucose (mg/100 ml)	HbA1C (%)	Other Information
Gregerson, 2004[2]	91%stevioside, 4% Reb A, 5% other steviol glycosides	1000 mg stevioside	Single dose	12 type2 diabetics	Time course reported: Average area under curve reduced by 18%±5%* (between 0 and 240 min)		Some subjects may have been receiving treatment for diabetes
Haebisch, 1992[3]	Crude stevia extract (13.9% stevioside)	Placebo	1 day	15 healthy volunteers	71.4 ± 2.0		Catabolic phase
		28 mg stevioside		20 healthy volunteers	75.1 ± 2.2		
		111 mg stevioside		25 healthy volunteers	67.8 ± 2.9*		
		Placebo		15 healthy volunteers	71.8 ± 2.6		Anabolic phase
		28 mg stevioside		20 healthy volunteers	73.1 ± 3.8		
		111 mg stevioside		25 healthy volunteers	68.3 ± 3.0*		

[2] Asterisk (*) in **this** study indicates statistical significance between pre and post treatment
[3] Haebisch et al measured blood glucose in a catabolic (fasting) and anabolic phase; significance was measured between placebo and control.
(*Indicates significantly different between placebo and stevioside and/or between baseline and post treatment);

Study	Substance tested	Doses	Duration	Subjects	Glucose (mg/100 ml)	HbA1C (%)	Other Information
Geuns et al., 2007[4]	>97% stevioside	Placebo, baseline	3 days	10 healthy volunteers	83.3 ± 2.2		
		Placebo, after 60 min			81.2 ± 2.0		
		250 mg/capsule 3x/day, baseline			85.0 ± 1.4		
		60 min after treatment			81.9 ± 1.6		
Ferri et al., 2006[5,6]	Stevioside (purity unspecified)	Placebo, baseline	24 weeks	7 subjects with mild hypertension	88.5 ± 6.2.	6.7 ± 0.2	
		Placebo, post-treatment			79.3 ± 5.0*	6.6 ± 0.4	
		3.75mg/kg bw/day for 7 weeks, 7.5 mg/kg bw/day for 11 weeks, 15 mg/kg bw/day for 6 weeks, baseline			91.8 ± 7.8	6.6 ± 0.3	Note individuals were given increasing doses over study period
		As above, post-treatment			80.7 ± 6.7*	6.6 ± 0.2	

[4] 10 subjects total for experiment. Subjects served as self controls three weeks after treatment.
[5] 14 subjects total, 7 each in placebo and treatment groups
[6] Significant differences between placebo at beginning and end of study (also true for stevioside group); NS between placebo and stevioside groups.

Study	Substance tested	Doses	Duration	Subjects	Glucose (mg/100 ml)	HbA1C (%)	Other Information
Jeppesen et al., 2006	Stevioside (purity unspecified)	Placebo	3 months	55 Type 2 diabetics on anti-hypertensive medication	Increased (value not reported	Increased (value not reported)	*Abstract of study*
		500 mg, 3x /day			No change (value not reported)	No change, value not reported	
Barriocanal et al., 2008[7]	Stevioside (>92%)	Placebo, baseline	3 months	15 Type 2 diabetics	131.3 ± 46.7	6.8 ± 1.6	
		Placebo, after treatment			118.9 ± 34.0	6.8 ± 1.0	
		250 mg, 3x /day, Baseline			151.2 ± 54	6.8 ± 1.2	
		250 mg, 3x /day, After treatment			133.8 ± 34.5	6.6 ± 1.1	
		Placebo, baseline		8 Type 1 diabetics	219.3 ± 74.1	8.2 ± 1.4	
		Placebo, after treatment			298.3 ± 58.8*	8.3 ± 1.6	
		250 mg, 3x /day, Baseline			144.9 ± 95.1	7.1 ± 1.6	
		250 mg, 3x /day, After treatment			155.3 ± 78.3	7.3 ± 1.1	

[7] Measurements were taken at baseline and post-treatment, significance within treatment group is denoted with an asterisk (*), significance between stevioside and placebo group noted with two asterisks (**)

Study	Substance tested	Doses	Duration	Subjects	Glucose (mg/100 ml)	HbA1C (%)	Other Information
Barriocanal et al., 2008[8]	Stevioside (>92%)	Placebo, baseline	3 months	17 Non-diabetics; normal/low normal BP	82.9 ± 10.2	5.3 ± 0.6	
		Placebo, after treatment		17 Non-diabetics; normal/low normal BP	83.9 ± 7.0	5.4 ± 0.7	
		250 mg, 3x /day, Baseline		13 Non-diabetics; normal/low normal BP	82.5 ± 6.6	5.3 ± 0.4	
		250 mg, 3x /day, After treatment		13 Non-diabetics; normal/low normal BP	82.9 ± 7.8	5.6 ± 0.6	

[8] Measurements were taken at baseline and post-treatment, significance within treatment group is denoted with an asterisk (*), significance between stevioside and placebo group noted with two asterisks (**)

Study	Substance tested	Doses	Duration	Subjects	Glucose (mg/100 ml)	HbA1C (%)	Other Information
Chan et al., 2000	Stevioside (purity unspecified)	Placebo 3x/day, baseline	1 year	46 subjects with mild to moderate hypertension	100.8 ± 14.4		
		Placebo 3x/day, after treatment		44 subjects with mild to moderate hypertension	97.2 ± 9.05		
		250mg 3x/day, baseline		60 subjects with mild to moderate hypertension	99.0 ± 12.6		
		250mg 3x/day, after treatment		56 subjects with mild to moderate hypertension	95.4 ± 12.6		
		Placebo 3x/day, baseline	2 years	89 subjects with mild hypertension	102.5 ± 12.6		
		Placebo 3x/day, post treatment		86 subjects with mild hypertension	108.2 ± 11.8		
		500 mg 3x/day, baseline		85 subjects with mild hypertension	100.8 ± 11.2		
		500 mg 3x/day, post treatment		82 subjects with mild hypertension	101.4 ± 10.8		

Blood Pressure Studies

The Panel carefully evaluated the potential effects of stevioside in normotensive and hypertensive individuals including hypertensive individuals with and without antihypertensive therapy. Inter-study comparisons were complicated by the following factors: different product formulations, diverse experimental designs, different subject populations and cohort sizes, and varying durations of treatment.

Human data were available for single dose, short-term (1-3days), intermediate (13-25 weeks) and long-term exposure periods (1-2 years). Doses of stevioside ranged from 28 mg/dose to 1500 mg/day in clinical studies (up to 30 mg/kg bw/day). These doses expressed in terms of Reb A Equivalents are up to 36 mg/kg bw/day. By comparison, anticipated 90th percentile dietary intakes from the proposed uses of Reb A would be no more than 5 mg/kg bw/day.

Blood pressure changes in normotensive individuals were small or absent and not meaningful or clinically significant. Barriocanal *et al.* (2008) conducted a 3-month, randomized, double-blind, placebo-controlled, parallel-design study employing type 2 diabetics (N=30), type 1 diabetics (N=16), and non-diabetics with normal to low normal blood pressure (N=30) with stevioside at 750 mg/day. No clinically significant differences between groups were observed in systolic or diastolic blood pressure measurements during the treatment period.

Hypertensive subjects were given stevioside for up to 2 years. Antihypertensive medication was discontinued before initiating the studies. Table 7 summarizes the blood pressure levels in control subjects and stevioside groups (250 mg/3x/day: 15 mg/kg bw/day) for 1 year (Chan, 2000). The baseline systolic blood pressure (BP) was 166.0 ± 9.4 (mean ±SD) mm Hg (placebo) and 166.5 ± 7.4 mm Hg (stevioside); the baseline diastolic blood pressure was 104.7 ± 5.2 mm Hg (placebo) and 102.1 ± 4.0 mm Hg (stevioside). At the end of 1 year there was a clinically significant decrease in systolic and diastolic blood pressure [systolic BP: 164.8 ± 8.7 mm Hg (placebo) vs. 152.6 ± 6.8 (stevioside) and diastolic blood pressure: 103.8± 5.4 (placebo) and 90.3 ± 3.6 (stevioside)]. In the 2-year study, the mean systolic blood pressure measurements were 149 ± 6.0 (placebo) and 150 ± 7.3 mm Hg (stevioside) pre-treatment and 150 ± 7.0 (placebo) 140 ± 6.8 mm Hg (stevioside) post-treatment. The mean diastolic blood pressure measurements were 96 ± 4.2 (placebo) and 95 ± 4.2 (stevioside) mm Hg pre-treatment and 95 ± 4.8 (placebo) and 89 ± 3.2 (stevioside) mm Hg post-treatment (Hsieh *et al*., 2003). Reductions of this magnitude were only seen in untreated individuals with significant hypertension. The Panel concluded that this magnitude of blood pressure reduction in these subjects would not present a risk.

Table 7: Summary of Blood Pressure Levels in Human Volunteers Taking Steviol Glycosides for periods of 1 day to 2 years

Study	Substance tested	Doses	Duration	Subjects	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	Other Information
Gregerson et al, 2004[9]	91% stevioside, 4% Reb A, 5% other steviol glycosides,	Placebo, baseline	Single dose	12 Type 2 diabetics	146 ± 4	86 ± 3	-30 to 0 minutes, pretreatment
		Placebo, after treatment			143 ± 4	87 ± 2	15 to 240 minutes, post-treatment
		1000 mg stevioside, baseline			143 ± 5	88 ± 3	-30 to 0 minutes, pretreatment
		1000 mg stevioside, after treatment			138 ± 4	84 ± 3	15 to 240 minutes, post-treatment

[9] 12 subjects total, subjects served as self-controls

Study	Substance tested	Doses	Duration	Subjects	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	Other Information
Haebisch, 1992[10]	Crude stevia extract (13.9% stevioside)	Placebo	1 day	15 healthy volunteers	109.3 ± 3.3	77.0 ± 3.7	Catabolic phase; supine
		28 mg stevioside[11]		20 healthy volunteers	108.3 ± 3.2	76.5 ± 2.5	
		111 mg stevioside		25 healthy volunteers	106.0 ± 3.0 *	73.2 ± 1.6*	
		Placebo		15 healthy volunteers	111.3 ± 3.1	84.0 ± 2.5	Anabolic; upright (15 min)
		28 mg stevioside		20 healthy volunteers	105.3 ± 3.1*	80.5 ± 2.4*	
		111 mg stevioside		25 healthy volunteers	108.4 ± 3.1*	83.8 ± 2.2	
Geuns et al., 2007[12]	>97% Stevioside	Placebo, baseline	3 days	10 healthy volunteers	115 ± 4	75 ± 1.33	
		Placebo, after 60 min			112 ± 3.33	73 ± 1.66	
		250 mg/capsule 3x/day, baseline			116 ± 4	74 ± 2.33	
		60 min after treatment			112 ± 3.33	72 ± 2.33	

[10] Haebisch et al measured BP in the supine and upright positions in a catabolic (fasting) and anabolic phase; significance was measured between placebo and treated (*indicates significantly different between placebo and control); Significant differences were also seen in the catabolic, low-dose group in the upright position and in the anabolic, high-dose group in the supine position for systolic BP. Significant differences were not seen in other groups.
[11] Total dose apparently divided into 4 doses during the day (paper is not clear: dose could possibly be 4X this value)
[12] 10 subjects total for experiment. Subjects served as self controls three weeks after treatment.

Study	Substance tested	Doses	Duration	Subjects	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	Other Information
Ferri et al., 2006[13]	Stevioside (purity unspecified)	Placebo, baseline	24 weeks	7 subjects with mild hypertension	133 ± 12	94 ± 8	
		Placebo, post treatment			124 ± 6	82 ± 4[14]	
		3.75mg/kg bw/day for 7 weeks, 7.5 mg/kg bw/day for 11 weeks, 15 mg/kg bw/day for 6 weeks, baseline			140 ± 13	94 ± 8	Note individuals were given increasing doses over study period
		As above, post treatment			123 ± 12	84 ± 8[15]	
Jeppesen et al., 2006	Stevioside (purity unspecified)	Placebo	3 months	55 (in placebo and control groups) type 2 diabetics on anti-hypertensive medication	NS difference (Values not reported)	NS difference (Values not reported)	Abstract of study
		500 mg, 3x /day					

[13] 14 subjects total, 7 each in placebo and treatment groups
[14] Significant differences compared to baseline placebo.
[15] Significant differences compared to stevioside.

Study	Substance tested	Doses	Duration	Subjects	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	Other Information
Barriocanal et al, 2008[16]	Stevioside (>92%)	Placebo, baseline	3 months	15 Type 2 diabetics	127.9 ± 13.7	76.7 ± 5.6	
		Placebo, after treatment			124.9 ± 13.3	77.4 ± 9.5	
		250 mg, 3x /day, Baseline			127.3 ± 15.1	77.3 ± 9.1	
		250 mg, 3x /day, after treatment			124.3 ± 13.5	74.7 ± 8.3	
		Placebo, baseline		8 Type 1 diabetics	108.3 ± 3.0	70.7 ± 4.4	
		Placebo, after treatment			105.7 ± 2.8*	69.7 ± 3.3	
		250 mg, 3x /day, Baseline			117.1 ± 6.6**	72.6 ± 6.9	
		250 mg, 3x /day, after treatment			115.9 ± 8.6	68.9 ± 7.2	

[16] Measurements were taken at baseline and post-treatment, significance within treatment group is denoted with an asterisk (*), significance between stevioside and placebo group noted with two asterisks (**)

Study	Substance tested	Doses	Duration	Subjects	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	Other Information
Barriocanal et al, 2008[17]	Stevioside (>92%)	Placebo, baseline	3 months	17 Non-diabetics; normal/low normal BP	111.7 ± 10.4	68.8 ± 5.5	
		Placebo, after treatment			112.2 ± 11.9	69.9 ± 8.1	
		Baseline		13 Non-diabetics; normal/low normal BP	111.0 ± 8.9	69.9 ± 7.2	
		After treatment			113.3 ± 10.8	69.8 ± 7.1	
Chan et al, 2000[18]	Stevioside (purity unspecified)	Placebo 3x/day, baseline	1 year	46 subjects with mild to moderate hypertension	166.0 ± 9.4	104.7 ± 5.2	
		Placebo 3x/day, after treatment		44 subjects with mild to moderate hypertension	164.8 ± 8.7	103.8 ± 5.4	
		250mg 3x/day, baseline		60 subjects with mild to moderate hypertension	166.5 ± 7.4	102.1 ± 4.0	
		250mg 3x/day, after treatment		56 subjects with mild to moderate hypertension	152.6 ± 6.8*	90.3 ± 3.6*	

[17] Measurements were taken at baseline and post-treatment, significance within treatment group is denoted with an asterisk (*), significance between stevioside and placebo group noted with two asterisks (**)
[18] For Chan et al, 2000 and Hseih et al, 2003, * = significant difference between baseline and endpoint; ** = significant difference between baseline and endpoint and treated and placebo.

Study	Substance tested	Doses	Duration	Subjects	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	Other Information
Hsieh et al., 2003[19]	Stevioside (purity unspecified)	Placebo 3x/day, baseline	2 years	89 subjects with mild hypertension	149 ± 6.0	96 ± 4.2	
		Placebo 3x/day, post treatment		86 subjects with mild hypertension	150 ± 7.0	95 ± 4.8	
		500 mg 3x/day, baseline		85 subjects with mild hypertension	150 ± 7.3	95 ± 4.2	
		500 mg 3x/day, post treatment		82 subjects with mild hypertension	140 ± 6.8**	89 ± 3.2**	

[19] For Chan et al, 2000, Ferri et al, 2006 and Hseih et al, 2003, * = significant difference between baseline and endpoint; ** = significant difference between baseline and endpoint and treated and placebo.

The Panel discussed potential concerns about the effects of stevioside use on hypertensive individuals who are on antihypertensive medications. The concern primarily related to reductions in blood pressure and possible orthostatic hypotension; however, one study using crude stevia extract did not reveal any changes in carefully measured blood pressure between the supine and upright positions (Haebisch, 1992).

It is noteworthy that the largest blood pressure reductions (i.e. ~10-14 mm Hg) were from studies in which subjects received either 750 (15 mg/kg bw/day) or 1500 mg/day (30 mg/kg bw/day) stevioside. These doses expressed in terms of Reb A Equivalents are 18 mg/kg bw/day or 36 mg/kg bw/day. These Reb A Equivalent doses are approximately 3.5 - 7 times the anticipated upper 90th percentile of intake from the proposed uses of Reb A. Furthermore, hypertensive individuals who were being treated with antihypertensive medications did not exhibit significant reductions in blood pressure (Barriocanal, 2008).

The Panel concluded that stevioside administration was associated with clinically relevant reductions in blood pressure for individuals with moderately severe hypertension who were not on antihypertensive therapy. However, clinically relevant reductions in blood pressure were not observed in individuals with normal blood pressure or in individuals with hypertension receiving antihypertensive medication.

ACCEPTABLE DAILY INTAKE (ADI) for Reb A

Based on the NOAEL for stevioside of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol) in a 2-year rat study, JECFA (2006) established a temporary ADI for steviol glycosides of 2 mg/kg bw/day (expressed as steviol). JECFA used a safety factor of 200 (100 for inter- and intra-species differences and additional factor of 2 because JECFA required additional information on pharmacological effects in humans). More recently, Food Standards Australia New Zealand (FSANZ, 2007) established an ADI for steviol glycosides of 4 mg/kg bw/day (expressed as steviol). FSANZ used a safety factor of 100 for inter- and intra-species differences. FSANZ provided the following rationale for applying a safety factor of 100:

> "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 [applied by JECFA] for uncertainty related to effects in normotensive or diabetic individuals is no longer required."

The Panel accepted the NOAEL used by JECFA and FSANZ (970 mg/kg bw/day). The default uncertainty factor typically used is 100 which is based on an inter-species factor of 10 and an intra-species factor of 10. Human data - including a study in which subjects were treated with 30 mg/kg for 2 years - are available for steviol glycosides as well as data that demonstrate the similarity of metabolism and kinetics between humans and animals. Therefore, a compound-specific determination of the uncertainty factor is

appropriate. The Panel could not use the human clinical trials to derive an ADI. However, the Panel concluded that because of the similarity in human and animal metabolism, a reasonable value for the intra-species factor would be 3-4 instead of 10 (WHO, 1994)[20]. The overall uncertainty factor would be 30-40.

Table 8. Acceptable Daily Intake from Totality of Evidence

Basis of ADI (Uncertainty Factor)	Expressed as Steviol (mg/kg bw/day)	Expressed as Reb A[22] (mg/kg bw/day)
JECFA temporary (200)	2	6
FSANZ (100)	4	12
Corroborating Reb A studies (100)	7	20
Panel ADI (100)	4	12
Panel ADI (40)	10	30
Conclusion for Reb A	**4-10**	**12-30**

22 Calculated using a conversion factor of 3.04 based on the molecular weights of 318.45 and 967.04 for steviol and Reb A, respectively.

The EDI of Reb A was calculated using the most recent food consumption survey results from the National Health and Nutrition Examination Survey (NHANES). Reb A intake was determined by multiplying each NHANES respondents' 2-day average food intake by the maximum proposed use level for each category. The total EDI from all proposed uses is 1.95 mg/kg bw/day at the mean and 4.72 mg/kg bw/day at the 90th percentile of intake among users. A user is defined as having consumed a food that would contain Reb A during the 2 days of the NHANES survey. Consideration of only 2 days of food consumption data results in a conservative upper estimate of potential Reb A intake.

In conclusion, even using conservative upper estimates of intake (5 mg/kg bw/day) and the lowest potential ADI (12 mg/kg bw/day), consumer intakes of Reb A from the proposed uses would not exceed the ADI.

[20] WHO International Programme on Chemical Safety. 1994. Environmental Health Criteria 170. Assessing Human Health Risk of Chemicals; Derivation of Guidance Values for Health-Based Exposure Limits.

BASIS FOR GRAS DETERMINATION

Regulatory Framework

The regulatory framework for determining whether a substance can be considered generally recognized as safe (GRAS) in accordance with section 201(s) (21 U.S.C. § 321(s)) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 et. Seq.) ("The Act"), is set forth at 21 CFR 170.30, which states:

"General recognition of safety may be based only on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. 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 scientific procedures shall ordinarily be based upon published studies which may be corroborated by unpublished studies and other data information."

There is an extensive database on steviol glycosides including specific studies for which Reb A was the test material. All steviol glycosides are metabolized by gastrointestinal microflora via the same intermediates and hydrolysis pathways; therefore, the safety data for all characterized steviol glycosides are relevant to the safety evaluation of Reb A. However, data for other steviol glycosides provide a conservative safety evaluation for Reb A since they all hydrolyze to steviol more rapidly than Reb A. There are no outstanding toxicology issues, and the metabolism and pharmacokinetic data for humans are well understood.

These criteria are applied below in an analysis of whether the use of Reb A as a sweetener for selected foods (tabletop sweeteners, sweetened ready-to-drink teas, diet carbonated soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal and sweetened cold cereals) is GRAS based upon scientific procedures.

The published literature supported by unpublished study results clearly demonstrate the lack of toxicity of Reb A and other steviol glycosides in animals and humans from oral ingestion at levels that are well above those anticipated from Reb A use as a sweetener in tabletop sweeteners, beverages, and cereals.

The Panel concluded that the totality-of-the-evidence satisfies the safety standard of reasonable certainty of no harm for the intended conditions of uses of Reb A. In addition because the information supporting safety is widely known and accepted by qualified experts, the Panel has concluded that it is not only safe, but generally recognized as safe (GRAS) for the intended conditions of use of Reb A.

Expert Panel Conclusion

We, the members of the Expert Panel have independently and collectively critically evaluated the available published and unpublished information on Reb A, other steviol glycosides and steviol, and unanimously conclude that Reb A, produced consistent with Good Manufacturing Practice and meeting the specifications described herein, is safe for use as a natural sweetener in selected beverages (including sweetened teas, diet soft drinks, energy drinks and flavored waters), cereals (oatmeal, cold cereal, cereal bars) and for tabletop use at the proposed use levels. We, the Expert Panel, further unanimously conclude that the uses of Reb A proposed above are GRAS based on scientific procedures. It is our opinion that other experts qualified to assess the safety of food and food ingredients would concur with these conclusions.

Signatures

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17th April 2008.
Date

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EXHIBIT II.

REB A GRAS DOSSIER

SAFETY EVALUATION DOSSIER

FOR

EXPERT PANEL DETERMINATION

OF THE

GRAS STATUS OF REBAUDIOSIDE A (REB A)

April 17, 2008

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Executive Summary

Rebaudioside A (common name Reb A) is the subject of the Generally Recognized as Safe (GRAS) Notice which this dossier supports. The conclusions are based on an evaluation using scientific procedures.

The regulatory framework for determining whether a substance is GRAS is in 21 CFR 170.30[1], which states that GRAS status through scientific procedures shall ordinarily be based upon published studies, which may be corroborated by unpublished studies and other data and information. These criteria have been applied to the existing data for Reb A. This analysis confirms that the use of Reb A as a sweetener in beverages, in cereals and in tabletop uses is GRAS. In the past, FDA has expressed concerns about the use of stevia and related materials as food ingredients; these concerns are addressed in this document.

Source, Characteristics, Manufacturing, and Intended Uses

Reb A, a natural sweetener, is a pure diterpene glycoside extracted from the plant *Stevia rebaudiana* (Bertoni), which belongs to the Asteraceae family and is native to Brazil and Paraguay.

Reb A is the second most abundant glycoside in stevia and is considered more suitable for use to sweeten foods and beverages than stevioside because of its greater solubility in water and better taste profile.

Reb A tastes about 200 to 300 times sweeter than sugar and is non-nutritive. It is structurally similar to other steviol glycosides, including stevioside. In many parts of the world, including Japan, South Korea, Israel, Mexico, Paraguay, Brazil, and Argentina,

[1] On April 17, 1997, the Food and Drug Administration (FDA) published a proposal in the *Federal Register* (62 *FR* 18938-18964) to reform the process for establishing the generally recognized as safe (GRAS) status of food ingredients. Since 1997 the agency has operated according to the new proposed 21 CFR 170.30, 170.35 and 170.36 that govern the GRAS process. This document contains the scientific basis for asserting the GRAS status of uses of Reb A as a sweetener in food, and serves as the primary focus of review for a Reb A GRAS Panel of Experts whose opinion concerning the safety of Reb A will form an integral part of the assertion that Reb A is GRAS for its intended uses under 21 CFR 170.30 and related regulations as currently administered by the FDA.

stevia glycosides, including extracts of the leaves, stevioside and Reb A, are used to sweeten food products and beverages.

Reb A is obtained by extraction of the leaves with water, ethanol or methanol and passed through an adsorption resin to concentrate the desired steviol glycosides, followed by an ethanol or methanol wash to release the glycosides. Highly pure (≥95% Reb A) is obtained by decoloration, desalting and recrystallization.

Stability and Degradation

Studies have been conducted to assess stability of Reb A in two model test systems and to identify and quantitate degradation products formed under extreme storage conditions at temperature ≥ 90ºF and pH < 3. No degradation products were identified in tabletop powder at conditions up to 105ºC for 96 hours. At pH 3.0 and ambient temperature (i.e. 70ºF) or refrigeration (40ºF) in an aqueous citric acid matrix, simulating diet soft drinks, no significant degradation of Reb A was seen over 25 weeks. In aqueous citric acid solutions held under extremely high temperatures, 2 main and 4 minor compounds were identified. All 6 compounds are structurally very similar to other steviol glycosides and/or were present in extremely low concentrations. Potential degradation products occurring under extreme storage conditions of Reb A in aqueous citric acid solutions were evaluated and determined to present no safety concerns.

Opinions of Authoritative Bodies on the Safety of Reb A

To the best of our knowledge, this is the first GRAS notice to FDA for a food use of Reb A[2]. However, steviol glycosides have been reviewed by other authoritative bodies, including the World Health Organization Joint Expert Committee on Food Additives (JECFA) and the Food Standards Authority of Australia and New Zealand (FSANZ).

[2] Historically FDA has received requests to classify botanical stevia and its various extracts as GRAS for use as a sweetener or flavoring agent in food. This GRAS notice is the first time an assertion of GRAS status for a stevia-related material has focused on a well-defined, single chemical entity of high purity, namely the steviol glycoside Reb A and used the current FDA process for GRAS notification proposed in 1997 (see previous footnote).

In 2006, JECFA established a temporary acceptable daily intake (ADI) for steviol glycosides of 2 mg/kg bw/day (expressed as steviol) based on the no-observed-adverse-effect level (NOAEL) for stevioside of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol or 1165 mg/kg bw/day Reb A) in a 2-year rat study. The corresponding ADI for Reb A (adjusting for the difference in molecular weight between steviol and Reb A) is 6 mg/kg bw/day. JECFA used a safety factor of 200 in calculating its ADI (100 for inter- and intra-species differences and an additional factor of 2 because at that time JECFA required additional information on pharmacological effects in humans). Data have now been published that address the data gap for pharmacological effects in humans. These data are scheduled for review by JECFA at its next meeting and have been included in this determination of the GRAS status of Reb A.

In 2007 FSANZ published an ADI for steviol glycosides of 4 mg/kg bw/day (expressed as steviol). Adjusting for molecular weight, the equivalent Reb A ADI is 12 mg/kg bw/day. FSANZ used a safety factor of 100 for inter- and intra-species differences.

FSANZ provided the following rationale for applying a safety factor of 100:

> "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 [applied by JECFA] for uncertainty related to effects in normotensive or diabetic individuals is no longer required."

Summary of Safety Database for Reb A

An extensive and widely known safety database exists for Reb A, steviol glycosides and steviol to support the GRAS status of Reb A for use in the proposed food categories, i.e. beverages, cereals and as a tabletop sweetener. The data cover the standard range of safety studies for determination of safety of food ingredients including acute/subchronic/chronic toxicity, genotoxicity, reproductive/developmental toxicity, and carcinogenicity, as well as absorption, distribution, metabolism and excretion (ADME). In addition, there have been a number of special studies in animals and humans that

address issues related to the safety of steviol glycosides including potential effects on blood pressure and blood glucose control.

The results of safety studies are summarized below. The ADME of Reb A and other steviosides has been extensively studied and is key to understanding the relevance of the available toxicology data. Therefore, the ADME is discussed first; followed by summaries of the toxicology data related to safety evaluation. The studies have been grouped by period of exposure and/or type of study.

Absorption, Distribution, Metabolism and Excretion (ADME)

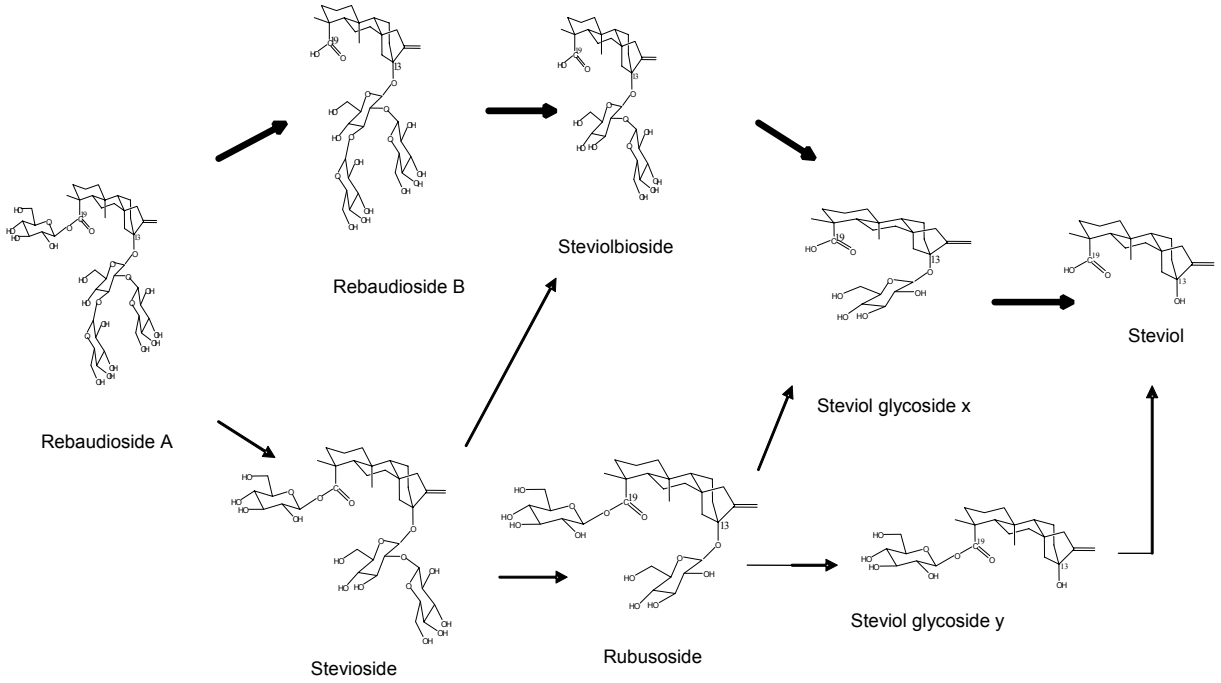
No absorption or structural modification of steviol glycosides in the stomach was found in stevioside-dosed test animals or human volunteers. The principal steviol glycosides, Reb A and stevioside are metabolized in experimental animals and humans by intestinal microflora by successive hydrolysis of glucose moieties. Human fecal microflora was found to completely hydrolyze stevioside and Reb A to their common aglycone, steviol, in 10 and 24 h, respectively. There was no further degradation of steviol. Stevioside and Reb A did not influence significantly the human intestinal microflora composition.

Besides steviol glucuronide and extremely small amounts of administered test article or free steviol, the steviol glycosides have not been detected in blood. When Reb A and total steviol were detected in peripheral blood of rats during daily administration of 2000 mg/kg bw/day of Reb A it was at extremely low levels, with mean plasma concentrations of approximately 0.6 and 12 µg/mL, respectively. Estimates of absorbed dose for Reb A and total steviol were approximately 0.02% and 0.06%, respectively, based on the amounts measured in urine collected over 24 hours in comparison to daily administered dietary dose to rats. It has been determined that free steviol that is absorbed from the lower intestines is transported to the liver via the portal vein where it is converted into steviol glucuronide, which is excreted in urine or bile.

Based on the results of all of the metabolism studies, ingested steviol glycosides are eliminated in the feces or hydrolyzed to steviol prior to absorption from the gut. Because of the common hydrolysis pathways (see figure below) and toxicokinetics of steviol

glycosides and steviol, any study in which the test material was a steviol glycoside is relevant in evaluating safety of Reb A. However, some studies have been conducted on uncharacterized extracts, and those studies may have reported effects due to other non-glycosidic substances that were present. The studies in which the test material was characterized as a high purity steviol glycoside are thus more likely to be reliable and were relied on for this determination of the GRAS status of Reb A.

Common Hydrolysis Pathways of Reb A to the Aglycone, Steviol



The toxicology results are summarized below by study type. Human test results have been grouped together to facilitate the evaluation of those studies.

Acute Toxicity

Reb A and stevioside (purity >96%) were not acutely toxic to mice, rats, or hamsters at doses as high as 15,000 mg/kg bw/day. Similarly, steviol is not acutely toxic to rats and mice at doses as high as 14,000 mg/kg bw/day.

Subchronic Toxicity Studies

Three subchronic studies are summarized for Reb A and stevioside. Dietary administration of Reb A to rats and dogs for 90-days and 6-months, respectively, at doses as high as 2000 mg/kg bw/day resulted in no adverse systemic toxicity. In the 90-day rat study, lower body weight gains were reported only in the high-dose (2000 mg/kg bw/day) group males. The study authors concluded that the lower body weights were due to the large proportion of the diet represented by the non-nutritive test material. Similar results have been reported with other intense sweeteners administered at high dietary concentrations and in that research it was concluded that body weight gain decreases are not an appropriate basis for a No-Observed-Adverse-Effect-Level (NOAEL) for intense sweeteners; therefore, the NOAEL for the subchronic Reb A study was ≥2000 mg/kg bw/day.

No dose-related effects were observed in rats that were administered stevioside in the diet at concentrations up to 5% for 13 weeks.

Chronic Toxicity

Two chronic toxicity/carcinogenicity studies have been conducted with stevioside. Both studies were 2-year studies that examined the effect of oral stevioside in rats. In the first study, stevioside administration in the diet showed no carcinogenic effects in the rat, and a NOAEL of 1.2% (600 mg/kg bw/day) in the diet was reported. In a more recent and more robust study, stevioside administered in the diet was not carcinogenic in rats, and a NOAEL of 2.5% (970 mg/kg bw/day in males) was established.

Genotoxicity

Reb A was not mutagenic with and without metabolic activation when tested *in vitro* in bacterial reverse mutation, bacterial forward mutation, and mammalian cell gene mutation assays. In addition, Reb A was negative in an *in vivo* mouse micronucleus assay in bone marrow. Stevioside was not mutagenic with and without metabolic activation in several experiments using *in vitro* bacterial and mammalian cell mutation, and negative in chromosomal aberration, sister chromatid exchange, and micronucleus

assays. Also, stevioside did not increase the micronucleus rate in an *in vivo* mouse micronucleus test in bone marrow and hepatocytes. Steviol was not mutagenic *in vitro* without metabolic activation in bacterial cell mutation, mammalian gene cell mutation, and chromosomal aberration studies, but with metabolic activation steviol was positive in some of these assays. However, in three *in vivo* mouse, one hamster and one rat micronucleus assays, chromosomal damage was not observed at dose levels as high as 8000 mg/kg bw.

Reproductive/Developmental Toxicity Studies

Eleven studies have been reported in the literature in which reproductive/developmental toxicity was evaluated. Studies have been conducted using Reb A, stevioside and steviol as the test material in hamsters and rats at doses up to 3000 mg/kg bw/day.

Reb A was tested in a two-generation dietary reproduction toxicity study in rats. There were no effects on reproduction (estrus cycles, mating, fertility, conception or copulation indices, number of days between pairing and coitus, gestation length, and spermatogenic endpoints). A dose level of at least 2000 mg/kg bw/day (highest dose administered) was considered to be the NOAEL for parental systemic and reproductive toxicity, and an exposure level ≥2000 mg/kg bw/day was considered to be the NOAEL for neonatal toxicity of Reb A in rats.

Reb A was tested in an oral (gavage) embryo/fetal development study in rats. Intrauterine growth and survival were unaffected by the administration of Reb A and there were no Reb A 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 when Reb A was administered by oral gavage to pregnant rats.

Stevioside had no effect on reproduction or development in a 3-generation hamster study with doses up to 2500 mg/kg bw/day and no effects in a rat fertility study with doses up

to 3000 mg/kg bw/day or in a developmental study in pregnant rats with doses up to 1000 mg/kg bw/day.

Steviol was not teratogenic in hamsters; however, decreased weight gain and increased mortality in dams and fetuses at doses ≥ 500 mg/kg bw/day, and a dose-dependent effect on kidneys in dams were observed. The effects with steviol (but not Reb A and stevioside) in hamsters are likely due to the rapid absorption of steviol, whereas Reb A and stevioside must first be hydrolyzed in the gut before steviol can be absorbed.

Human Studies

The effects of the oral administration of stevioside in humans have been evaluated in several studies for durations of one day to two years. These studies indicate that steviol glycosides are unlikely to cause any effects on blood pressure in normal healthy humans. Steviol glycosides may decrease blood pressure slightly in hypertensive individuals who are not taking antihypertensive medications; however, this effect is not considered to be adverse. FSANZ also reached this conclusion in their draft risk assessment, which was published in 2007.

The human study results confirm that there are unlikely to be treatment-related effects of steviol glycosides (including Reb A and stevioside) on blood glucose at doses that could result from its use as a sweetener in normal or diabetic individuals.

FSANZ has reviewed the same studies and concluded the following:

> "…there is a relatively low risk of hypoglycaemia in normal healthy human subjects, particularly at concentrations that may be encountered in the diet.[…] In summary, the weight-of-evidence indicates that stevioside would be unlikely to produce hypoglycaemia in humans at concentrations encountered in the diet."

In conclusion, there is an extensive safety database on steviol glycosides including specific studies in which Reb A was the test material. All steviol glycosides are metabolized via the same intermediates and same hydrolysis pathways; therefore, the safety data for all such steviol glycosides are relevant regardless of which test substance

was administered to test animals. There are no outstanding toxicology issues, and the metabolism and pharmacokinetic effects in humans is well understood. NOAELs ≥2000 mg/kg bw/day (*expressed as Reb A)* have been established based on rat 90-day and dog 6-month subchronic, rat embryo/fetal developmental and rat 2-generation reproduction toxicity studies conducted with Reb A. These data corroborate the extensive published toxicology database for the steviol glycosides.

Proposed Uses

Reb A would be used at the levels proposed in the following table:

Proposed Uses and Levels of Reb A (ppm)

Food Category	Reb A (ppm)
Tabletop sweeteners	30,000[3]
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

[3]Reb A content of sachet prior to dilution and not representative of "as consumed"

Basis for ADI and Estimated Daily Intake

As shown in the table below, the totality-of-evidence related to the safety evaluation of Reb A allows one to conclude that an acceptable daily intake (ADI) of 12-20 mg/kg bw/day (expressed as Reb A) is supported by the data and is appropriate for use in assessing safety of Reb A for use in food and the determination that these uses of Reb A are GRAS.

Basis of ADI (Safety Factor)	Expressed as Steviol (mg/kg bw/day)	Expressed as Reb A (mg/kg bw/day)
JECFA temporary (200)	2	6
FSANZ (100)	4	12
Corroborating Reb A studies (100)	7	20
Conclusion for Reb A	**4-7**	**12-20**

The estimated daily intake (EDI) of Reb A from the proposed uses including tabletop sweeteners, sweetened ready-to-drink iced teas, diet carbonated soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal, and cold cereals (sweetened), was estimated using the maximum proposed use rates and data from the most recent National Health and Nutrition Examination Survey (2003-2004). The EDI on a per "user" basis is approximately 2 mg/kg bw/day at the mean and 5 mg/kg bw/day at the 90th percentile of intake. The assumptions that were used in estimating consumer intakes from all proposed uses (beverages, cereals and as a tabletop sweetener) are conservative and, therefore, the estimates most certainly overestimate potential consumer intakes of Reb A. Even with these conservative assumptions, all calculated EDIs for the food categories of interest are well below the ADI (12-20 mg/kg bw/day). This demonstrates that there is a reasonable certainty of no harm from the proposed addition of Reb A to these products.

In conclusion, the GRAS status of Reb A for use as a sweetener in beverages, in cereals, and in tabletop uses is confirmed.

Introduction

Rebaudioside A (common name Reb A), a natural sweetener, is a pure diterpene glycoside extracted from the plant *Stevia rebaudiana* (Bertoni) that belongs to the Asteraceae family and is native to Brazil and Paraguay. Reb A is the second most abundant glycoside in stevia and is considered more suitable for use to sweeten foods and beverages than stevioside because of its greater solubility in water and better taste profile. Reb A has a sweetness potency of 200-250 times that of sugar on a weight basis and is non-nutritive. It is structurally similar to other steviol glycosides, including stevioside.

Historically, FDA has received requests to classify botanical stevia and its extracts as Generally Recognized as Safe (GRAS) for use as a sweetener or flavoring agent in food. The GRAS Notice which this dossier supports represents the first time an assertion of GRAS status for a stevia-related material has focused on a well-defined, single chemical entity of high purity, namely the steviol glycoside, Reb A. To our knowledge, this is the first submission to FDA since additional research has defined the common pathways for steviol glycoside absorption, distribution, metabolism, and excretion (ADME) of steviol glycosides. Based on that research, new clinical studies and previously published literature, several authoritative bodies have concluded that steviol glycosides are safe for use as a sweetener. Specifically the Food and Agriculture Organization/World Health Organization Joint Expert Committee on Food Additives (JECFA) has established a temporary ADI (expressed as steviol) of 0-2 mg/kg bw/day. Food Standards Australia New Zealand (FSANZ) reviewed the same data plus new research in human volunteers and have proposed an ADI (also expressed as steviol) of 0-4 mg/kg bw/day. Based on molecular weight of steviol and Reb A, these ADIs would be 6 and 12 mg/kg bw/day, respectively, expressed as Reb A.

FDA has received numerous requests, inquiries, and petitions over the years for the use of stevia and stevia-related and derived chemicals as sweeteners in food. The FDA record (including a memorandum by Whiteside and Hollingsworth, 1990) dating from at least 1972, contains numerous references to stevia-related actions and positions. In these references, FDA expressed a range of concerns about the use of stevia and related materials as food ingredients. These

concerns are responded to below in the context of the present-day widely known and accepted information about the steviol glycosides including, in particular, Reb A.

- **Product Characteristics**

Issue: Available records indicate that FDA requested information about the identity, purity, and specifications of stevia and related products of interest numerous times in the past. FDA reviewers mention the need for identification and characterization of any "residual substances." Source documents request analysis of, and assays and characterization of, the intended ingredient and its impurities; its manufacturing process; its stability in foods; its planned food uses; and adequately documented characterization of any residuals or impurities. Because composition has varied among ingredients of interest and substances tested, FDA has considered this issue to be crucial in establishing the relevance of various safety studies.

Resolution: This notification is for a single chemical Rebaudioside A (Reb A) (≥ 95%) as detailed in Section I. Specifications, manufacturing method, stability in foods, proposed food uses, discussion of potential degradants, analytical methods and analysis of several manufactured lots are included.

- **Stability**

Issue: Hydrolysis conditions, hydrolysis rates and hydrolysis products forming in food from the use of stevia and steviol glycosides have been cited as concerns by FDA in the past. For example, for carbonated beverage use, FDA requested information in the 1990s on stability of stevia-derived food ingredients in aqueous medium down to pH of 2.5 over a period of at least three months at temperatures of up to 40 ºC so as to assess the safety of any degradation products.

Resolution: Comprehensive stability studies have been conducted to confirm stability under normal use conditions. In addition, studies have been conducted for long-term storage under extreme temperature (up to 110ºF) and pH (2.0 to 7.0) conditions. The safety of resulting degradation products has been confirmed (See Sections III and V.C).

- **Adequacy of Toxicological Data**

Issue: Among the publicly available records on stevia and its glycosides, FDA has expressed concern about the lack of toxicity data and/or the lack of quality in the existing data at that time to support safe use of stevia-derived ingredients in food. For example:

  - Existing studies were not consistent with FDA's guidelines or standards outlined for toxicological testing.
  - Toxicological studies at the time lacked complete characterization of the substance fed and the identity of the substances fed varied among the various studies.
  - There was an inadequate characterization of the ADME of stevia and related compounds in animals or humans. In particular, FDA wanted evidence whether stevia or its glycosides were metabolized to steviol in the GI tract by human gut microflora, as appeared to be the case in rats.
  - FDA expressed concern that the carcinogenic potential of stevia-derived ingredients was not well determined in animals or humans.
  - FDA has commented that steviol is "highly mutagenic" in salmonella mutation assays in the presence of metabolic activation, increasing the importance of good carcinogenicity and ADME studies.
  - Most studies were of short duration (3 months or less).
  - FDA cites inadequate reproduction studies.

Resolution: The toxicology database has been expanded and strengthened by the completion of numerous studies conducted according to FDA GLPs and test guidelines with Reb A as the test material (rat and dog subchronic, *in vitro* and *in vivo* genotoxicity, toxicokinetics and rat development/reproductive studies). New clinical studies have been conducted using human volunteers to evaluate potential effects of steviol glycosides on blood pressure and blood glucose. The results of these new toxicology studies conducted with Reb A (presented in Section V), are corroborative of published toxicology studies with stevioside and together with the new published clinical studies, uniformly support the GRAS status of Reb A.

- **Anti-fertility Effects or Other Reproductive Effects in Animals and Humans**

Issue: FDA expressed concern about the presence of some historical data pointing to the possibility that stevia-derived products can induce reproductive effects, including a pronounced antifertility effect when administered in the drinking water of test animals and a no-effect level had not been determined. FDA was also concerned about a report that steviol induced androgenic and anti-androgenic effects in chickens and its potential relevance to humans. Cited often was [Mazzei] Planas and Kuc (1968), apparently confirmed by Nunes *et al*. (1988), where it was reported that an aqueous extract of stevia decreased fertility in female rats. Also cited are anecdotal reports of Guarani of Paraguay using stevia and its aqueous extracts as an oral contraceptive.

Resolution: Published studies reported no reproductive or developmental effects of stevioside in hamsters and rats, as presented in Section V.A.2.e. These studies are corroborated by new FDA GLP and test guideline studies with Reb A that demonstrate a lack of reproductive or developmental effects even at the extremely high doses tested (2000 mg/kg bw/day).

- **Hypoglycemia**

Issue: FDA has cited the potential for stevia and its extracts to produce hypoglycemia in animals and humans.

Resolution: Sufficient data now available, including several new studies in which steviol glycosides were administered to human volunteers, demonstrate that there will not be an adverse effect on blood glucose by use of Reb A under conditions of intended use. A separate section of this document consolidates the results of testing in human volunteers (Section V.B.).

- **Blood Pressure Effects**

Issue: FDA has expressed concern about the potential of stevia and its extracts to produce adverse effects on the cardiovascular system in animals and, potentially, in humans.

Resolution: This notification specifically evaluated potential effects on blood pressure in animals and humans (results are presented in Sections V.A.3 and V.B). The data demonstrate that in some studies there are effects (albeit small) on blood pressure in animals, particularly with stevia extracts. Although stevioside administration was associated with clinically relevant reductions in blood pressure for individuals with moderately severe hypertension who were not on antihypertensive therapy, clinically relevant reductions in blood pressure were not observed in individuals with normal blood pressure or in individuals with hypertension receiving antihypertensive medication.

In summary, this submission contains a complete review of the literature as well as presentation of the intended uses. It is organized to first describe the chemical identity of Rebaudioside A (Reb A), its technological function in the proposed uses and estimated daily intakes (EDI) from those uses. This is followed by the safety evaluation including ADME, toxicology and an in-depth analysis of research in human volunteers. The acceptable daily intake (ADI) is determined and compared to the EDI. This comprehensive analysis confirms the GRAS status of Reb A based on scientific principles.

I. Notified Substance Rebaudioside A (Reb A) Identification

Reb A is a diterpenoid glycoside obtained from the leaves of *Stevia rebaudiana* Bertoni (hereafter referred to as "stevia"). Stevia, a plant native to South America (notably Brazil and Paraguay), has been used for many years as a sweetener in Latin America and some Asian countries. Although impure stevia was used since the early part of the 20th century, it became more popular when several Japanese manufacturers developed ways to purify it in the 1970s. The ability of sweeteners from stevia to withstand the elevated temperatures of pre-sweetened hot beverages held in vending machines has driven the Japanese innovation in this area. Japan is currently the largest market for stevia, producing more than 100 foods and beverages that contain stevia and steviol glycosides. Stevia and stevia extract are also very popular in Korea, occupying 40% of the sweetener market. Currently, most of the stevia for the Japanese market is grown in China, although some is also grown in Taiwan, Thailand, and Vietnam. Interest in growing stevia also has come from Canada, the Czech Republic, India, and Russia (Kinghorn *et al*., 2001) and cultivation of stevia in Paraguay continues.

Stevia derives its sweetness from several glycosides (see Appendix I.-1 entitled Structures of Steviol Glycosides Found in *Stevia rebaudiana* (Bertoni) Leaf), with stevioside and Reb A the component glycosides of principal sweetening interest. In addition to sweetness, from an organoleptic standpoint, stevioside has some bitterness and aftertaste.

Reb A is the second most abundant glycoside in stevia and is considered more suitable for use in foods and beverages than stevioside because of its greater solubility in water and better taste profile (Bakal *et al.*, 1986). Reb A is a white, crystalline, odorless, water soluble powder approximately 200 and 300 times sweeter than a 10% and 2-4% sucrose solution, respectively. Although sweetness potency values are dependent on the concentration used, Reb A with a sweetness potency of 200-250 times that of sugar on a weight basis can be compared with cyclamate, aspartame, acesulfame-K, saccharin, and sucralose which have potency values of 35, 200, 200, 350-400, and 600, respectively (Fry, 2007).

The chemical name, CAS number, chemical formula, formula weight and structural formula for Reb A follow.

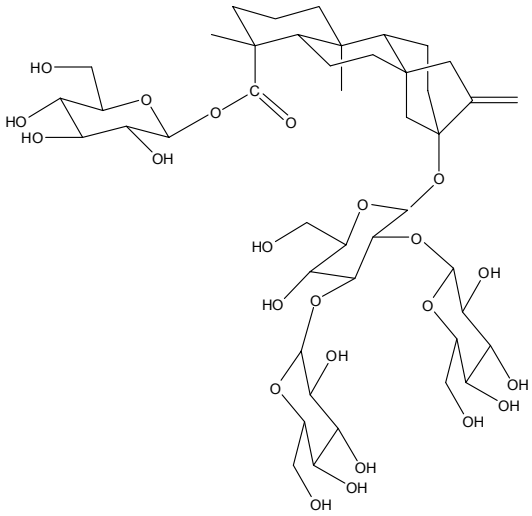
Chemical Name: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] kaur-16-en-18-oic acid β-D-glucopyranosyl ester

CAS Number: 58543-16-1

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

Formula Weight: 967.03

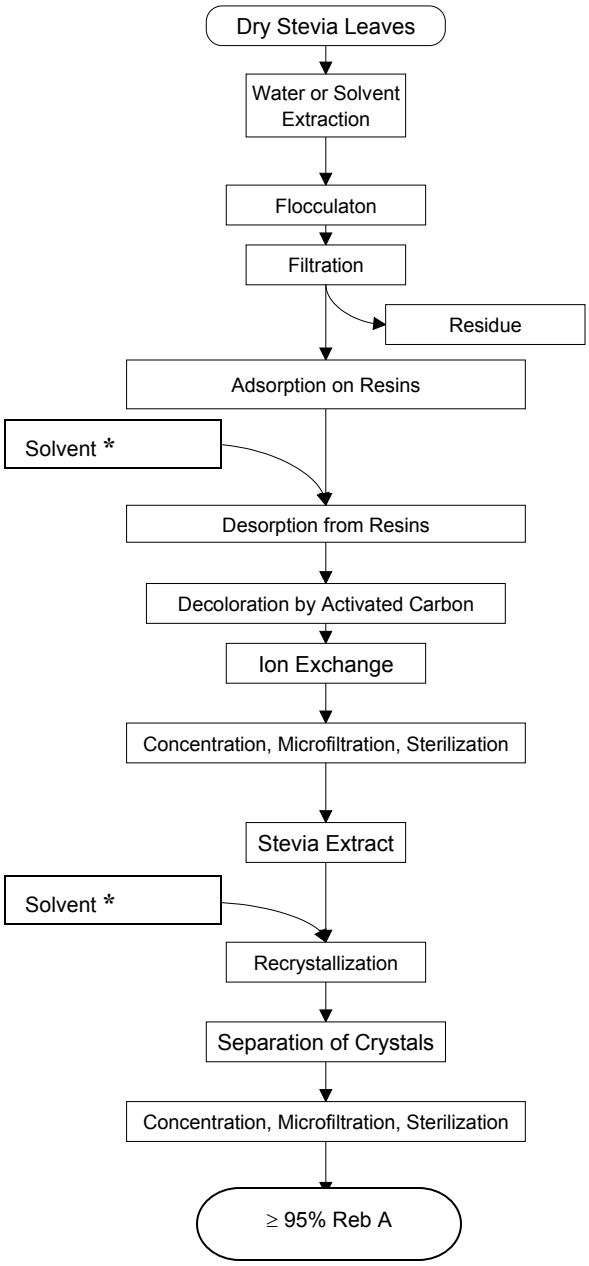
Structural formula:



Typical Method of Manufacture of Reb A

The source of Reb A, the leaves of *Stevia rebaudiana* (Bertoni), is a botanical and as such the steviol glycoside content is slightly influenced by the temperature in the year of cultivation, the latitude, and the time of harvesting. Stevia leaves contain 2 – 4% (w/w) Reb A and the highly purified material is obtained by extraction of the leaves with water, ethanol or methanol and the extract is passed through an adsorption resin to trap and concentrate the desired steviol glycosides. The resin is washed with ethanol or methanol to release the glycosides. Through decoloration and desalting by ion exchange and microfiltration, consistently higher purity Reb A is obtained. Recrystallization with alcohol/water mixtures results in very high purity Reb A which may be spray-dried or granulated and residual alcohol removed (Figure I.-1). Typical NMR, FTIR, Mass Spectra, and HPLC are presented in Appendix I.-2 (entitled Reb A NMR, FTIR, Mass Spectra, and HPLC).

Figure I.-1: Typical Method of Manufacture of Reb A



* Aqueous ethanol or methanol.

Specifications for Food Grade Reb A

Reb A is a single chemically defined substance (≥ 95%) and should not be confused with stevia leaves or stevia extracts which are natural product "mixtures." The food grade specifications for Reb A summarized in Table I.-1 are comparable to the JECFA 2007 specifications for steviol glycosides (Appendix I.-3 entitled JECFA Monograph (2007) for Steviol Glycosides). Consistency of Reb A manufacturing is demonstrated by the analyses of 3 lots of Reb A and the certificates of analysis from the vendor (Appendix I.-4 entitled Reb A Lot Analyses and Certificates of Analysis).

Table I.-1: Purity and Specifications for Food Grade Reb A

Test	Acceptable Target/Range	Test Method (Appendix I.-5)
Purity Reb A (wt/wt %)	≥ 95% Reb A (on dry basis)	JECFA, 2007 (modified)
Total Steviol Glycosides (wt/wt %)	≥ 95% (on dry basis)	JECFA, 2007 (modified)
Stevioside (wt/wt %)	2% (on dry basis) maximum	JECFA, 2007 (modified)
Steviol (wt/wt %)	< 0.005% (on dry basis)	Bazargan *et al*., 2007 (LC-MS/MS)
Moisture Content (%) by Loss on Drying	5.0% maximum	FCC, 2003 (p. 855)
Optical Rotation	-29 to -37 degrees	FCC, 2003 (p. 844)
Arsenic (as As)	1.0 mg/kg maximum	FCC, 2003 (p. 861)
Lead	1.0 mg/kg maximum	FCC, 2003 (p. 867)
Cadmium	1.0 mg/kg maximum	FCC, 2003 (p. 863)

Quality Specifications for Reb A

Reb A is derived from a botanical product, *Stevia rebaudiana* (Bertoni) and, therefore, quality specifications have been set to address microbiological contamination, residual solvent, and pesticide residues (see Table I.-2). There are no pesticide maximum residue limits (MRL) set for the raw agricultural commodity, stevia, and the vendor has declared that no pesticides are used on the stevia plants. Therefore, there should be no detectable pesticide residues in the multi-residue screen (Appendix I.-6 entitled Pesticide Declaration and Analyses).

Table I.-2: Quality Specifications for Reb A

Test	Acceptable Target/Range	Test Method
Total Aerobic Plate Count	1000 cfu/g maximum	Downes and Ito, 2001a
Total Aerobic Mold Count	100 mold cfu/g maximum	Downes and Ito, 2001b
Total Aerobic Yeast	100 yeast cfu/g maximum	Downes and Ito, 2001b
Heat Resistant Mold	Non-detectable (< 1 cfu/50 g)	Downes and Ito, 2001c
Coliform	< 10 cfu/g	Downes and Ito, 2001d
E. coli	< 3 MPN/g	Downes and Ito, 2001d
Salmonella ssp	Negative in 25g	Downes and Ito, 2001e
Staphylococcus aureus	Non-detectable (< 1 cfu/g)	Downes and Ito, 2001f
Alicyclobacillus acidoterrestris	Non-detectable (<1 cfu/50 g)	JFJA, 2007
Residual Solvents	Methanol < 300 ppm Ethanol < 1000 ppm	U.S. Pharmacopoeia, 2007
Pesticide Residues*	Non-detectable	U.S. FDA, 2004; CDFA, 1988

*See Appendix I.-4.

II. Technical Function and Self-limiting Use Information

Study of Sweetness Levels of Reb A in Tabletop Sweetener

The purpose of this study was to demonstrate the functionality of Reb A as a tabletop sweetener and to identify the concentrations of Reb A that deliver the typical, minimum and maximum sweetness levels as judged by a sensory panel when tabletop sweetener is served in brewed coffee.

Samples of brewed coffee sweetened with tabletop sweetener at seven different concentrations of Reb A were evaluated in sensory panels. Each sweetener concentration was served in random sequence in a separate sensory panel session, where 21 to 32 panelists were asked to evaluate the sweetness in coffee. Panelists used the following 5-point category scale to rate sweetness:

5 = "Much Too Sweet"
4 = "Slightly Too Sweet"
3 = "Just About Right"
2 = "Not Quite Sweet Enough"
1 = "Not At All Sweet Enough"

Frequency distribution data from all panels were collected and compiled to confirm that the coffee was sweet, and to determine the typical, minimum and maximum sweetness levels for a tabletop sweetener when served in coffee.

The typical sweetness level, defined as that concentration which received the largest proportion of "Just About Right" responses, was found to be 147 ppm. The minimum sweetness level, defined as the predicted concentration where 75% of the responses fell into the lower two categories, "Not At All Sweet Enough" and "Not Quite Sweet Enough" combined, was determined to be 79 ppm. The maximum sweetness level, defined as the predicted concentration where 75% of the responses fell into the upper two categories, "Slightly Too Sweet" and "Much Too Sweet" combined, was determined to be 547 ppm.

In conclusion, Reb A functions effectively as a tabletop sweetener and in coffee the typical sweetness level is 147 ppm which correlates to 26 mg Reb A/gram serving. There was an increase in sweetness ratings as the concentration of Reb A increased from 74 ppm to 735 ppm. Based on the criteria for this study, coffee sweetened with tabletop product containing Reb A is considered "Not Sweet Enough" at or below 79 ppm and "Too Sweet" at or above 547 ppm or 97 mg Reb A/gram serving.

Study of Functionality of Reb A in Beverages

The sweetness potency of Reb A in beverages is represented as the sweetness imparted by a particular Brix solution of sugar. Sweetness potency curves for nutritive sweeteners, e.g. sugar and high fructose corn syrup, are linear and the potency does not depend on the sweetener concentration. As with other potent sweeteners, the Reb A sweetness potency varies when compared against a low sugar solution versus a high sugar solution. Reb A potency may be as much as 300X when compared against a 2-4% sugar solution but is 200X when compared against a 10% sugar solution.

The sensory stability of Reb A was investigated by comparing the time-dependent taste degradation of Reb A against an aspartame (APM) control using bench-produced beverages stored at 70ºF and double triangle design methodology, randomized complete block design with 48 panelists (Appendix II.-1 entitled Reb A Beverage Sensory Study Methodology). Table II.-1 illustrates that the sensory stability was statistically significantly different ($P<0.05$) between Reb A and APM after 10 weeks with each time point compared against the 4-week results. Reb A was more stable than APM from 10 to 24 weeks.

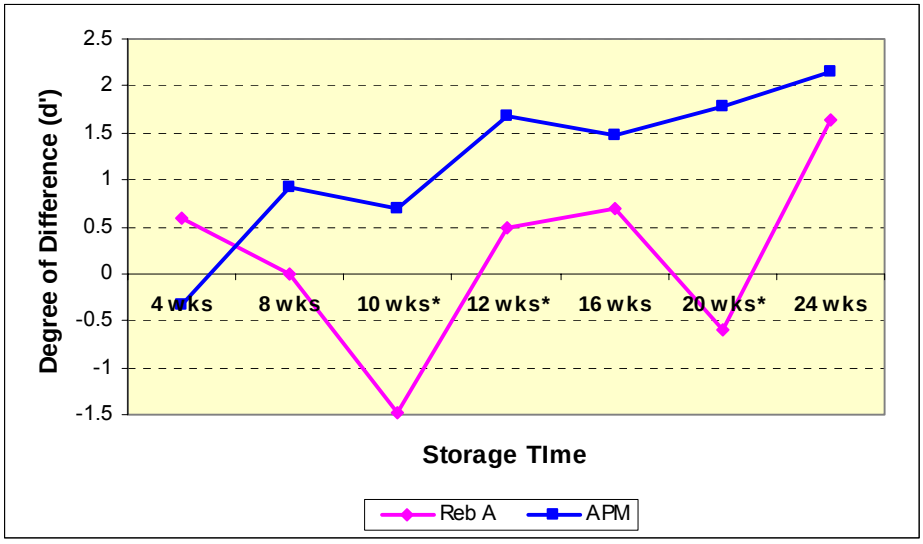
Table II-1: Sensory Stability of Reb A and APM Evaluated by Beta-Binomial Analysis

	Reb A[1]		APM[1]
4 weeks	0.24	4 weeks	0.47
8 weeks	Not sampled	8 weeks	0.056
10 weeks	0.96	10 weeks	0.32
12 weeks	0.43	12 weeks[2]	0.002
16 weeks	0.17	16 weeks[2]	0.001
20 weeks	0.36	20 weeks[2]	0.0000
24 weeks[2]	0.00000	24 weeks[2]	0.00000

[1] P value for statistical significance compared to 4-week results
[2] A significant difference @95% CL. Criterion is set at P<0.05.

Sensory perceptual differences between Reb A and APM over 24 weeks of storage were investigated (Figure II.-1). When compared to week 4, APM and Reb A exhibited significant differences after 12 weeks and 24 weeks, respectively. The data indicates that there was no perceptual difference in Reb A for up to 20 weeks.

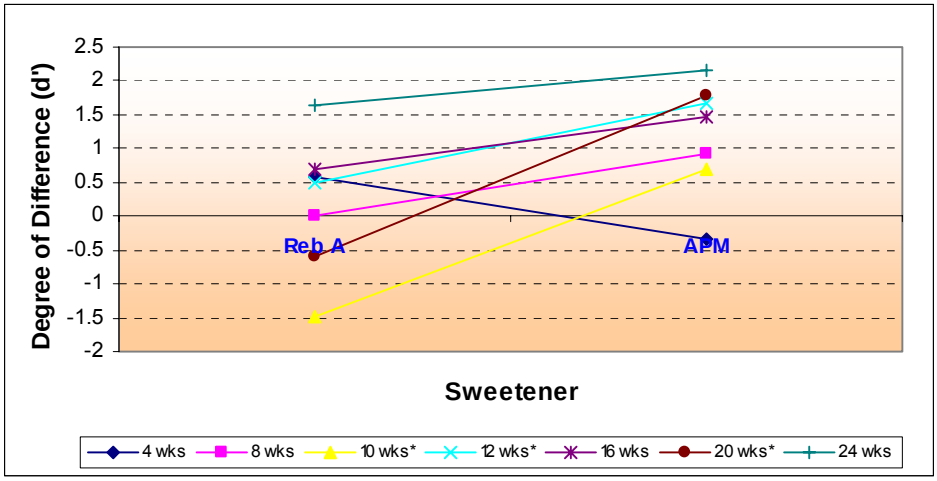
Figure II.-1: Sensory Perceptual Differences of Reb A and APM



The studies also provided information on taste degradation difference between Reb A and APM (Figure II.-2). The data indicated that except for 4 weeks, APM was more different

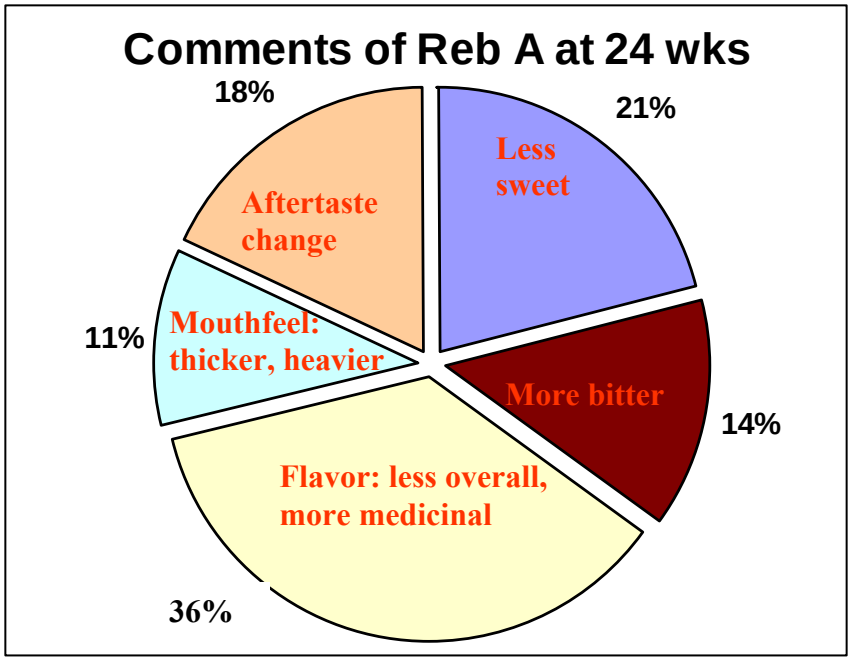
from 4 weeks than Reb A. A noticeable difference in Reb A was shown at 24 weeks as compared to a noticeable difference shown after 12 weeks for APM.

Figure II.-2: Taste Degradation Differences Between Reb A and APM



The Reb A sensory property changes at 24 weeks were investigated (Figure II.-3). Comments on the beverage taste indicated that Reb A may interact with sensory properties including sweet, bitter, flavor, mouthfeel, and aftertaste; all resulting in more intense chemical and medicinal aroma, less sweet and more bitter taste, and/or heavier and thicker mouthfeel.

Figure II.-3: Sensory Property Change of Reb A after 24 Weeks



In summary, the perception of sweetness is beverage and concentration dependent. However, qualitative assessments indicate that 20 ppm is the lowest sweetness threshold in a beverage system. Qualitative assessments also indicate that 14 Brix is considered a "too sweet" beverage, which in a sweetness equivalency study it was determined that it takes approximately 1200 ppm Reb A in water to match the sweetness of a 14% sugar solution. Most beverages are sweetened to approximately 10 Brix or 10% sugar solution. Experimentation with 500 ppm Reb A indicated that the sweetness potency is 200X, therefore the potency is presented as: 10%/0.05% = 200X. The overall sensory stability of Reb A is significantly better than APM over the beverage shelf-life period of 24 weeks. Taste degradation of Reb A did not occur until 20 weeks.

III. Reb A Stability

Twenty-Six Week Stability and Functionality Study of Reb A in Tabletop Powder

The purpose of this study was to assess the chemical stability and functionality of Reb A as a sweetener in a prototype tabletop powder formulation stored under accelerated conditions set to 40 ± 2°C and 75 ± 5% relative humidity (RH). Chemical stability was determined by monitoring content levels using validated HPLC methodology (Appendix III.-1, entitled HPLC Assay for the Analysis of Reb A in Tabletop Products).

Functionality was demonstrated by sensory panel ratings of coffee sweetened with tabletop powder. The sensory panel consisted of 20 - 31 employees, who used a 5-point sweetness rating scale to evaluate the samples. The category values were:

5 = "Much Too Sweet"
4 = "Slightly Too Sweet"
3 = "Just About Right"
2 = "Not Quite Sweet Enough"
1 = "Not At All Sweet Enough"

The Reb A tabletop powder was considered to be functionally sweet provided no more than 75% of the panelists rated the sweetness of the coffee in categories 2 and 1.

Reb A was combined with Unidex® to provide approximately 26.10 mg (26,100 ppm) of Reb A per serving. Process capability and isosweetness data analysis supports target Reb A level of 26.10 mg/serving. Unidex® is a blend of dextrose and maltodextrin that is commonly used as a carrier for high intensity sweeteners in tabletop products. Coffee was sweetened targeting final Reb A concentration of 147 ppm. Samples were periodically evaluated over a 26-week period for stability and functionality.

Twenty-six-week data indicates there is no statistically significant degradation of Reb A. After 26 weeks under accelerated conditions, 26.65 mg (26,650 ppm) of Reb A remained

in the tabletop powder. These results are within the variability of the manufacturing of dry blended powders which always have variability from batch to batch due to individual particle sizes, static, etc. Content Uniformity run on the blend (12 samples taken from throughout the blender) showed a range of 2.60 to 2.74% Reb A, with an average of 2.66%. Target formula, given some dusting losses in production was 2.61%.
At 26-weeks, 55% of the panelists rated the sweetness in the upper three categories, "Just About Right," "Slightly Too Sweet" and "Much Too Sweet" while 45% of the panelists rated the sample in the lower two categories, "Not Quite Sweet Enough" and "Not At All Sweet Enough". Therefore, Reb A in a tabletop powder is stable and functional as a sweetener through 26 weeks under accelerated storage conditions, 40 ± 2°C, 75 ± 5% RH.

Forced Degradation of Reb A Powder

A forced degradation study was conducted to assess the chemical stability of Reb A powder when exposed to prolonged heat conditions. The study (described in Appendix III.-2, entitled Forced Degradation of Reb A Powder) compared the amount of Reb A, stevioside, rebaudioside C, and dulcoside A present in samples of Reb A before and after exposing the samples to high heat 105°C for 4 days (96 hours).

The assay values for Reb A, stevioside, rebaudioside C, and dulcoside A are presented in Table III.-1. No rebaudioside C or dulcoside A were detected in any of the samples. Data for the heated samples indicates a slight increase in the mean % Reb A, and a slight decrease in the mean % stevioside; however, a two tailed t-test (α=.05) analysis suggests the slight changes are not statistically significant. Consequently, no significant degradation occurred in samples held at a constant temperature of 105°C for 96 hours.

Table III.-1: Forced Degradation Results: Reb A

FRESH (AMBIENT) SAMPLE ANALYSIS				
	% Reb A	**% Stevioside**	**% Rebaudioside C**	**% Dulcoside A**
Sample #1	90.97%	0.43%	0.00%	0.00%
Sample #2	91.44%	0.00%	0.00%	0.00%
Sample #3	92.83%	0.00%	0.00%	0.00%
Sample #4	93.17%	0.00%	0.00%	0.00%
Mean	**92.10%**	**0.11%**	**0.00%**	**0.00%**

HEATED (OVEN) SAMPLE ANALYSIS				
	% Reb A	**% Stevioside**	**% Rebaudioside C**	**% Dulcoside A**
Sample #1	94.16%	0.14%	0.00%	0.00%
Sample #2	92.75%	0.00%	0.00%	0.00%
Sample #3	92.15%	0.00%	0.00%	0.00%
Sample #4	92.12%	0.19%	0.00%	0.00%
Mean	**92.80%**	**0.08%**	**0.00%**	**0.00%**

In addition to showing no degradation, Table III.-2 illustrates the peak purity values for each Reb A peak detected via spectral analysis. Using the default purity limit set by Perkin Elmer Iris software of <1.5, all Reb A peaks were shown to be pure. Contour maps for all eight samples did not reveal additional peaks when analyzing wave lengths from 190nm to 400nm.

Table III.-2: Peak Purity Results from Spectral Analyses (Purity limit <1.5)

Sample	Reb A Peak Purity
Fresh Bag 1-1	1.11
Fresh Bag 1-2	1.13
Fresh Bag 2-1	1.11
Fresh Bag 2-2	1.05
Heated Bag 1-1	1.23
Heated Bag 1-2	1.11
Heated Bag 2-1	1.13
Heated Bag 2-2	1.23

In conclusion, no additional degradation components were found after exposing samples to a constant temperature of 105°C heat for 96 hours.

<u>Stability of Reb A in Carbonated Beverages and Model Solutions</u>

(Chang and Cook, 1983)

Introduction

Studies were conducted to determine the stability of Reb A in both pure sweetener solutions and carbonated beverages under various storage conditions. A robust summary of the study including details of the study design are included in Appendix III.-3 (entitled Stability of Reb A in Carbonated Beverages and Model Solutions).

Results for Pure Sweetener Solutions

Results of the HPLC and TLC analyses indicated that prolonged heating of the neutral Reb A solution, 6.5 mg/mL, at 100ºC resulted in a decrease in the Reb A concentration (i.e. 68.5 % recovery at 48 hours) and the appearance of rebaudioside B and glucose as the degradation products.

Results for Acidic Solutions

Acidic Reb A solutions heated to 60ºC for up to 137 hours did not demonstrate appreciable degradation of Reb A; however, heating to 100ºC resulted in considerable degradation, i.e. 13.2 and 24.1 % recovery after 13 hours in the phosphoric or citric acid systems, respectively.

Citric acid Reb A solutions heated to 100ºC resulted in one unknown degradation product at 4 hours and two unknown degradation products at 10 and 14 hours. A third unknown degradation product was detected during 4-10 hours of heating in the phosphoric acid system, but not at 13 hours. Rebaudioside B and glucose were present during heating of both acidic solutions.

Results for Carbonated Beverages

Reb A showed no significant degradation during 4 months of storage at 4ºC, 3 months at room temperature (22ºC), or 1 month at 37ºC in carbonated citric or phosphoric acid beverages. Although a decrease in Reb A concentration was observed by HPLC (i.e. 13-

25% degradation after 4 months of storage at 37ºC), no degradation products were observed.

Minimal reduction in Reb A concentration was observed after storage at 60ºC for 137 hours (i.e. 3% and 6% degradation in citric and phosphoric acid beverages, respectively); however, substantial degradation did occur when beverage samples were exposed to 3000 langleys of sunlight (i.e. 22 and 18% degradation in the phosphoric and citric acid beverages, respectively). A single unidentified degradation product was detected after storage of the Reb A beverage samples in sunlight for one week.

Summary

Reb A in carbonated citric or phosphoric acid beverages showed no significant degradation during prolonged storage at refrigeration, normal ambient or elevated ambient temperatures. However, minimal reduction in Reb A concentration was observed after storage at extremely high temperature for carbonated citric or phosphoric acid beverages. In model neutral or acidic test solutions, prolonged heating at 100ºC resulted in a decrease in the Reb A concentration, but for prolonged heating at 60ºC degradation of Reb A was minimal.

<u>Stability of Reb A in an Aqueous Citric Acid Matrix</u>

(Stetson, 2008a-c [Unpublished])

Introduction

The pH range for the beverages for which Reb A is foreseen as a sweetener is from pH 2.8 to 3.2 and recommended storage temperature is ambient (i.e. ≈70°F). Therefore, the experiments conducted at pH 3 and 70°F in the following summary are most pertinent to the intended condition of use for these products. However, results also are reported for extreme environmental conditions of temperature ≥ 90ºF and pH < 3. The Reb A commercial products are referred to by their brand names throughout this summary in order to distinguish between the results for each product.

Methods

In an FDA GLP (21 CFR Part 58) compliant study (Stetson, 2008a [Unpublished]), Reb A concentrations in aqueous solutions containing two purified Reb A commercial products (Chrysanta® 99-P or Rebaudioside A-98%) at an initial concentration of 600 µg/mL were determined analytically. Additional details of the study design are included in Appendix III.-4 (entitled Stability of Reb A in an Aqueous Citric Acid Matrix).

Purity and Composition of Three Reb A Products

Reb A, rebaudioside B, stevioside, steviolbioside, and steviol concentrations in three purified Reb A commercial products (Chrysanta® 99-P, Rebaudioside A-92% and Rebaudioside A-98%) were identified and quantitated (Stetson, 2008a [Unpublished]). Rebaudioside B and stevioside were the contaminants present at the highest concentrations. Steviol was present in the preparations in only trace amounts (Table III.-3).

Table III.-3: Purity of Analyzed Reb A Products

	Concentration, µg/mL and (% of total)				
Reb A Commercial Product	**Reb A**	**Rebaudioside B**	**Stevioside**	**Steviolbioside**	**Steviol**
Chrysanta® 99-P	579 (96.90)	11.3 (1.89)	7.03 (1.18)	0.17 (0.028)	0.00534 (0.00089)
Rebaudioside A-98%	618 (98.70)	6.00 (0.96)	2.08 (0.33)	0.064 (0.010)	*0.00073 (0.00012)*
Rebaudioside A-92%	608 (99.03)	4.78 (0.78)	1.16 (0.19)	*0.019 (0.0031)*	*0.00021 (0.000034)*

Note: Italics indicate extrapolated values < LLOQ (lower limit of quantification).

Stability in an Aqueous Citric Acid Matrix

Solutions of two purified Reb A commercial products (Chrysanta® 99-P and Rebaudioside A-98%), prepared at nominal concentrations of 0.60 mg Reb A/mL in aqueous citric acid solutions (50mM) at various pH values (2.0, 4.0 and 7.0 ± 0.5), were sterile filtered using 0.22 µm filters and stored for up to 15 weeks at approximately 40, 70,

90 or 110 ± 5 ºF. Samples were collected and analyzed at 0, 1, 3, 5, 7, 9, 11, 13 and 15 weeks of incubation (Stetson, 2008a [Unpublished]). In a second study, solutions of Chrysanta® 99-P prepared at nominal concentrations of 0.60 mg Reb A/mL in aqueous citric acid (50mM) solutions at various pH values (2.5, 3.0 and 3.5 ± 0.5), were sterile filtered using 0.22 µm filters and stored for up to 25 weeks at approximately 40, 70, 90 or 110 ± 5 ºF. Samples were collected and analyzed at 0, 1, 3, 5, 7, 9, 11, 13, 15, 20 and 25 weeks of incubation (Stetson, 2008c [Unpublished]). In both studies the rates of Reb A degradation, as well as the concentrations of rebaudioside B, stevioside, steviolbioside, and steviol contaminants/degradants, were monitored analytically.

During incubation for 15 weeks, no significant Reb A degradation was detected in

1) the Chrysanta® 99-P and Rebaudioside A-98% pH 7 solutions at 40, 70, 90 or 110ºF,
2) the Chrysanta® 99-P and Rebaudioside A-98% pH 4 solutions at 40, 70 or 90ºF and
3) the Chrysanta® 99-P and Rebaudioside A-98% pH 2 solutions at 40ºF.

However, solutions prepared at pH 4 and incubated at 110ºF, or at pH 2 and incubated at 70, 90 and 110ºF, resulted in significant Reb A degradation.

The expected effect of increasing incubation temperature on Reb A degradation rate was apparent, i.e. the half-life values for Reb A in the Chrysanta® 99-P solutions at pH 2 and stored at 70, 90 and 110ºF were 67.3, 12.3 and 3.2 weeks, respectively. The solution pH also had a significant effect on Reb A degradation. The half-life values for Reb A in the Chrysanta® 99-P solutions at pH 2 and pH 4 and stored at 110ºF were 3.2 and 126 weeks, respectively. There were no significant differences in the stability (or instability) of Reb A observed with solutions of Chrysanta® 99-P or Rebaudioside A-98% (Stetson, 2008a [Unpublished]). In addition, during incubation for 25 weeks, Chrysanta® 99-P solutions prepared at pH 2.5, 3.0 and 3.5 and incubated at 70, 90 and 110ºF resulted in Reb A degradation (Stetson, 2008c [Unpublished]).

Just as in the previous study (Stetson, 2008a [Unpublished]), the expected effect of increasing incubation temperature on Reb A degradation rate occurred, i.e. the half-life values for Reb A in the Chrysanta® 99-P solutions at pH 2.5 and stored at 70, 90 and 110ºF were 128, 21.9 and 4.7 weeks, respectively. As in the previous study, pH had a significant effect on Reb A degradation. The half-life values for Reb A in the Chrysanta® 99-P solutions at pH 2.5, 3.0 and 3.5 and stored at 110ºF were 4.7, 10.3 and 29.9 weeks, respectively (Stetson, 2008c [Unpublished]). However, the half-life values for Reb A decreased in the Chrysanta® 99-P solutions at pH=2.5, 3.0 and 3.5 stored at 40ºF, which was opposite to the trend demonstrated with all other data. Since the data collected in the previous study (Stetson, 2008a [Unpublished]) during incubation at 40ºF of Chrysanta® 99-P solutions at pH=2.0, 4.0 and 7.0 resulted in positive degradation constants (no detectable Reb A degradation), it is likely that the trend of the data for the conditions of pH=2.5, 3.0 and 3.5 stored at 40ºF was the result of assay variability, rather than decreasing half-lives. The results are summarized in Table III.-4 below (Stetson, 2008c [Unpublished]).

Table III.-4: Degradation of Analyzed Reb A Commercial Products

		Degradation Constant and ($T_{½}$ in weeks)			
Reb A Commercial Product	**pH**	**40ºF**	**70ºF**	**90ºF**	**110ºF**
Chrysanta® 99-P[1]	2.0	NSD	-0.0103 (67.3)	-0.0562 (12.3)	-0.2179 (3.18)
Rebaudioside A-98%[1]	2.0	NSD	-0.0122 (56.8)	-0.0614 (11.3)	-0.2552 (2.72)
Chrysanta® 99-P	2.5	-0.0012 (578)	-0.0054 (128)	-0.0317 (21.9)	-0.1462 (4.74)
Chrysanta® 99-P	3.0	-0.0021 (330)	-0.0047 (147)	-0.0144 (48.1)	-0.0674 (10.3)
Chrysanta® 99-P	3.5	-0.0028 (248)	-0.0002 (347)	-0.0039 (178)	-0.0232 (29.9)
Chrysanta® 99-P[1]	4.0	NSD	NSD	NSD	-0.0055 (126)
Rebaudioside A-98%[1]	4.0	NSD	NSD	NSD	-0.0036 (192)
Chrysanta® 99-P[1]	7.0	NSD	NSD	NSD	NSD
Rebaudioside A-98%[1]	7.0	NSD	NSD	NSD	NSD

NSD = No significant degradation of Rebaudioside A detected
[1] = Data from previous study (Stetson, 2008a [Unpublished])

For test article solutions prepared at pH 2.0, 2.5, 3.0 and 3.5 and incubated at 70, 90 and 110ºF or prepared at pH 4 and incubated at 110ºF, Reb A degradation resulted in two main unidentified degradant peaks in the HPLC/UV chromatograms (Stetson, 2008a,c [Unpublished]). The main degradant peak (i.e. Compound X; identified as iso-Reb A, see p. III-13) eluted within 0.3 minutes following the elution of Reb A. The second

degradant peak (i.e. Compound Y; iso-rebaudioside B, see p. III-13) eluted within 0.3 minutes following the elution of rebaudioside B. Initially, Reb A degradation appears to be quantitatively accounted for by the accumulation of Compound X, suggesting that the initial step in Reb A degradation under severe pH and temperature conditions is the formation of Compound X. However, there is a delay in Compound Y appearance and significant accumulation, which appears to be related to the accumulation of Compound X to significant concentration levels. The accumulation of Compound X in solutions at pH 3 during incubation at 110ºF, for example, appears to plateau by weeks 15 to 20. These data would suggest that Compound Y is the direct product of Compound X degradation. The four following figures (Figures III.-1, 2, 3, and 4) demonstrate the effect of low vs. high temperature at pH 3.0 (Stetson, 2008c [Unpublished]). At pH 3.0 and ambient temperature (i.e. 70°F) or refrigeration (i.e. 40°F) over the 25-week period of the stability study, Reb A degradation is insignificant as illustrated in Figures III.-1 and III.-2. It is not until an extremely high temperature of 90°F or 110°F is sustained for several weeks that significant degradation of Reb A occurs as illustrated in Figures III.-3. and III.-4 below.

Figure III.-1: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 3 and 40°F

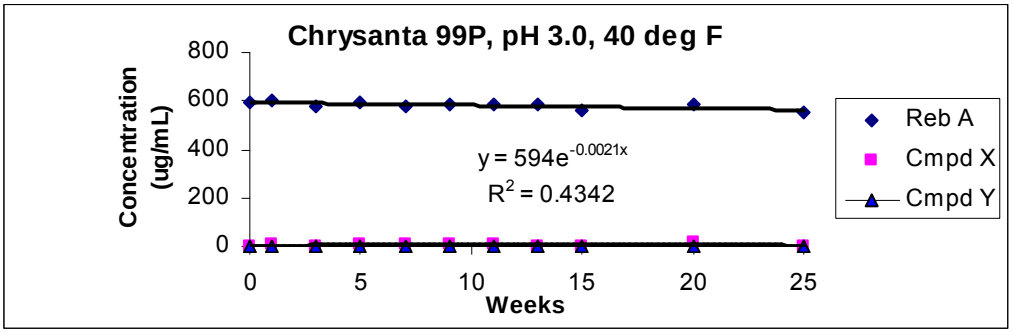


Figure III.-2: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 3 and 70°F

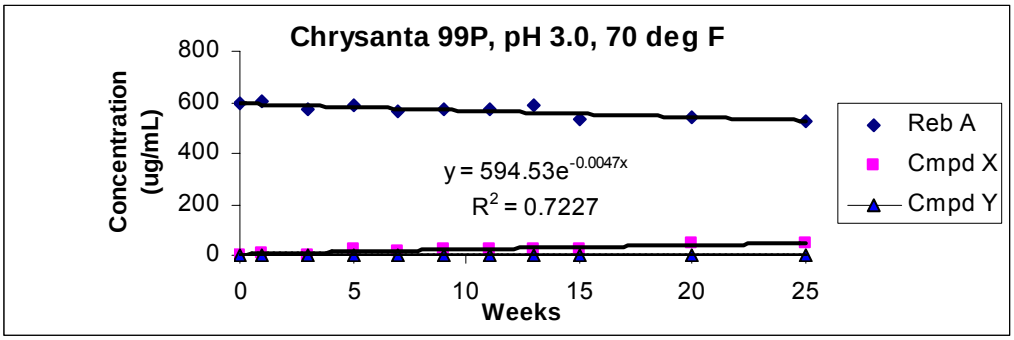


Figure III.-3: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 3 and 90°F

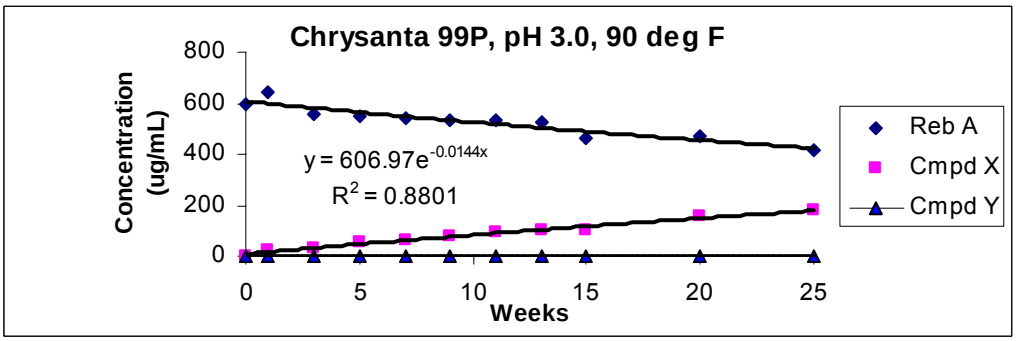
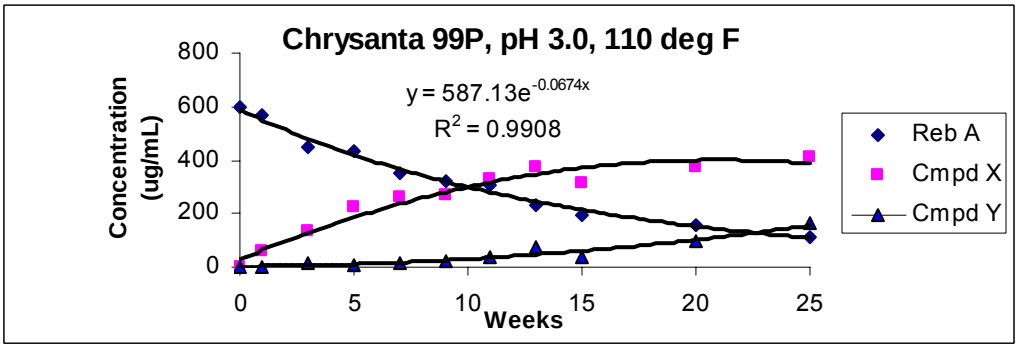
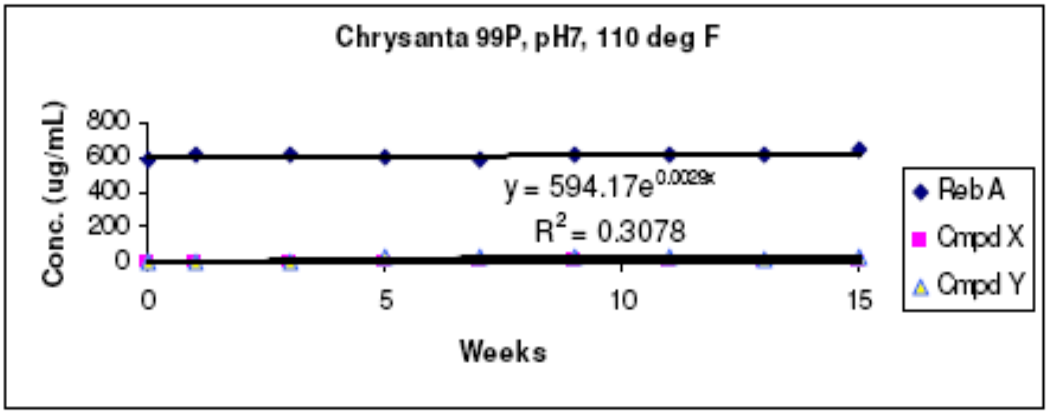


Figure III.-4: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 3 and 110°F



The following figure (Figure III.-5), in comparison to Figure III.-4, illustrates that Reb A degradation is more dependent on low pH than on high temperature (Stetson, 2008a [Unpublished]).

Figure III.-5: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 7 and 110°F



In addition to monitoring Reb A degradation over time, the concentrations of rebaudioside B, stevioside, steviolbioside, and steviol degradants/contaminants were analyzed. The results of these analyses and the range of concentration values (expressed as µg/mL) for rebaudioside B, stevioside, steviolbioside, and steviol generated from samples collected throughout the 15-week incubation period are summarized in Table III.-5 below (Stetson, 2008a [Unpublished]).

Table III.-5: Concentration of Degradants in Analyzed Reb A Commercial Products

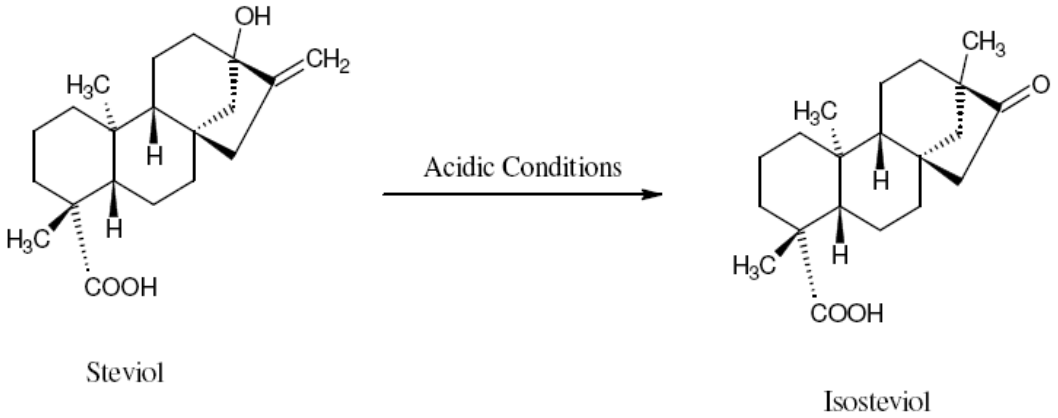
			Concentration Range (µg/mL)			
Reb A Commercial Product	**pH**	**Temp (ºF)**	**Reb B**	**SS**	**SBS**	**Steviol**
Chrysanta® 99-P	2.0	40	9.45-16.8	6.73-11.1	0.173-0.351	*0.0046*-0.0093
		70	11.0-20.7	6.53-11.3	0-1.23	0.0059-0.0133
		90	11.6-23.6	4.13-10.6	0.198-0.526	0.0058-0.0496
		110	11.6-28.4	*0*-4.12	0.190-0.518	0.0061-0.177
Chrysanta® 99-P	4.0	40	10.1-15.2	7.24-9.69	0.191-0.393	*0.0045*-0.0089
		70	10.4-14.6	7.65-9.92	0.191-0.380	0.0058-0.0102
		90	11.0-17.5	6.54-11.7	0.205-0.386	0.0066-0.0117
		110	10.4-27.3	6.09-9.85	0.208-0.556	0.0061-0.0168
Chrysanta® 99-P	7.0	40	11.3-14.0	6.93-10.2	0.190-0.344	0.0067-0.0124
		70	9.26-14.8	6.46-10.7	0.193-0.370	0.0063-0.0134
		90	9.40-17.4	6.03-10.7	0.193-0.367	0.0068-0.0212
		110	12.0-28.5	7.01-10.7	0.203-0.516	0.0067-0.0151
Rebaudioside A-98%	2.0	40	5.34-7.39	2.25-3.30	0.074-0.127	*0*-0.0054
		70	5.80-16.8	1.78-3.42	0.067-0.156	*0.00094*-0.0078
		90	6.45-20.9	1.27-3.20	0.077-0.232	*0.0012*-0.0522
		110	6.55-21.7	0.320-3.50	0.066-0.290	*0.00093*-0.214
Rebaudioside A-98%	4.0	40	4.86-7.91	1.76-3.49	0.068-0.129	*0-0.0041*
		70	5.34-8.00	1.98-3.20	0.067-0.129	*0*-0.0063
		90	5.65-9.95	1.90-3.20	0.066-0.135	*0.00085*-0.0092
		110	6.90-22.2	2.02-3.35	0.083-0.204	*0.00141*-0.0125
Rebaudioside A-98%	7.0	40	4.79-7.35	2.18-3.45	0.0716-0.121	*0.00086*-0.0051
		70	5.23-8.00	2.20-3.25	0.076-0.126	*0.00081-0.0043*
		90	5.15-10.4	2.33-4.10	0.069-0.128	*0.00051*-0.0054
		110	7.35-23.7	1.98-3.35	0.079-0.223	*0.00098*-0.0093

Note: Italics indicate extrapolated values < LLOQ (lower limit of quantification).
Reb B = Rebaudioside B
SS = Stevioside
SBS = Steviolbioside

These data indicate that rebaudioside B, stevioside, steviolbioside and steviol, initially present in both Chrysanta® 99-P and Rebaudioside A-98% in trace amounts, failed to accumulate to significant levels even during incubation of pH 2 test article solutions at 110ºF, suggesting that the degradation of Reb A, which occurs under acidic conditions at elevated temperatures, proceeds primarily via the Compound X, Compound Y pathway (Stetson, 2008a [Unpublished]).

Four unidentified and minor (in apparent concentration) degradant peaks appeared in the LC/MS/MS chromatograms (Stetson, 2008a [Unpublished]). Similar to what was observed with Reb A and Compound X, and with rebaudioside B and Compound Y, three of the four unknown peaks chromatographically eluted immediately following the chromatographic peak of one of the steviol glycosides (or steviol). In these cases, the unknown peaks eluted immediately following the stevioside, steviolbioside and steviol chromatographic peaks and were detected with the same m/z mass ion as the compound eluting immediately before. This consistent chromatographic retention time and mass spectrometric ionization relationship between the known steviol glycosides and the unknown Reb A degradation products suggests that the initial step in Reb A degradation is the formation of a structural isomer probably within the steviol core of the molecule, and that further degradation occurs as the successive loss of glucosyl residues. Since Compound X is an isomer of Reb A (i.e. iso-Reb A) and Compound Y is an isomer of rebaudioside B (i.e. iso-rebaudioside B), then the minor degradant peaks may be designated as iso-stevioside, iso-steviolbioside and isosteviol II. The fourth unknown peak was detected at $(m/z)^- = 317.3$, the $(M-1)^-$ mass ion of steviol and isosteviol II, and eluted about 2 minutes after steviol. This unknown peak has been designated isosteviol, and is presumed to be the isomer previously described in the published literature as the product of the acid-catalyzed Wagner-Meerwein rearrangement of steviol (Hanson and De Oliveira, 1993), as follows.

Figure III.-6: Degradation of Steviol to Isosteviol under acidic conditions



At test conditions of pH 2.0 and 70°F, the most relevant test conditions for which concentration data are available for the minor degradants, they were detected at the following concentrations over the 15-week stability study (Stetson, 2008a [Unpublished]): iso-stevioside [≈0 – 1.55 µg/mL]; iso-steviolbioside [≈0 – 0.150 µg/mL]; isosteviol II [≈0 – 0.0173 µg/mL]; and isosteviol [≈0 – 0.0470 µg/mL] in comparison to the initial nominal concentration of 600 ug/mL for Reb A. The concentrations for the minor degradants peaks are approximate (≈) because they are estimated based on the peaks of the known Reb A components immediately preceding them, rather than reference standards.

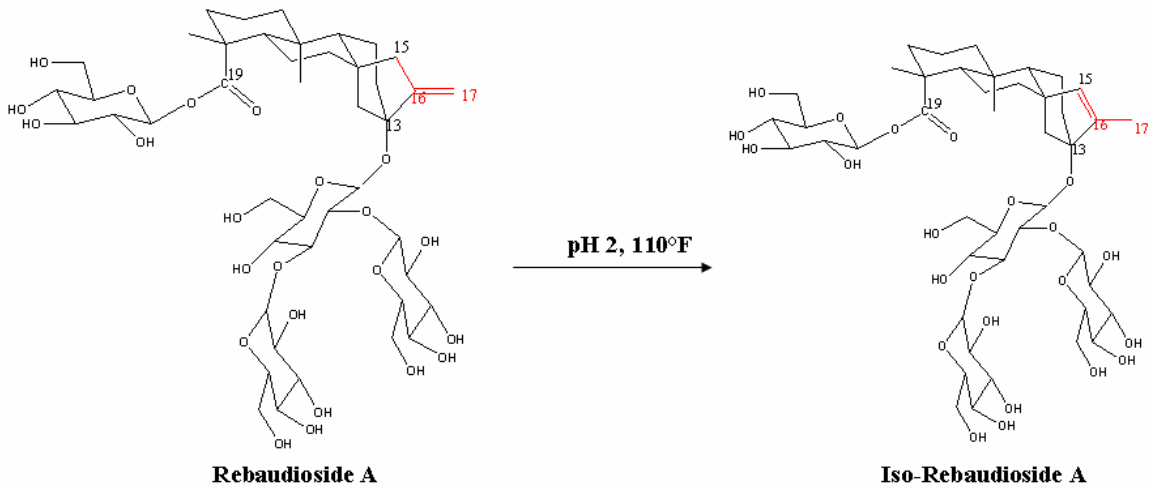
Identification of Compound X

As follow up to the stability studies, Compound X was prepared, isolated, purified and crystallized for characterization and identification at ChemPharma Int'l., LLC (Eisenberg, 2007 [Unpublished]). Experiments were conducted to prepare quantities of the Compound X from Reb A using high temperature and low pH conditions as used in the stability studies. After approximately 70% conversion of Reb A to Compound X, the material was desalted, and Compound X was isolated using preparative column chromatography and crystallized. A crystal structure of Compound X was obtained and the chemical structure elucidated. The ChemPharma report (Eisenberg, 2007 [Unpublished]) concludes:

"Rebaudioside A has been shown to undergo an alkene isomerism in acidic conditions at elevated temperatures from an external alkene in Rebaudioside A to an internal double bond in iso-Rebaudioside A. The isomer has been characterized by HPLC/MS, 1H-NMR, and single crystal x-ray crystallography. Small amounts were isolated and purified by semi-preparative reversed-phase HPLC and further crystallized to produce a single crystal suitable for x-ray analysis. The x-ray structure clearly established the three-dimensional structure of iso-Rebaudioside A as having an internal double bond in the five-membered ring, with an external methyl group. Rebaudioside A is reported as having an exocyclic double bond. The x-ray crystal structure obtained for Rebaudioside A confirmed the structure, and the position of the exocyclic double bond."

Reb A and the elucidated structure of Compound X (iso-Reb A) are shown in the following figure.

Figure III.-7: Degradation of Reb A to iso-Reb A at low pH with heat



Summary

Reb A, rebaudioside B, stevioside, steviolbioside, and steviol concentrations in three purified Reb A products (Chrysanta® 99-P, Rebaudioside A-92% and Rebaudioside A-98%) were identified and quantitated using validated assays. The results

support the reported test article purity values for Chrysanta® 99-P (Certificate of Analysis (C of A) purity = 95.7%), Rebaudioside A-98% (C of A purity = 99.5%) and Rebaudioside A-92% (C of A purity = 96.2%).

During incubation in aqueous citric acid solutions (50mM) at various pH values (2.0, 4.0 and 7.0 ± 0.5) for 15 weeks, no significant Reb A degradation was detected in 1) the Chrysanta® 99-P and Rebaudioside A-98% pH 7 solutions at 40, 70, 90 or 110ºF, 2) the Chrysanta® 99-P and Rebaudioside A-98% pH 4 solutions at 40, 70 or 90ºF and 3) the Chrysanta® 99-P and Rebaudioside A-98% pH 2 solutions at 40ºF. However, solutions prepared at pH 4 and incubated at 110ºF or at pH 2 and incubated at 70, 90 and 110ºF, resulted in significant Reb A degradation. Reb A degradation rate increased with increasing incubation temperature and with decreasing solution pH. At intermediate pH values (2.5, 3.0 and 3.5 ± 0.5), aqueous citric acid solutions (50mM) incubated at 40, 70, 90 and 110ºF for 25 weeks demonstrated results fitting logically between those for pH 2 and 4. There were no significant differences in the stability (or instability) of Reb A observed with solutions of Chrysanta® 99-P or Rebaudioside A-98%.

Reb A degradation under severe pH and temperature storage conditions resulted in two main degradant peaks (i.e. Compounds X and Y) in the HPLC/UV chromatograms. The data indicate that the initial step in Reb A degradation at low pH and high temperature is the formation of Compound X, and that Compound Y is the direct product of Compound X degradation. The data and other observations associated with the degradation of Reb A at low pH and high temperature conditions indicate that Compound X (iso-Reb A) and Compound Y (iso-rebaudioside B) are structural isomers of Reb A and rebaudioside B, respectively.

During incubation for 15 weeks, rebaudioside B, stevioside, steviolbioside and steviol, which initially were present in both Chrysanta® 99-P and Rebaudioside A-98% in trace amounts, failed to accumulate to significant levels even under severe incubation conditions of test article solutions at 110ºF and pH 2.

Under severe pH and temperature storage conditions, four minor (in apparent concentration) degradant peaks appeared in the LC/MS/MS chromatograms. Three of the four unknown peaks chromatographically eluted immediately following the chromatographic peaks for stevioside, steviolbioside and steviol, and were designated iso-stevioside, iso-steviolbioside and isosteviol II. The fourth unknown peak was detected at the mass ion of steviol and isosteviol II, and eluted about 2 minutes after steviol. This unknown peak is concluded to be isosteviol, the isomer identified in the published literature as the product of the acid-catalyzed Wagner-Meerwein rearrangement of steviol.

A safety evaluation of the possible Reb A degradation products is presented in Section V.C.

IV. Estimated Human Intake of Reb A

Available Data and Methods

The estimated daily intake (EDI) of Reb A from the proposed uses including tabletop sweeteners, sweetened ready-to-drink iced teas, diet soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal, and sweetened cold cereals, was estimated using the proposed use rates provided in Appendix IV.-1 (entitled NHANES 2003-2004 Food Codes and Reb A Use Level Included in EDI Analysis), Exponent® Inc.'s Foods and Residue Evaluation Program (FARE™ 8.09) software, and data from the most recent National Health and Nutrition Examination Survey (NHANES 2003-2004). The NHANES 2003-2004 (NCHS, 2007) is a complex multistage probability sample designed to be representative of the civilian U.S. population. The survey collects two days of food intake data, in addition to nutrition, demographic, and health information. The NHANES survey over-samples minorities, low-income groups, adolescents (12-19 years), and adults 60 years of age and older and statistical weights are provided by the National Center for Health Statistics (NCHS) to adjust for the differential probabilities of selection. Participants included 10,122 subjects in 2003-2004. Only individuals with complete and reliable 2-day dietary records were included in the analysis; therefore, N=8,354.

Exponent® used the statistically weighted values from the survey in the analyses. The statistical weights compensate for variable probabilities of selection, adjust for non-response, and provide intake estimates that are representative of the U.S. population and the selected age-gender subgroups.

Exponent® estimated the daily intake on a per "user" basis. In this analysis, a "user" is anyone who reported consuming at least one Reb A containing food (Reb A food) on either of the survey days, i.e. United States Department of Agriculture's (USDA's) "user" definition. Each individual who reported consuming a Reb A food on either of the survey days was identified, and that individual's responses for both survey days was used. Zero consumption days were included in calculating that individual's average daily intake.

Given the lack of acute effects for Reb A, it is appropriate to average exposures over a longer period than one day. Therefore, Exponent® used each respondent's food consumption averaged over the two days of the NHANES 2003-2004 survey. For example, if someone reported consuming 100 grams of bread on day 1 and 150 grams of bread on day 2, his/her 2-day average bread consumption would be 125 grams, i.e. [100+150]/2.

A 2-day average typically overestimates long-term, chronic daily intake; however, only two nonconsecutive days' worth of food consumption data are available in the most recent NHANES 2003-2004 survey database. The 2-day average is typically a poor estimator of lifetime average daily intake, especially for foods eaten infrequently. It is well known that food consumption data collected over longer periods of time, e.g., 14 days as in Market Research Corporation of America (MRCA) consumer surveys, yield estimates of daily intake that may be significantly lower than 2-day averages (Lambe *et al*., 2000).

The EDI of Reb A was calculated by multiplying each NHANES respondents' 2-day average food intake by the use levels described in Table IV.-1. Results are presented on a bodyweight basis, i.e. mg Reb A/kg bw/day. Each individual's intake of Reb A was divided by his/her bodyweight to provide the per capita and per user intakes on a bodyweight basis. Analyses were conducted using Exponent®'s proprietary software program FARE™ version 8.09. Mean and 90th percentile daily intakes on a per user basis, mg/kg bw/day, were estimated for the proposed uses of Reb A for the U.S. population (individuals 2+ years old).

Appendix IV.-1 lists all NHANES food codes for the proposed food categories and the corresponding Reb A use level.

Proposed Use Level of Reb A

Reb A that is the subject of this GRAS notification is proposed for use as a natural sweetener in tabletop sweeteners, sweetened ready-to-drink iced teas, diet soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal, and sweetened cold cereals. Reb A would be used at the levels proposed in Table IV.-1.

Table IV.-1: Proposed Uses and Levels of Reb A (ppm)

Food Category	Reb A (ppm)
Tabletop sweeteners	30,000[1]
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

[1]Reb A content of sachet prior to dilution and not representative of "as consumed"

Estimated Daily Intake

The per user intake estimates of Reb A for the U.S. population from the proposed uses are presented in Table IV.-2 in mg/kg bw/day.

Table IV.-2: Estimated Daily Intake of Reb A from Proposed Uses (U.S. Population: 2+ Age Group)

		Per User (mg Reb A/ kg bw/day)	
Food Category	**N (Unweighted)**	**Mean**	**90th Percentile**
Tabletop sweeteners	665	0.78	1.73
Sweetened ready-to-drink teas	964	1.02	2.43
Diet soft drinks	1182	3.32	7.36
Fruit juice drinks	2801	0.95	2.15
Energy drinks	37	0.40	0.66
Flavored Water	16	1.14	1.57
Cereal bars	336	0.06	0.13
Oatmeal	600	0.42	0.78
Cold cereals	284	0.10	0.16
Total EDI (beverages, cereals, tabletop)	**4959**	**1.95**	**4.72**

The total EDI from all proposed uses is 1.95 mg/kg bw/day at the mean and 4.72 mg/kg bw/day at the 90th percentile of intake among users (rounded to 2 and 5 mg/kg bw/day, respectively). Intakes by age/sex subgroups are similar (Table IV.-3).

Table IV.-3. Estimated Daily Intake of Reb A from Proposed Uses (U.S. Population: Children, Teenagers and Adults)

Population	Unweighted Users	Per User (mg/kg bw/day)	
		Mean	90th Percentile
Children 2-5 years	438	2.4	5.1
Male teenagers	542	1.6	3.4
Female teenagers	567	1.2	2.4
Male >20 years	1207	2.0	4.8
Females >20 years	1416	2.2	5.6

For the entire 2+ age group, cereal products have the lowest EDI, with mean per user estimates ranging from 0.06 mg/kg bw/day for cereal bars to 0.42 mg/kg bw/day for oatmeal; energy drinks also fall within this low range. Paradoxically, the proposed use in diet soft drinks would appear to result in a higher EDI than the total, with mean and 90th percentile per user estimates at 3.32 mg/kg/day and 7.36 mg/kg/day, respectively. The higher estimate for diet soft drinks is due to the fact that the consumers who consume diet soft drinks are not necessarily the same consumers who consume cereals or tabletop sweeteners, and in many cases will not be the same consumers. Thus, the population of "users" changes for the individual food categories and for the total across all categories. The soft drink category has fewer consumers than the total for all categories. Also the serving size for diet soft drinks, because they contain a large proportion of water, is larger than the serving size for products such as cereals and tabletop sweeteners. Since the use level of Reb A is on a concentration (ppm) basis, estimated intake of Reb A is directly dependent on the amount of food consumed in a day as well as the levels of Reb A in those foods. When the intake for a consumer of diet soft drinks is combined with the intake for a consumer of cereals, the estimated intake among the population of consumers of all foods containing Reb A is lower than soft drink consumers and higher than cereal consumers due to the averaging effect of the two consumers. For example, the average intake per user for two individuals who each consumed a different food containing

Reb A: the first individual weighs 70 kg and consumed 355 g of diet soft drink a day with Reb A at a use level of 150 ppm resulting in an intake of 0.76 mg Reb A/kg bw/day. The second individual weighs 50 kg and consumed 40 g of cereal with Reb A at a use level of 150 ppm resulting in an intake of 0.12 mg Reb A/kg bw/day. Neither individual consumed other foods containing Reb A. Therefore, the average Reb A consumption/user would be 0.44 mg Reb A/kg bw/day.

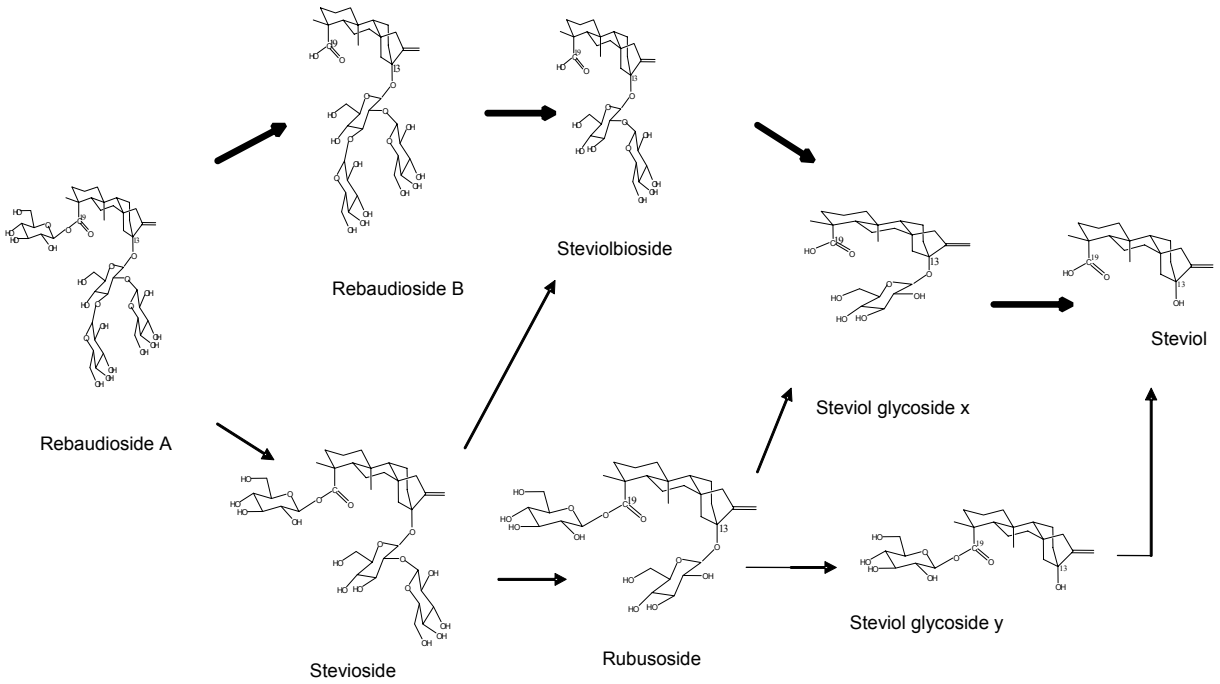
In summary, the total EDI from all proposed Reb A uses is less than or equal to 2 mg/kg bw/day at the mean and 5 mg/kg bw/day at the 90th percentile of intake among users in the U.S. population (2+ years). The range of exposures among population subgroups is similar, e.g, 1.2 to 2.4 mg/kg bw/day (mean) and 2.4 to 5.6 mg/kg bw/day (90th percentile).

V. Safety Evaluation

There is an extensive published and unpublished database for Reb A and steviol glycosides, including stevioside and steviol. Different test substances have been used in various studies by researchers. Some studies have been conducted with extracts of the leaves of the stevia plant without purification. The extracts have non-steviol glycosides which could cause effects that are not due to the glycosidic compounds. Therefore, studies on stevia leaf extracts are of less relevance than the studies conducted with purer test article. In addition, different routes of administration have been studied; however, the oral (dietary) route is of most relevance to this evaluation of the use of Reb A in food.

Metabolism and pharmacokinetic studies have identified a common hydrolysis pathway for all steviol glycosides (see ADME, Section V.A.1.). This observation provides the basis for the relevance of study results in which the material tested was a steviol glycoside, including Reb A and stevioside. Steviol is the final hydrolysis product for all the steviol glycosides. The hydrolysis pathways of Reb A and other steviol glycosides are shown in the figure below.

Common Hydrolysis Pathways of Reb A to the Aglycone, Steviol



A comprehensive dataset for Reb A was developed that includes subchronic, genotoxicity, reproductive/developmental toxicity studies as well as pharmacokinetic data/evaluations. These Reb A studies were conducted in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practice Regulations (21 CFR Part 58) and the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice [C (97) 186/Final] using purified Reb A (≥95% Reb A). The study protocols were written to comply with the relevant FDA Redbook 2000 test guidelines (U.S. FDA, 2000a-g). Because of their relevance to this GRAS notification, these studies are described in greater detail in the Appendices.

In addition to the Reb A studies, there are published literature that address ADME, acute toxicity, subchronic toxicity, chronic/carcinogenicity, genotoxicity, and developmental/reproductive effects of stevioside and steviol in animals. In addition there are metabolism, short term, long term and special studies in human volunteers.

In order to organize the diverse data, Reb A studies are presented first, followed by studies for stevioside and then steviol. Each section contains summary tables that identify relevant studies and provide the conclusions of each study.

V. Safety Evaluation

A. Preclinical Studies

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

The structures of Reb A, stevioside and important hydrolysis products are shown in Figure V.-1. All steviol glycosides share the same aglycone, steviol, and glycosidic bonds. Research has shown the steviol glycosides to be hydrolyzed by common pathways, but at different rates. Reb A is hydrolyzed the slowest of the tested steviol glycosides.

An overview of the potential common metabolic and elimination pathways for steviol glycosides is presented in Figure V.-2. Briefly, the ester and ether linkages are prone to hydrolysis by bacteria in the cecum. The double bond in the aglycone, steviol, may be epoxydated rapidly followed by hydration to form the glycol as shown in Figure V.-2. Research has shown that all orally administered steviol glycosides, including Reb A, are hydrolyzed in the gut with the amount of degradation dependent on hydrolysis rates and residence time. Because of their large molecular weight and high polarity, the uptake of steviol glycosides by the intestinal tract is extremely low, as expected. This has been confirmed by numerous studies, including experiments with everted gastrointestinal sacs of rats (Koyama *et al.*, 2003a) and Caco-2 cell layers (Geuns, *et al.* 2003a). A number of *in vitro* and *in vivo* studies indicated that stevioside is not absorbed across the digestive tract in rats or humans and is not metabolized by any of the normal digestive enzymes or by the acid conditions of the stomach (Wingard *et al.*, 1980; Nakayama *et al.*, 1986; Hutapea *et al.*, 1997; Koyama *et al.*, 2003a, b; Geuns and Pietta 2004). This suggests Reb A would not be absorbed or metabolized either and was confirmed in a more recent study in rats (Sloter, 2008a [Unpublished]). Numerous investigators have demonstrated that most of the stevioside and Reb A administered is hydrolyzed by bacteria of the colon, resulting in free steviol that is then excreted in the feces or absorbed (Koyama, *et al.*, 2003a; Geuns *et al.*, 2003a; Hutapea *et al.*, 1997; Wingard *et al.*, 1980; Sloter, 2008a [Unpublished]). Since steviol is inefficiently absorbed, the greatest percentage of free steviol is found in the feces. The fraction of steviol that is absorbed is transported via the

portal vein to the liver where it undergoes glucuronide conjugation and excretion in the urine and bile.

Figure V.-1: Structures of Reb A and Important Hydrolysis Products

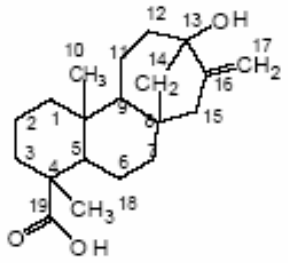
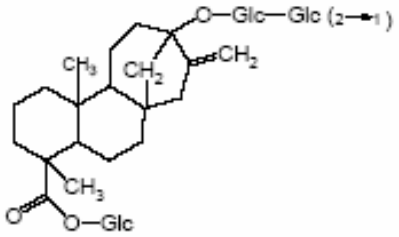
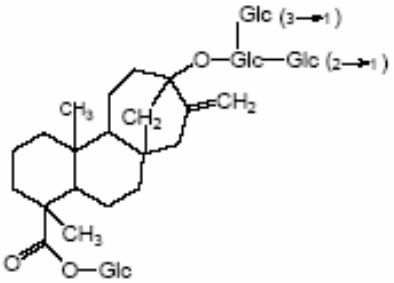
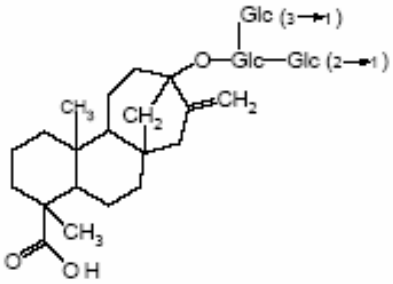
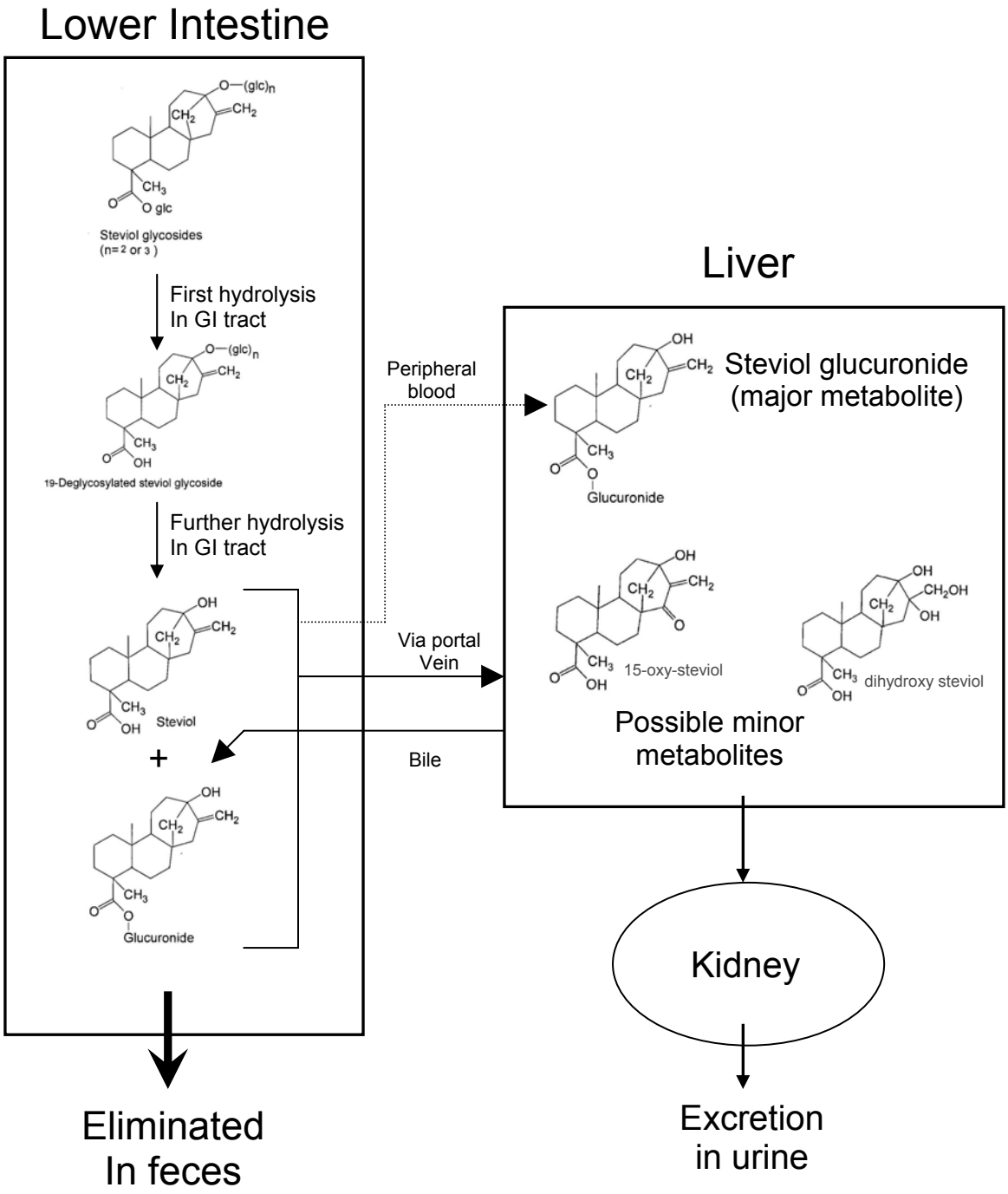
STRUCTURE	NAME AND COMMENTS
	**Rebaudioside A** Triglycoside at the alcoholic group and monoglycoside at the carboxylic group
	**Stevioside** Triglycosylated steviol, diglycoside at the alcoholic group and monoglycoside at the carboxylic group
	**Rebaudioside B** Triglycoside at the alcoholic group and free carboxylic group
	**Steviol** The aglycone of all the other glycosylated products

Figure V.-2. Summary of possible metabolic reactions and elimination pathways for steviol glycosides



In contrast to steviol glycosides, free steviol can be directly absorbed from the GI tract. The maximum concentration (approximately 18 µg/mL) of free steviol in rat portal plasma was reached 15 minutes after its oral administration at 45 mg/kg, reflecting rapid absorption from the stomach and upper intestine. The portal plasma concentration of steviol was maintained at 2–3 µg/mL for 8 hours (Koyama *et al.*, 2003a). This finding indicates that steviol was absorbed at the lower part of intestine, and that steviol glucuronide was reabsorbed by the enterohepatic circulation pathway (Nakayama *et al.*, 1986).

No mammalian species differences have been reported in the ADME of steviol or steviol glycosides.

Nakayma *et al.* (1986) estimated that 5 days after administering ^{3}H-stevioside 68% of the administered dose (AD) was excreted in the feces, 2.3% in urine and 24% in expired air. The ^{3}H measured in expired air seems to represent glucose metabolism since steviol does not undergo complete metabolism based on results from other investigators. The results of this study are presented in Table V.-4. (see section entitled 'Excretion in Urine and Feces').

Additional research was undertaken to confirm the findings in the published literature and specifically to confirm that the ADME for Reb A is corroborative of the data for the other steviol glycosides. A dietary 2–generation reproduction study (Sloter, 2008a [Unpublished]) was expanded to include a toxicokinetic phase for this purpose. Blood, urine and feces were collected from a subgroup of the F_1 test animals. The results of these analyzes provide important information to describe the ADME of Reb A and are presented in the next section.

ADME and Toxicokinetics of Reb A

Despite more than a year of experimental effort at SRI International using multiple approaches, ^{14}C-radiolabeling of Reb A was infeasible. Therefore, bioanalytical approaches were developed to investigate ADME and toxicokinetics of Reb A in rats. In order to provide information about the toxicokinetics and ADME of Reb A, blood, urine and feces samples were collected during a dietary 2-generation reproduction study (Sloter, 2008a [Unpublished]). In addition to confirming that Reb A follows the same general ADME pathways as the other steviol glycosides, this study provides additional details about the specific hydrolysis pathway for Reb A and quantifies the levels of Reb A, its steviol glycoside hydrolysis products, steviol, and steviol glucuronide in blood, feces and urine.

Methods

The study design is presented in detail in Section V.A.2.e and Appendix V.-4 (entitled Two-Generation Dietary Reproduction Toxicity Study in Rats). The toxicokinetic (TK) phase measured Reb A and its hydrolysis and metabolic products in plasma, urine and feces samples collected from animals administered Reb A at 500, 1000 or 2000 mg/kg bw/day in the diet. In this phase, 1 pup/sex/litter was selected from 25 litters in each of the test article treated groups, Groups 2 through 4 (total of 25 F_1 males and 25 F_1 females in each group). Twelve F_1 animals/sex/group were randomly selected for urine collection over 24 hours and fecal sample collection at approximately 6 weeks of age. Twelve different F_1 animals/sex/group were randomly selected for urine collection over 24 hours and fecal sample collection at approximately 12 weeks of age. Animals were placed in plastic metabolic cages without a food jar to minimize cross-contamination of urine and fecal samples with the test diet. Urine and feces samples were collected on ice and after collection were stored frozen at approximately -70°C until analysis. After the 24-hour sampling period, the animals were returned to their home wire-mesh cages and administration of the test diet was resumed until euthanasia. Blood samples were collected from these same animals (alternating among 4 sub-groups of 3/sex/group/time point) 2-3 days following the collection of urine and fecal samples at approximately 6:00

A.M., 9:00 A.M., noon (12:00 P.M.), 3:00 P.M., 6:00 P.M., 9:00 P.M., and midnight (12:00 A.M.) as well as at approximately 3:00 A.M. on the following day (designated as nominal times of 0, 3, 6, 9, 12, 15, 18 and 21 hours, respectively). Plasma was isolated after centrifugation under refrigerated conditions and stored frozen at approximately -70°C until analysis.

Plasma, urine and aqueous extracts of the fecal samples were analyzed for Reb A and its major hydrolysis products, rebaudioside B, stevioside, steviolbioside and steviol using a validated LC-MS/MS method. The lower limit of quantitation (LLOQ) was 5 ng/mL for all analytes except for steviol with a LLOQ of 10 ng/mL. Plasma, urine and aqueous extracts of the fecal samples were reanalyzed following pretreatment with glucuronidase and sulfatase as a measure of conjugated steviol. For fecal extracts, an apparent instability of steviol during storage and/or glucuronidase and sulfatase pretreatment was observed resulting in total steviol levels lower than steviol concentration before pretreatment; therefore, for feces the results are presented only for (free) steviol. The results of these analyses were used for toxicokinetic evaluation.

Results

The amounts of Reb A and its hydrolysis and metabolic products including the *Total Reb A Equivalents* are summarized in Tables V.-1 (plasma), V.-2 (urine) and V.-3 (feces). The use of the term *Reb A Equivalent* means that the measured mass of the other steviol glycosides and/or steviol has been adjust by their respective molecular weight relative to the molecular weight of Reb A. As an example, 100 mg of steviol would be equivalent to:

100 mg × 967.03 (MW of Reb A)/318.45 (MW of steviol) = 303.7 mg *Reb A Equivalent*.

This transformation was performed uniformly in the study results so that the measured masses or concentrations of Reb A, the other steviol glycosides and/or steviol could all be compared and totaled on a common relative basis. Therefore, *Total Reb A Equivalents*

means that measured masses or concentrations of all detected Reb A, steviol glycosides and steviol have been summed to present the total amount in the sample.

TABLE V.-1: SUMMARY OF REB A AND HYDROLYSIS PRODUCTS IN PLASMA OF F_1 MALE AND FEMALE RATS ADMINISTERED REB A FOR 6 and 12 WEEKS VIA THE DIET

	Males						Females					
	Week 6			Week 12			Week 6			Week 12		
Dosage Level [mg/kg/day]	500	1000	2000	500	1000	2000	500	1000	2000	500	1000	2000
	Reb A											
C_{avg} [ng/mL]	271	509	811	202	390	570	226	451	618	194	354	477
Dose Normalized C_{avg}	0.542	0.509	0.405	0.403	0.390	0.285	0.452	0.451	0.309	0.388	0.354	0.239
AUC_{last} [ng*hr/mL]	5709	10338	16179	4213	8203	11887	4653	8891	12629	3913	7172	9895
Dose Normalized AUC_{last}	11.4	10.3	8.09	8.43	8.20	5.94	9.31	8.89	6.31	7.83	7.17	4.95
	Steviol (as *Reb A Equivalents*)											
C_{avg} [ng/mL]	1637	6679	11525	1637	2460	6058	4115	4466	8055	3150	5287	8245
Dose Normalized C_{avg}	3.27	6.68	5.76	3.27	2.46	3.03	8.23	4.47	4.03	6.30	5.29	4.12
AUC_{last} [ng*hr/mL]	99682	146586	224287	45441	52334	124065	83853	90726	167552	65511	109909	170107
Dose Normalized AUC_{last}	199	147	112	90.9	52.3	62.0	168	90.7	83.8	131	110	85.1
	Total Steviol[1](as *Reb A Equivalents*)											
C_{avg} [ng/mL]	4973	8608	18834	2086	3511	8798	5433	5713	10776	5092	6558	11451
Dose Normalized C_{avg}	9.95	8.61	9.42	4.17	3.51	4.40	10.9	5.71	5.39	10.2	6.56	5.73
AUC_{last} [ng*hr/mL]	160211	191151	366916	69691	74756	181138	115499	118503	226303	105902	134149	234140
Dose Normalized AUC_{last}	320	191	183	139	74.8	90.6	231	119	113	212	134	117
	Total Reb A Equivalents											
C_{avg} [ng/mL]	7983	9143	19689	3504	3903	9374	5672	6188	11434	5286	6915	11931
Dose Normalized C_{avg}	16.0	9.14	9.84	7.01	3.90	4.69	11.3	6.19	5.72	10.6	6.91	5.97
AUC_{last} [ng*hr/mL]	166280	202011	383989	73911	82999	193144	120434	127833	239733	109810	141373	244084
Dose Normalized AUC_{last}	333	202	192	148	83.0	96.6	241	128	120	220	141	122

[1]Steviol concentration after glucuronidase and sulfatase treatment of plasma samples. NC = Not calculable. N= 3/sex/group.

C_{avg} = The average concentration of the compound in plasma at steady state. The average of the mean plasma concentration values at all time points over the 21-hour collection interval.

AUC_{last} = The area under the plasma compound concentration vs. time curve from nominal time 0 to the last time point with quantifiable plasma compound concentration (T_{last}).

Calculation of the toxicokinetic parameters was performed in WinNonlin 5.2 and Microsoft® Excel 2002 using non-compartmental analysis.

TABLE V.-2: *TOTAL REB A EQUIVALENTS* AND PERCENT OF DOSE EXCRETED IN URINE OVER 24-HOURS OF F_1 MALE AND FEMALE RATS ADMINISTERED REB A FOR 6 and 12 WEEKS VIA THE DIET

Dosage Level	Males						Females					
	Week 6			Week 12			Week 6			Week 12		
[mg/kg/day]	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%
	Reb A (µg) in Urine over 24 hours											
500 (Group 2)	23.9	24.7	103	35.0	17.5	50.0	16.6	14.9	89.7	16.2	11.7	72.4
1000 (Group 3)	27.9	19.5	69.8	56.9	30.3	53.2	29.3	21.6	73.8	33.5	11.7	35.0
2000 (Group 4)	103	183	177	118	62.5	52.8	36.2	39.4	109	55.5	33.8	60.9
	Steviol (µg *Reb A Equivalents*) in Urine over 24 hours											
500 (Group 2)	2.93	4.56	156	0.265	0.545	206	11.6	22.0	189	7.42	8.53	115
1000 (Group 3)	1.22	1.39	114	0.699	0.905	129	15.1	38.5	254	37.5	113	301
2000 (Group 4)	2.00	1.99	100	4.60	9.14	199	9.71	14.4	149	6.52	8.85	136
	Total Steviol[1] (µg *Reb A Equivalents*) in Urine over 24 hours											
500 (Group 2)	104	191	182	4.26	7.25	170	67.2	45.4	67.5	165	278	169
1000 (Group 3)	40.1	49.0	122	3.69	6.72	182	169	284	168	119	79.6	66.7
2000 (Group 4)	136	291	214	51.1	104	202	350	288	82.4	279	246	88.1
	***Total Reb A Equivalents* (mg) in Urine over 24 hours**											
500 (Group 2)	0.129	0.193	149	0.040	0.020	49.8	0.088	0.053	61.0	0.181	0.277	153
1000 (Group 3)	0.070	0.050	71.3	0.063	0.033	52.2	0.200	0.286	143	0.159	0.076	47.7
2000 (Group 4)	0.243	0.473	195	0.174	0.115	66.0	0.388	0.288	74.3	0.336	0.241	71.7
	Percent of Estimated[2] Dose Excreted in Urine over 24 hours											
500 (Group 2)	0.146	0.209	143	0.018	0.008	45.6	0.120	0.071	59.6	0.142	0.221	156
1000 (Group 3)	0.042	0.033	80.1	0.014	0.007	49.8	0.136	0.180	132	0.060	0.030	49.5
2000 (Group 4)	0.090	0.202	224	0.019	0.013	67.0	0.134	0.100	74.5	0.063	0.045	71.3

[1]Steviol concentration after glucuronidase and sulfatase treatment of urine samples.
[2]Dose was estimated based on the individual animal weight on the day of sample collection and the targeted test article concentration in the diet.
N = 12 sex/group.
SD = Standard deviation.
CV% = Coefficient of variation.

TABLE V.-3: *TOTAL REB A EQUIVALENTS* AND PERCENT OF DOSE RECOVERED IN FECES OVER 24-HOURS OF F_1 MALE AND FEMALE RATS ADMINISTERED REB A FOR 6 and 12 WEEKS VIA THE DIET

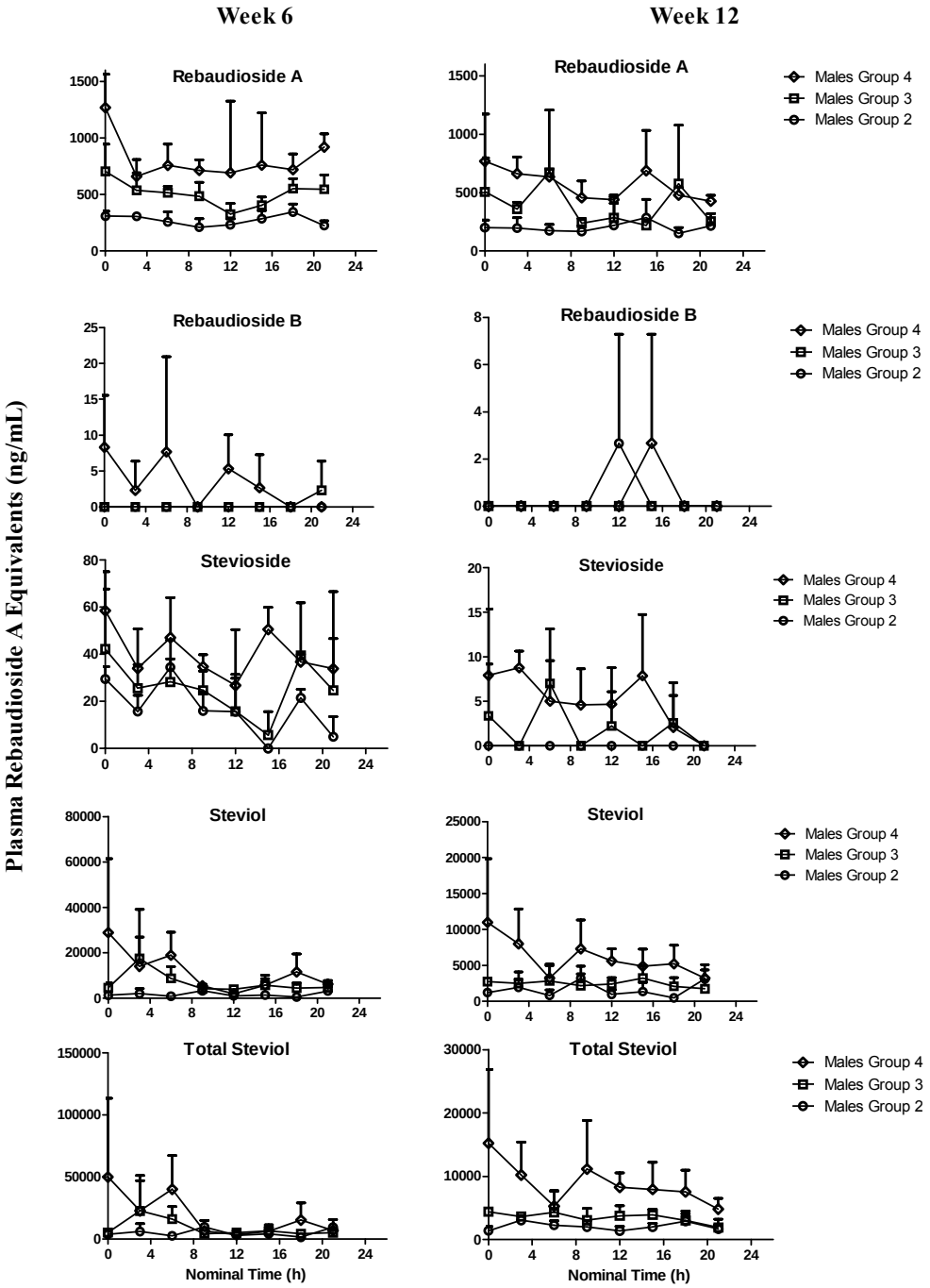
Dosage Level	Males						Females					
	Week 6			Week 12			Week 6			Week 12		
[mg/kg/day]	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%
	Reb A (µg) in Feces over 24 hours											
500 (Group 2)	3311	4109	124	13440	29726	221	9552	15061	158	3868	7518	194
1000 (Group 3)	11533	18902	164	27645	72047	261	41960	51265	122	3888	7163	184
2000 (Group 4)	16321	24000	147	7432	14096	190	13570	23849	176	829	1117	135
	Steviol (µg *Reb A Equivalents*) in Feces over 24 hours											
500 (Group 2)	101270	74334	73.4	171939	56992	33.1	75592	23551	31.2	111078	64847	58.4
1000 (Group 3)	65849	22823	34.7	332076	194792	58.7	62215	31793	51.1	186883	96010	51.4
2000 (Group 4)	68923	69505	101	643239	338535	52.6	65621	50647	77.2	223841	208380	93.1
	***Total Reb A Equivalents* (mg) in Feces over 24 hours**											
500 (Group 2)	122	71.8	59.1	201	65.3	32.5	94.9	32.8	34.6	121	69.0	56.9
1000 (Group 3)	113	45.9	40.6	388	240	61.9	124	74.2	59.8	211	93.4	44.3
2000 (Group 4)	131	108	82.5	855	449	52.5	136	176	129.3	252	234	92.8
	Percent of Estimated[1] Dose Recovered in Feces over 24 hours											
500 (Group 2)	139	80.4	57.7	90.6	31.6	34.9	131	51.4	39.1	89.9	43.2	48.1
1000 (Group 3)	65.0	27.3	41.9	89.0	54.2	60.9	92.2	60.9	66.1	80.1	38.5	48.0
2000 (Group 4)	38.1	29.3	76.7	90.5	44.5	49.2	48.4	65.2	135	53.2	45.3	85.1

[1]Dose was estimated based on the individual animal weight on the day of sample collection and the targeted test article concentration in the diet.
N = 12 sex/group.
SD = Standard deviation.
CV% = Coefficient of variation

Animals receiving Reb A via the diet for 6 or 12 weeks were only systemically exposed to very low levels of Reb A and its final hydrolysis product, steviol in free and conjugated form (Table V.-1). Systemic exposure to Reb A ($C_{avg} \approx 600$ mg/mL, or 0.6 μg/mL) was similar between male and female rats for both evaluation periods (6 and 12 weeks). Systemic exposure to total steviol (as *Reb A Equivalents*) in terms of C_{avg} was 9- to 26-fold higher than exposure to Reb A regardless of dosage level, gender or sampling period, confirming that Reb A itself is very poorly absorbed. Systemic exposure to very low levels of Reb A and total steviol in terms of C_{avg} (ng/mL) and AUC_{last} (hr*ng/mL) increased as the test article levels in diet increased; however, the increase in exposure was less than proportional to the increase in Reb A dosage over the 500 to 2000 mg/kg/day range.

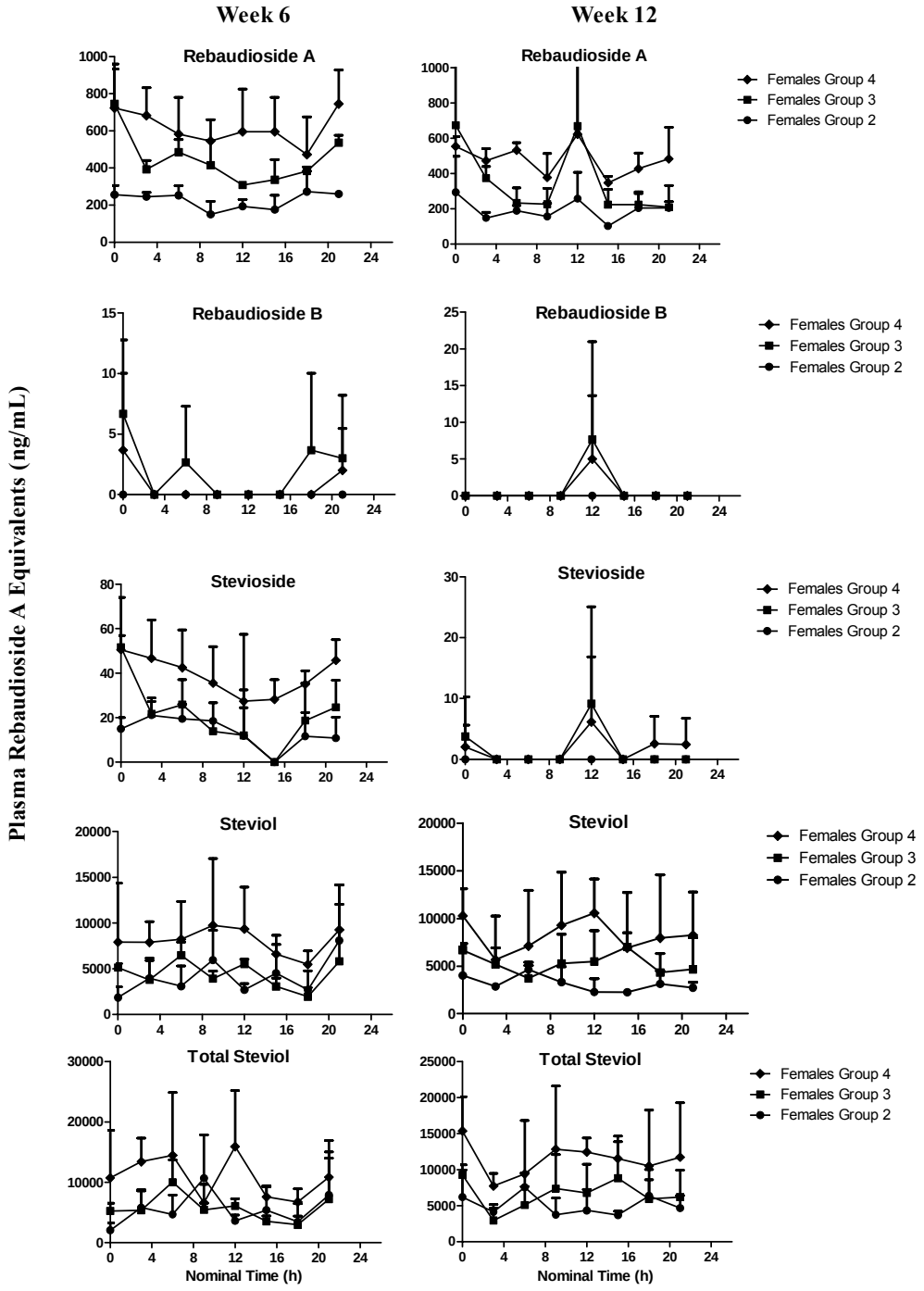
Plasma concentrations of Reb A, steviol and total steviol were measurable in all samples on both sampling days with relatively small fluctuations among the time points, suggesting that steady state was reached (Figures V.-3 and V.-4). Also, systemic exposure in plasma (ng/mL) to the other Reb A hydrolysis products was extremely low or below levels of quantitation. Systemic exposure to stevioside at week 6 was more than an order of magnitude lower than Reb A; and at week 12, stevioside was measurable sporadically in only a few animals regardless of Reb A dosage level. Rebaudioside B was quantifiable in only a few samples for either evaluation period. No steviolbioside was quantifiable in plasma of male or female rats.

Figure V.-3: Concentration of Reb A and Hydrolysis Products in Plasma of F_1 Male Rats at 6 and 12 Weeks of Age Administered Reb A via the Diet



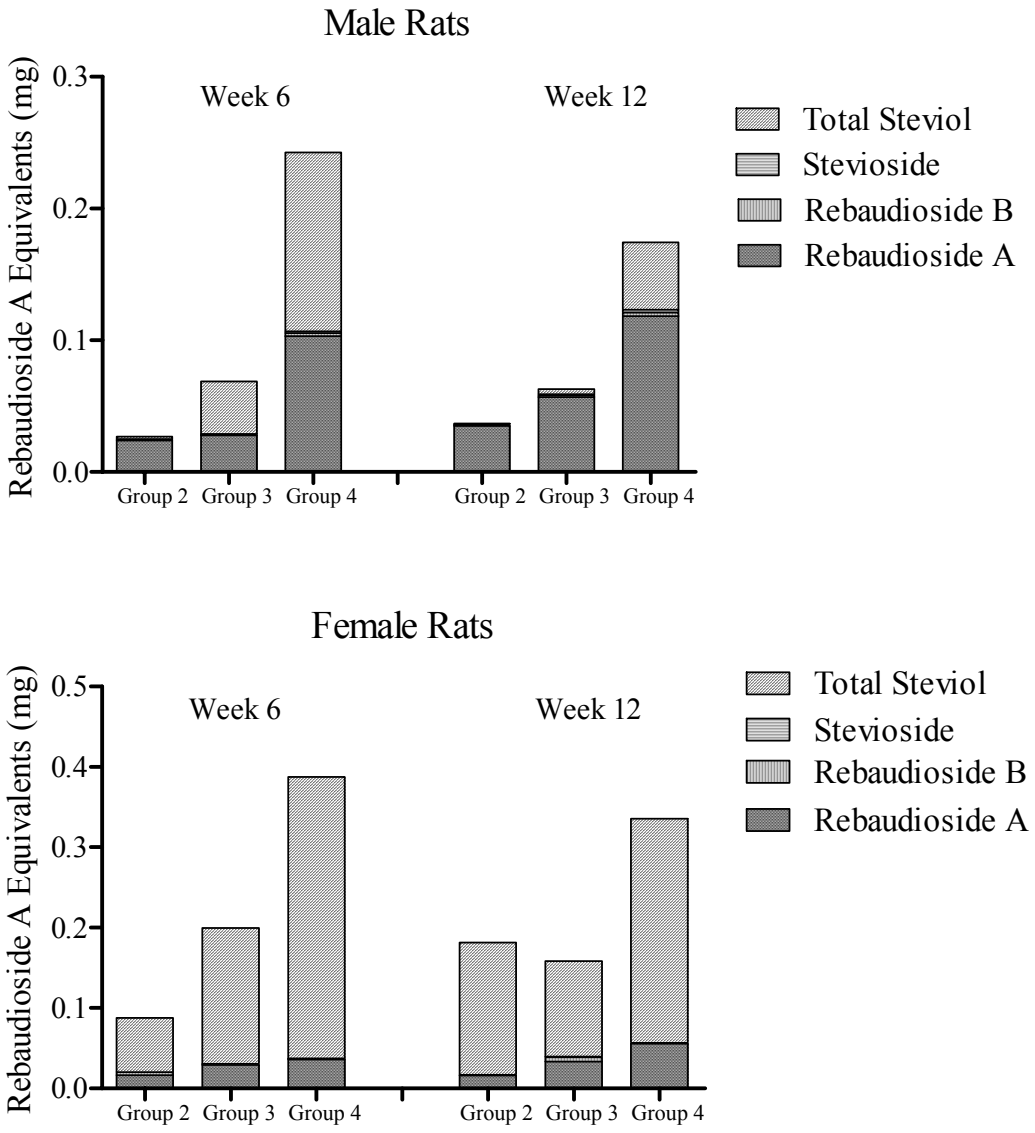
Note: Midpoint of values at each time point is presented along with a bar for the maximum value. Group 2 = 500 mg/kg/day; Group 3 = 1000 mg/kg/day; Group 4 = 2000 mg/kg/day

Figure V.-4: Concentration of Reb A and Hydrolysis Products in Plasma of F_1 Female Rats at 6 and 12 Weeks of Age Administered Reb A via the Diet



Note: Midpoint of values at each time point is presented along with a bar for the maximum value. Group 2 = 500 mg/kg/day; Group 3 = 1000 mg/kg/day; Group 4 = 2000 mg/kg/day

Figure V.-5: Amounts of Reb A Equivalents Excreted in Urine over 24 Hours of F_1 Male and Female Rats at 6 and 12 Weeks of Age Administered Reb A via the Diet



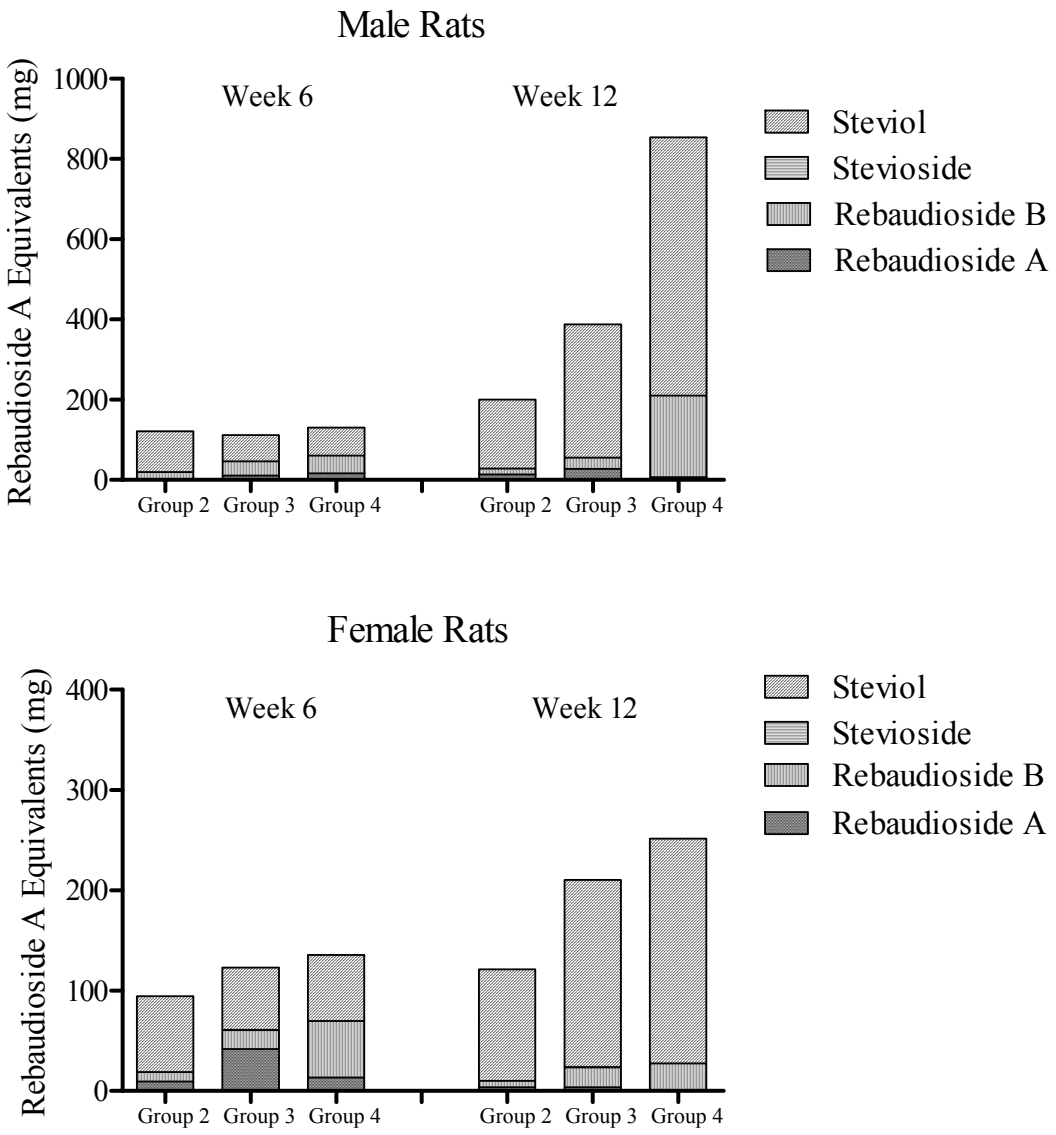
Note: Group 2 = 500 mg/kg/day; Group 3 = 1000 mg/kg/day; Group 4 = 2000 mg/kg/day

Reb A and free/conjugated steviol were the two main constituents detected in urine as they were in plasma (Figure V.-5). However, the higher than expected proportion of Reb A excreted in the urine in part may be an artifact resulting from the dietary route of dose administration employed in this study. Because the dietary route was used, Reb A/food particle dust was undoubtedly present on fur of the treatment animals. The Reb A/food particle dust easily could be dispersed

during urine collections in metabolism cages, thereby contaminating urine samples with Reb A. Also, steviol glucoronide may be excreted in bile as well as urine, thereby reducing the total steviol:Reb A ratio in urine as compared to this ratio in plasma. Expressed in terms of administered Reb A dose levels, only 0.014% to 0.146% (mean 0.08% for both genders and all dose groups) of the estimated dose excreted in urine collected over 24 hours was accounted for as *Total Reb A Equivalents* at weeks 6 and 12 (see Percent of Estimated Dose Excreted in Urine over 24 hours in Table V.-2). The amount of Reb A excreted in urine collected over 24 hours at weeks 6 and 12 in terms of administered Reb A dose levels was on average about 0.02% for both genders and all dose groups. A conclusion of very low systemic exposure to Reb A and its hydrolysis products is supported by the low amounts of *Reb A Equivalents* excreted in urine over 24 hours (Figure V.-5).

Approximately 40% to 140% (mean 83.9% for both genders and all dose groups) of the estimated daily doses were recovered as *Total Reb A Equivalents* in feces over 24 hours (see Percent of Estimated Dose Recovered in Feces over 24 hours Table V.-3). Variability in recovery of *Total Reb A Equivalents* from feces in this study comes from at least the following sources: 1) dietary intake of Reb A occurring *ad libitum* before food was removed during the sampling period as opposed to an oral gavage bolus dosing method normally used in ADME studies, 2) the amount and rate of dietary consumption prior to the sampling period varied with each animal, 3) transit time through the lower gastrointestinal tract varied with each animal, 4) hydrolysis rates of Reb A and its hydrolysis products in the lower gastrointestinal tract varied with each animal, and 5) absorption of steviol from the lower gastrointestinal tract varied with each animal. Steviol was the main constituent accounting for 50 to 92% of the *Total Reb A Equivalents* recovered in feces over 24 hours (Table V.-3; Figure V.-6). Substantial amounts of rebaudioside B were detected in feces (Figure V.-6), in contrast to rat plasma and urine where only extremely low levels of rebaudioside B were detected and there was no relationship to dosage.

Figure V.-6: Amounts of Reb A Equivalents Recovered in Feces over 24 Hours of F_1 Male and Female Rats at 6 and 12 Weeks of Age Administered Reb A via the Diet



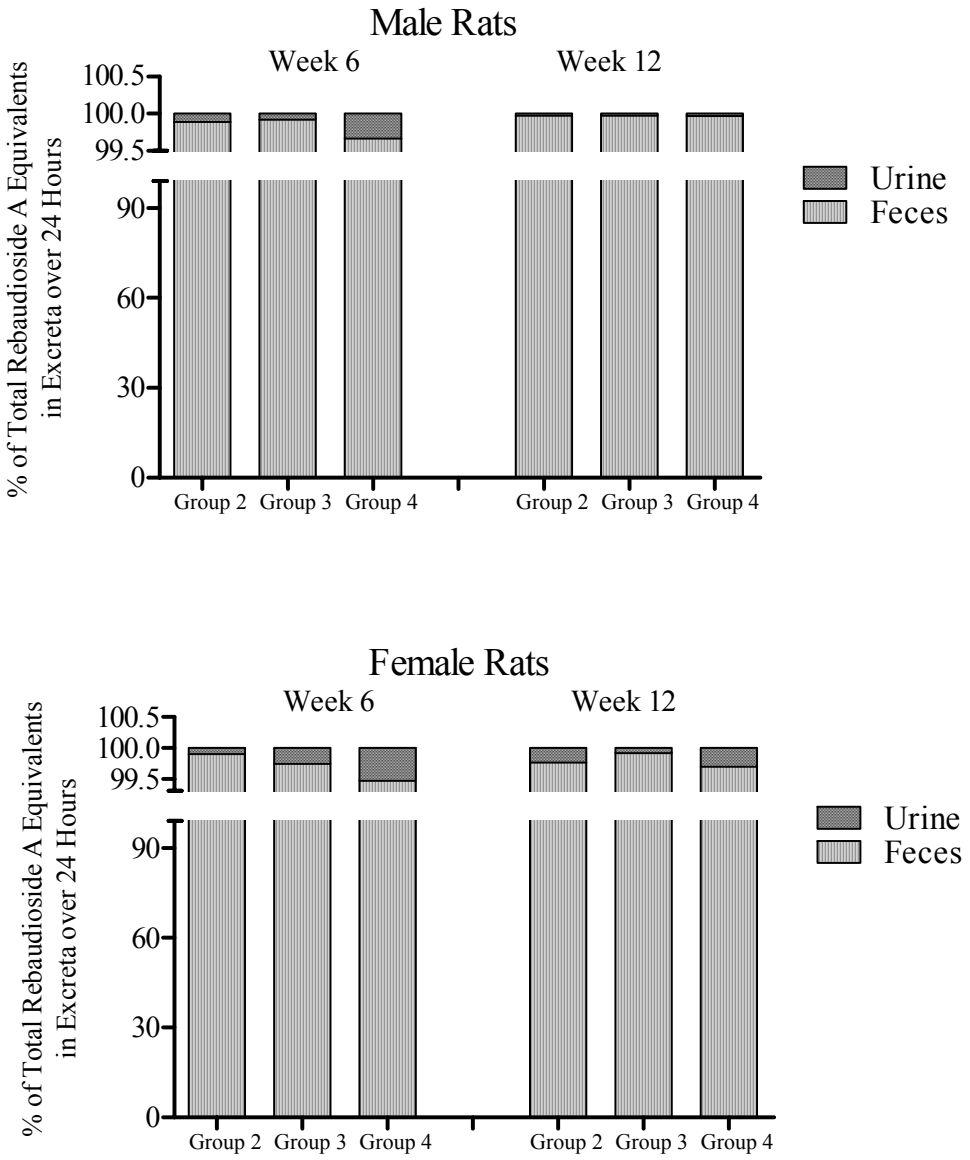
Note: Group 2 = 500 mg/kg/day; Group 3 = 1000 mg/kg/day; Group 4 = 2000 mg/kg/day

Rebaudioside B has a structure close to that of Reb A (minus one glucose residue) and is formed by hydrolysis in the large intestine by resident microflora, as are the other steviol glycosides depicted in Figure V.-8. The practical absence of rebaudioside B in plasma and urine indicates that limited or no absorption of Reb A and hydrolysis products, except steviol, occurs in the

distal parts of the rat gastrointestinal tract; otherwise, the high levels of rebaudioside B in feces would be reflected in plasma and urine.

Examination of the distribution of *Total Reb A Equivalents* collected over 24 hours in urine and feces of F_1 male and female rats administered Reb A via the diet for 6 and 12 weeks corroborates the work of Geuns *et al.* (2007) that Reb A and its hydrolysis products are excreted almost exclusively in the feces (Figure V.-7).

Figure V.-7: Distribution of Total Reb A Equivalents Collected over 24 Hours in Urine and Feces of F_1 Male and Female Rats Administered Reb A via the Diet



Note: Group 2 = 500 mg/kg/day; Group 3 = 1000 mg/kg/day; Group 4 = 2000 mg/kg/day

Conclusions

The results of this study (Sloter, 2008a [Unpublished]) corroborate the published literature on the ADME for Reb A and other steviol glycosides and allow one to conclude that all steviol glycosides follow the same pathways of hydrolysis (Figure V.-8), albeit at different rates of hydrolysis.

Regarding toxicokinetics for plasma:

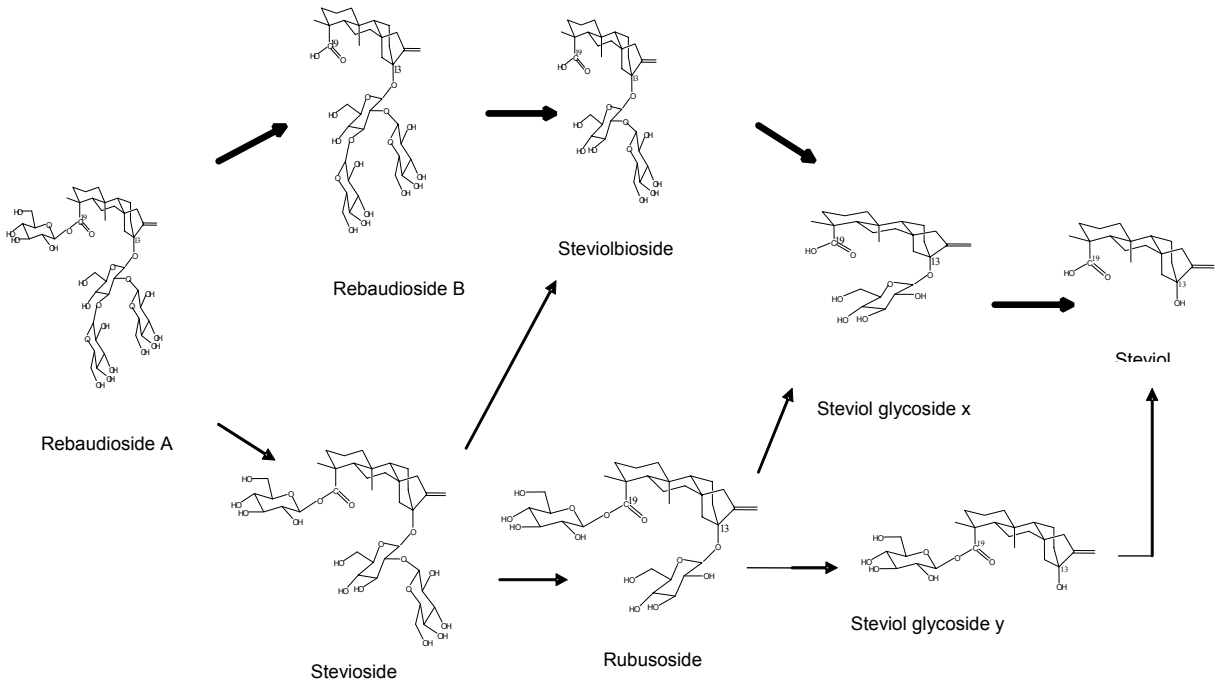
- Mean plasma concentrations (C_{avg}) of Reb A and total steviol (as *Reb A Equivalents*) are extremely low (≈ 0.6 and 12 µg/mL for high-dose, respectively)
- Mean plasma concentrations of other measured Reb A hydrolysis products (as *Reb A Equivalents*) are below the limits of the analytical methods or are insignificant (< 0.06 µg/mL for high-dose)
- Mean plasma concentrations of Reb A and steviol (as *Reb A Equivalents*) for the 2000 mg/kg/day dose group are less than dose-proportional in comparison to mean plasma concentrations at 500 and 1000 mg/kg/day, suggesting that dose levels higher than 2000 mg/kg/day would not result in significantly higher mean plasma concentrations

Regarding excretion for urine and recovery for feces collected over 24 hours:

- Mean urinary Reb A and measured hydrolysis products expressed as *Total Reb A Equivalents* are low (≈ 0.285 mg for high-dose) and accounted for almost exclusively by free and conjugated steviol
- Mean urinary Reb A and *Total Reb A Equivalents* amounts in comparison to the daily administered dose results in estimates of dose absorbed of ≈ 0.02% and ≈ 0.08%, respectively
- Mean fecal Reb A and measured hydrolysis products expressed as *Total Reb A Equivalents* are high (≈ 344 mg for high-dose)
- Mean fecal Reb A and measured hydrolysis products expressed as *Total Reb A Equivalents* compared to daily administered dose results in an estimate of percent of dose recovered ≈ 84%
- Mean fecal levels of rebaudioside B (expressed as *Reb A Equivalents*) are relatively high and stevioside is very low leading to the conclusion that hydrolysis of Reb A goes predominantly

via a rebaudioside B pathway rather than stevioside and/or hydrolysis of stevioside to steviolbioside occurs rapidly.

Figure V.-8: Common Hydrolysis Pathways of Reb A to the Aglycone, Steviol



Absorption, Distribution, Metabolism, and Excretion of Steviol Glycosides and Steviol

The published literature provides data that are in agreement with those reported above for Reb A. These data are presented below.

Absorption and/or Metabolism in the Stomach

No absorption or structural modification of steviol glycosides in the stomach as was found in stevioside-dosed rats (Nakayama *et al.*, 1986). Stevioside incubated in human stomach juice for 6 hours was not altered (Geuns *et al.*, 2007).

Absorption and/or Metabolism in the Small Intestine

The intestinal absorption of stevia mixture (Reb A, stevioside, rebaudioside C, and dulcoside A) and steviol was studied *ex vivo* using everted gastrointestinal sacs from rats. The everted gastrointestinal sacs were incubated in stevia mixture or steviol for 30 minutes. Salicylic acid was used as a positive control to demonstrate the integrity of the test system. The components of

the stevia mixture were poorly absorbed with more than 93% remaining in the mucosal fluid, whereas greater than 70% of the steviol was absorbed. Steviol was transported in both the duodenum-jejunum and ileum at similar rates (Koyoma *et al.,* 2003a).

Koyoma *et al.* (2003a) administered a stevia mixture, including Reb A orally to humans and reported that the steviol glycosides were first hydrolyzed by intestinal microflora (i.e. absorption of steviol glycosides does not occur in the small intestine). Steviol was then absorbed into blood of the portal vein. The inability to absorb steviol glycosides is not surprising given their high molecular weights (Reb A: 967.03; rebaudioside C: 951.03; stevioside: 804.88; dulcoside A: 788.88) and hydrophilic nature due to the glucose units. The glucose units are removed by hydrolysis from Reb A and other steviol glycosides to form steviol, which is much less hydrophilic and can be absorbed.

Orally administered steviol is rapidly absorbed in the stomach or the upper small intestine of rats, whereas steviol glycosides and stevia mixture are degraded to steviol by intestinal microflora and then absorbed in the lower large intestine (Koyoma *et al.*, 2003a; Gardana *et al.*, 2003; and Wingard *et al.*, 1980). No preferential site for steviol absorption between duodenum and ileum has been identified (Koyoma *et al.*, 2003a).

A very small fraction of the dosed ^{3}H-stevioside in rat may undergo hydrolysis in the lower small intestine to yield steviobioside (stevioside hydrolyzed at ester linkage at C-19 bond) and steviol, which accounts for 7.5-8% of the administered dose after 4 h in the lower small intestine at the cecum. Limited absorption of the produced free steviol takes place in the small intestine (Nakayama *et al.,* 1986).

Absorption and/or Metabolism in the Large Intestine

As noted above, the principal steviol glycosides, Reb A and stevioside are metabolized in experimental animals and humans by intestinal microflora by successive hydrolysis of glucose moieties. However, this process does not appear to be efficient. Based on its chemical structure, Reb A may be hydrolyzed by intestinal microflora to rebaudioside B or stevioside and given a long enough residence time in the lower intestine, could hydrolyze through intermediates to the aglycone, steviol.

When intestinal transport was investigated using the Caco-2 system, only a small fraction of stevioside and Reb A (apparent permeability coefficient, P_{app} of 0.16×10^{-6} and 0.11×10^{-6} cm/s, respectively) was transported through the cell layer; whereas, steviol was transported more readily ($P_{app}=38.6\times10^{-6}$ cm/s) (Geuns *et al.*, 2003a).

Reb A and stevioside were studied *in vitro* by anaerobic incubation with microbial whole-cell suspensions from rat cecum and aerobic incubation with sonic cell-free extracts prepared from rat cecal contents to determine if they are metabolized to steviol. After two days of incubation of a 2.5 mg/mL dose of stevioside in whole-cell suspensions, 107% of theoretical was recovered as steviol. However, after two days of incubation of a 3.0 mg/mL dose of Reb A in whole-cell suspensions, only 65% of theoretical was recovered as steviol. The experiment with Reb A was continued and it was determined that after four and six days 83 and 108% of theoretical, respectively, were recovered as steviol. Incubation with sonic cell-free extracts resulted in much slower rates of hydrolysis with only 50 and 2% of stevioside and Reb A, respectively, converted to steviol after seven days for (Wingard *et al.*, 1980).

Degradation of Reb A and stevioside at concentrations of 0.2 and 10 mg/mL was investigated *in vitro* by their incubation under anaerobic conditions with pooled human fecal homogenates from five healthy volunteers for 0, 8 and 24 hours. Reb A and stevioside were degraded in a time-dependent and concentration-dependent manner at both concentrations. At 0.2 mg/mL, Reb A had degraded approximately 30-35% after 8 hours and 100% after 24 hours; whereas at 10 mg/mL, Reb A had degraded only 5-10% at 8 hours and 56% after 24 hours of incubation. The conversion

of Reb A to steviol at substrate concentrations of 0.2 and 10 mg/mL after 24 hours was determined to be 109 and 22%, respectively. In contrast to Reb A, stevioside was determined to degrade more rapidly since 100 and 77% had degraded by 24 hours at 0.2 and 10 mg/mL, respectively. On the other hand, it was reported that the conversion of stevioside to steviol at 0.2 and 10 mg/mL in 24 hours was 84 and 63%, respectively. The 24-hour conversion rate to steviol at the 0.2 mg/mL incubation concentration of 109% for Reb A compared to 84% for stevioside is paradoxical since the authors propose the main route of Reb degradation goes through stevioside as its first metabolite. The authors concluded that there are apparently no species differences between humans and rats in anaerobic metabolism by intestinal microflora of Reb A and stevioside (Koyama *et al.*, 2003b).

Stevioside and Reb A were incubated for 72 hours under anaerobic conditions with fecal suspensions provided by six male and five female volunteers aged 20-50 years. Stevioside completely degraded to steviol in a 10-hour period. Steviolbioside (a metabolite of Reb A and stevioside) concentration peaked after 2-4 hours of incubation, and then decreased to zero with steviol detected after 3-4 hours of incubation. These results suggest that stevioside was initially hydrolyzed to steviolbioside and then this intermediate was subsequently metabolized to steviol. After a period of 6-7 hours, Reb A was hydrolyzed to steviolbioside and completely metabolized to steviol after 24 hours. The results of this study do not elucidate whether Reb A preferentially hydrolyzes to rebaudioside B or stevioside before further hydrolysis to steviolbioside. Steviol remained unchanged during the 72-hour incubation and no other metabolites were observed. No steviol epoxide derivatives were found after incubation of Reb A or stevioside samples with intestinal microflora from 11 human volunteers (Gardana *et al.*, 2003).

Stevioside was administered via the diet to pigs at approximately 70 mg/kg bw/day for 14 days and daily blood and fecal samples were collected starting after two days. Analysis of fecal samples indicated that stevioside was completely converted to steviol; however, no stevioside or steviol was detected in blood samples. The authors attribute the difference between the in vitro and in vivo results, i.e.relatively high absorptive transport in the Caco-2 system (discussed above) and the absence of steviol in blood samples following repeated oral intake by pigs, to the

fact that in the *in vitro* system steviol is in solution in direct contact with the Caco-2 cell layer whereas *in vivo* steviol probably is absorbed to the contents of the colon (Geuns *et al.*, 2003a).

In cultures of coliforms, bifidobacteria, enterococci and bacteroides from human fecal bacterial suspensions, only the bacteroides were efficient in hydrolyzing stevioside (purity 85%) and Reb A (purity 90%) to steviol. Both stevioside and Reb A were hydrolyzed initially to steviolbioside with the hydrolysis occurring more slowly for Reb A. Steviolbioside underwent further hydrolysis to steviol; however, steviol remained unchanged during the 72 h incubation period (Gardana *et al.,* 2003).

Stevioside and Reb A did not influence significantly the human intestinal microflora composition; however, stevioside weakly inhibited anerobic bacteria, whereas Reb A weakly inhibited activity on aerobic bacteria particularly coliforms (Gardana *et al*., 2003).

Excretion in Urine and Feces

Geuns *et al.* (2007) reported that between 13 and 40 mg free steviol could be detected in a 24-hr feces sample collected from human volunteers following 3 doses/day of 250 mg stevioside/dose. The lack of stevioside in the feces indicates that the bacterial flora degraded all the stevioside into steviol glycoside intermediates or into steviol, which itself was not further metabolized. This result has also been shown in pigs *in vivo* (Geuns *et al.*, 2003a) and in pig and human feces *in vitro* under anaerobic conditions (Geuns *et al.*, 2003a, Koyoma *et al.*, 2003b, Gardana *et al.*, 2003). Geuns *et al.* (2007) did not detect free steviol in the peripheral blood or urine of the study subjects.

In rats, stevioside is hydrolyzed to steviol via an intermediate, steviolbioside, with enterohepatic circulation producing unidentified conjugated metabolites of steviol in the bile. Excretion is via the feces, with 68% of the initial dose of stevioside excreted in the feces by day 5 post-dose, 2% in the urine and the remainder in expired air (Nakayama *et al.* 1986). Since the study used a uniform ^{3}H-stevioside radiolabel, the interpretation of quantitative results is limited.

Geuns *et al.* (2007) did not detect any free steviol in urine from human volunteers. After enzymatic hydrolysis of urine extracts by β-glucuronidase/sulfatase, steviol was found as the

only aglycone present. In terms of absorbed dose, the amount of Reb A excreted in the 24-hour urine was approximately 0.02% of the administered dose. There was no indication for the occurrence of other conjugated molecules such as steviol sulfates, even after large-scale extractions of urine (Geuns *et al.*, 2006). Conjugated steviol metabolites were readily filtered through the kidney and excreted in urine. No free steviol or stevioside was detected in urine of stevioside dosed human volunteers. Urine collected for 24 h from 750 mg stevioside-dosed volunteers for 3 days contained up to 318 mg of steviol glucuronide (Geuns *et al.*, 2007)

Steviol, Steviol Glycosides and Conjugated Steviol in Portal Blood or Peripheral Blood

Geuns *et al.* (2007) did not detect free steviol in the peripheral blood of human volunteers. These results are in agreement with the lack of free steviol in pig blood (Geuns *et al*., 2003a). These results are also in agreement with the metabolism studies recently performed with 9 human volunteers (Simonetti *et al.*, 2004). In this study free steviol was not detected in blood and urine using LC- MS (Simonetti *et al.*, 2004). The absence of free steviol in the peripheral blood of the volunteers and the presence of conjugated forms suggest that free steviol is converted into steviol glucuronide in the lower intestine and/or liver (Geuns *et al.,* 2007; Simonetti *et al.,* 2004). In bile duct cannulated rats that were intracecally dosed with 1 ml (0.7 mg) of steviol-17-[^{14}C], the radioactivity was excreted via the bile duct (105.5 ± 8.4% of dose, biliary excretion; 96.4 ± 0.4% of dose, fecal excretion) (Wingard *et al*., 1980). Besides steviol glucuronide, no free steviol or any of the other possible steviol metabolites have been detected in blood or urine from administration of stevioside. The results of a study that demonstrated in *in vitro* experiments that the steviol metabolism by human microsomes was 4 times lower than the already low metabolism of rat microsomes Koyoma *et al.* (2003a) are corroborated by Geuns (2007).

Time Course from Administration to Elimination

Although a study was conducted examining steviol glycoside transport and metabolism in rats (Nakayama *et al.* 1986), because of the method of radiolabeling (uniform ^{3}H), the interpretation of quantitative results is limited. Since steviol is not destructively metabolized, the radioactivity detected, in large part, may be attributed to glucose metabolism.

Summary

In summary, Figure V.-8 shows the common hydrolysis pathways of the steviol glycoside family of compounds. All steviol glycosides undergo glycosidic bond hydrolysis by the action of intestinal microflora yielding free steviol. Reb A is converted to free steviol much more slowly than stevioside. Extremely low concentrations (≈ 0.6 µg/mL) of free Reb A were detected in plasma during a dietary study in rats at the high-dose level of 2000 mg/kg bw/day. However, neither Reb A nor stevioside is absorbed effectively into the blood stream whereas steviol is absorbed in the blood stream. Steviol is then transported via the portal vein to the liver where it is glucuronidated. Steviol glucuronide is efficiently excreted in urine and to some degree bile. Intestinal steviol and other steviol glycosides that are not absorbed are eliminated in the feces. Figure V.-2 summarizes possible metabolic reactions and elimination pathways for steviol glycosides, including Reb A.

Reb A

Crude (purity not specified) Reb A was suspended in 1% carboxymethylcellulose and administered via gastric intubation to male Swiss-Webster mice (number not specified). The dose level was described as, "maximum quantities tested were determined by availability and physiological limits." No toxicity could be demonstrated in mice with crude Reb A at 2000 mg/kg body weight. Two weeks after dose administration, no significant differences were noted at necropsy in body or organ (heart, spleen, lung, pancreas, brain, kidney, and liver) weights in comparison to the control group (Medon *et al.*, 1982).

Stevioside

Stevioside (purity 96%) is not acutely toxic to mice, rats, or hamsters at doses as high as 15,000 mg/kg body weight (Toskulkao *et al.,* 1997; Medon *et al.,* 1982). Groups of 10-15 male and female mice, rats, and hamsters received a single oral administration of stevioside prepared in distilled water, followed by a 14-day observation period. Dosages ranged from 8000 – 15,000 mg/kg. No overt clinical signs of toxicity were noted following treatment and no gross or histopathological changes were observed in selected organs and tissues taken at necropsy (Toskulkao *et al.,* 1997). Medon *et al.* (1982) administered acute oral dosages of stevioside up to 2000 mg/kg followed by a 14-day observation period. No overt signs of toxicity were noted following treatment and no differences were noted in body or organ weights.

Steviol

Steviol (purity 90%) is not acutely toxic to rats and mice at doses as high as 14,000 mg/kg body weight (Toskulkao *et al.*, 1997). Groups of 10-15 male and female mice, rats, and hamsters received a single oral administration of steviol prepared in corn oil,

followed by a 14-day observation period. Dosages ranged from 3000–15,000 mg/kg. No overt signs of toxicity were noted in rats and mice at doses up to 14,000 mg/kg. However, signs of acute toxicity, including death (1/15 males and females for rats and mice) were observed in rats and mice at a dose of 15,000 mg/kg. No morphological or histopathological changes were noted. Hamsters treated with steviol had LD_{50} values of 5200 and 6100 mg/kg for males and females, respectively. Histopathological examination of the kidneys of hamsters treated with steviol revealed severe degeneration of the proximal tubular cells, pointing to acute renal failure as a possible cause of death (Toskulkao *et al.*, 1997).

Table V.-4: Acute Oral Toxicity Studies

Species	Compound	LD_{50}	Reference
Mouse	Reb A (purity not specified)	> 2000 mg/kg	Medon *et al.*, 1982
Mouse	Stevioside (96% purity)	> 15,000 mg/kg	Toskulkao *et al.*, 1997
Mouse	Stevioside (96% purity)	> 2000 mg/kg	Medon *et al.*, 1982
Rat	Stevioside (96% purity)	> 15,000 mg/kg	Toskulkao *et al.*, 1997
Hamster	Stevioside (96% purity)	> 15,000 mg/kg	Toskulkao *et al.*, 1997
Mouse	Steviol (90% purity)	> 15,000 mg/kg	Toskulkao *et al.*, 1997
Rat	Steviol (90% purity)	> 15,000 mg/kg	Toskulkao *et al.*, 1997
Hamster	Steviol (90% purity)	5200 mg/kg (males) 6100 mg/kg (females)	Toskulkao *et al.*, 1997

In summary, Reb A had a mouse oral LD_{50} > 2000 mg/kg, the highest dose tested. Stevioside demonstrated minimal or no acute oral toxicity in multiple species even at very high oral gavage doses. Steviol also demonstrated minimal or no acute oral toxicity in mice and rats, but in hamsters acute renal failure apparently caused deaths at high doses.

Reb A

Rat 90-day oral (dietary) toxicity study

A 90-day oral (dietary) toxicity study was conducted in Crl:CD(SD) rats with 99.5% pure Reb A (Eapen, 2007 [Unpublished]; Nikiforov and Eapen, 2008). A control and 3 treated groups were included; target dosage levels were 500, 1000 and 2000 mg/kg/day. Dietary concentrations were adjusted weekly based on expected average weight and current food consumption. The concentration of Reb A in the formulated test diet reached 35.93 and 30.42 g/kg (35,930 and 30,420 ppm) for high-dose group males and females, respectively, by study week 12. Each group consisted of 20/animals/sex. Additional details of the study design and results are presented in Appendix V.-1 (entitled Rat 90-Day Oral (Dietary) Toxicity Study of Reb A).

There were no test article-related effects on clinical observations, food consumption or functional observational battery or locomotor activity parameters. There were no test article-related macroscopic, organ weights or microscopic findings. Average daily test article consumption for the target dosage levels of 500, 1000 and 2000 mg/kg/day was 517, 1035 and 2055 mg/kg/day for males and 511, 1019 and 2050 mg/kg/day for females, respectively.

Lower body weight gains were noted in the 2000 mg/kg/day group males throughout the study (statistically significant at weeks 12 and 13). These lower mean body weight gains resulted in statistically significant lower cumulative body weight gains and a statistically significant mean body weight difference that was 9.1% lower compared to the control group at the end of the dosing period (study week 13). The authors concluded that this effect was due to the extremely high levels of test material which contained no caloric value in the diet (resulting in a decrease in total calorie consumption). A similar trend in mean body weights did not occur in the female test article-treated groups; however, this

may be explained by the fact that the overall inclusion rates of test article in the diet were lower for the females as compared to the males throughout the study. The food efficiency data for males demonstrate that body weight gained as a percent of feed consumed is statistically significantly decreased at 2000 mg/kg/day as compared to the control group for the following intervals: week 0 to 1, week 3 to 4, and week 7 to 8. In contrast, the food efficiency data for females demonstrate that body weight gained as a percent of feed consumed was the same for test article-treatment groups as compared to the control group.

Based on the results of this study, the NOAEL was considered to be at least 2055 and 2050 mg/kg/day for males and females, respectively, the highest average dosage levels examined.

As discussed in subsequent sections of this Safety Evaluation, decreases in food efficiency data, body weight and/or body weight gain compared to controls are observed at the highest dietary dose levels of Reb A and stevioside in rat subchronic toxicity (Eapen, 2007 [Unpublished]; Nikiforov and Eapen, 2008; Aze *et al*., 1991), rat chronic toxicity/carcinogenicity (Xili *et al*., 1992; Toyoda *et al*., 1997) and rat or hamster reproduction toxicity (Sloter, 2008a [Unpublished]; Yodyingyuad and Bungawong, 1991; Mori *et al*., 1981) studies. It may be concluded that decreases in body weight and/or body weight gain in these studies are due to the extremely high levels of Reb A or stevioside administered via the dietary route which provide no caloric value in the diet, resulting in a decrease in total calorie consumption. Effects on body weight, body weight gain, and/or feed efficiency have been reported for other high intensity sweeteners when they are administered in the diet to test animals at similarly high dietary concentrations. For example, the reductions in body weight gain that have been reported for neotame, sucralose, and saccharin ranged from 3.7 to >20% in comparison to controls. It was concluded that body weight gain decreases are not an appropriate basis for a no-observed-adverse-effect level (NOAEL) (Flamm *et al.,* 2003).

In the absence of toxicity, JECFA has concluded that changes in body weight gain are not appropriate for establishing NOAELs when they are associated with lower food consumption or food efficiency. It is the normal practice of JECFA to recognize when body weight is affected by reduced palatability of food containing high concentrations of test material. For example, JECFA noted that lower body weight gain for sucralose at the high dose in the long-term rat study (1500 mg/kg bw/day) was due to poor palatability of the diet and did not consider this finding adverse when setting the NOAEL and an ADI of 0-15 mg/k bw/day. Similarly, the highest dose of acesulfame-potassium, 1500 mg/kg bw/day was likewise associated with lower body weight gain. JECFA did not consider this an adverse finding when establishing an ADI for acesulfame-potassium of 0-15 mg/kg bw/day (WHO, 1983 as summarized in Flamm *et al*., 2003).

Dog 6-month oral (dietary) toxicity study

A 6-month oral (dietary) toxicity study was conducted in Beagle dogs with Chrysanta® 99-P as the test material (which is 95.7% Reb A) (Eapen, 2008 [Unpublished]). A control and 3 treated groups were included; target dosage levels were 500, 1000 and 2000 mg/kg/day. Dietary concentrations were adjusted weekly based on expected average weight and food consumption. Concentration of Reb A in the formulated test diet reached 61.70 and 68.15 g/kg (61,700 and 68,150 ppm) for high-dose group males and females, respectively, by study week 24. Each group consisted of 4 males and 4 females. Additional details of the study are presented in Appendix V.-2 (entitled Reb A Dog 6-Month Oral (Dietary) Toxicity Study).

There were no unscheduled deaths during the course of the study. No test article-related clinical observations were noted. In-cage, open field, and functional observations and/or measurements were unaffected by test article administration. No test article-related hematology findings, serum chemistry findings, or urinalysis findings were reported. No test article-related gross necropsy observations, alterations in final body weight, alterations in organ weights, or histologic changes were reported at the scheduled necropsy.

Based on the results of this study, no systemic toxicity of Reb A administered as a dietary admix to beagle dogs for a minimum of 182 days was observed at dosage levels up to 2000 mg/kg/day. Therefore, the NOAEL for oral (dietary) administration of Reb A was 2000 mg/kg/day.

Stevioside

In a 13-week study in F344 rats, no stevioside-related toxicity was reported at doses up to 2500 mg/kg bw/day (Aze *et al.,* 1991; JECFA, 1999). Stevioside was administered to 10 male and 10 female Fischer 344 rats in the diet at levels of 0, 0.31, 0.62, 1.25, 2.5, or 5% for 13 weeks. These levels were estimated to be equivalent to doses of 160, 310, 630, 1300, and 2500 mg/kg bw/day (JECFA, 1999; FDA, 1993). Signs of toxicity were recorded and changes in body weight and food consumption were measured. Hematology and clinical chemistry examinations were conducted during the course of the study. All animals were sacrificed and necropsies were performed at the end of the study. No mortality was observed during the course of the study and changes in body weight gain and food consumption were noted when stevioside-treated groups were compared with the control group. Statistically significant decreases in terminal body weights were observed in males (5% dose level) and females (2.5 and 5% dose levels). Lactate dehydrogenase (LDH) was reported to be increased in some groups of stevioside-treated male rats; however, there was not a dose related trend and there were no accompanying changes in any other cytoplasmic enzymes. The incidence of single-cell necrosis (graded minimal to slight) in the liver was increased in treated male groups. However, the lack of a dose-response relationship for both endpoints combined with the low severity of necrosis and occurrence of increased LDH only in male rats resulted in the authors' conclusion that the effects were nonspecific and not treatment related. Furthermore, in a subchronic (90-day) dietary study of Reb A in rats (Nikiforov and Eapen, 2008), there were no significant differences in glutamate dehydrogenase and sorbitol dehydrogenase in treated groups as compared to controls. A few statistically significant hematological and biochemical parameters were reported but were not dose related. Appendix V.-3 (entitled Data Tables from Aze *et al.* (1991)) presents the results for male and female rats.

Steviol

No published short-term toxicity studies of steviol in animals are available.

Table V.-5: Short-Term/Subchronic Oral Toxicity Studies

Species	Compound	Route	Duration	NOAEL	Reference
Rat (Crl:CD (SD) male and female rats)	Reb A (99.5% purity)	Oral (diet)	90 days	≥ 2000 mg/kg bw/day	Eapen, 2007 [Unpublished]; Nikiforov and Eapen, 2008
Dog (Beagle male and female)	Reb A (95.7% purity)	Oral (diet)	6 months	≥ 2000 mg/kg bw/day	Eapen, 2008 [Unpublished]
Rat (Fischer 344 male and female)	Stevioside (95.6% purity)	Oral (diet)	13 weeks	2500 mg/kg bw/day (5% in diet determined as the maximum tolerable dose for a 2-year carcinogenicity study)	Aze *et al.*, 1991, as cited in JECFA, 1999

In summary, dietary administration of Reb A to rats and dogs for 90-days and 6-months, respectively, at doses as high as 2000 mg/kg bw/day resulted in no adverse systemic toxicity. Stevioside administered to rats in the diet at concentrations up to 5% resulted in a few statistically significant changes at some doses for some biochemical parameters. However, the lack of a dose-response relationship resulted in the authors' conclusion that the effects were nonspecific and not treatment related. The JECFA panel reviewed this data and reported the same conclusions.

Reb A

Chronic toxicity/carcinogenicity studies have not been conducted with Reb A and for the reasons set forth below are not necessary according to FDA's Concern Level guidelines (U.S.FDA, 2006). However, there are studies for stevioside, which based on the common metabolic pathways (see Section V.A.1), are relevant to understanding the safety of long term exposures to Reb A.

Systemic exposure to Reb A would be lower than other steviol glycosides since Reb A is very poorly absorbed by animals or humans based on low apparent permeability coefficient results with the Caco-2 system and the toxicokinetics results presented in the section entitled "Absorption, Distribution, Metabolism, and Excretion." The hydrolysis of Reb A in the gut is even slower than stevioside and most ingested Reb A and its hydrolysis products are excreted in the feces. Since (1) systemic exposure to Reb A and its hydrolysis products is extremely limited (i.e. only approximately 0.08% of administered Reb A dose can be measured in 24-h urine of rats as *Total Reb A Equivalents*), (2) subchronic toxicity studies in rats and dogs demonstrate no adverse signs of toxicity at high doses, (3) genetic toxicity studies for Reb A and its possible hydrolysis products show no adverse effects, and 4) long-term studies are available for stevioside; therefore, even long-term toxicity potential is negligible and there is no need for further testing of Reb A to assess chronic toxicity/carcinogenicity.

As an example of the low systemic exposure potential for Reb A and its hydrolysis products, the following calculation shows human exposure potential at the 90th percentile of Reb A intake (see Section IV.) based on 0.08% as the percent of absorbed dose as *Total Reb A Equivalents* in urine (see (1) above):

[4.72 mg/kg bw/day × 0.0008] × 1000 µg/mg = 3.78 µg/kg bw/day.

Where: 4.72 mg/kg bw/day = 90th percentile of human Reb A intake

0.0008 = 0.08% converted to fraction of Reb A dose in rats absorbed and excreted in 24-hour urine as *Total Reb A Equivalents*.

In addition, because of the common hydrolysis pathways, poor absorption, efficient elimination and other factors discussed in detail in the section entitled "Absorption, Distribution, Metabolism, and Excretion," the chronic toxicity/carcinogenicity toxicology data for stevioside are relevant to Reb A. The available data for stevioside are discussed in the following sections for the safety evaluation of Reb A.

Stevioside

The effects of oral stevioside in rats have been examined in two 2-year studies (Xili *et al.,* 1992; Toyoda *et al.,* 1997). In the first study, Xili *et al.* (1992) administered stevioside (85% purity) in the diet of 45 male and 45 female Wistar rats at levels of 0, 0.2, 0.6, or 1.2% for two years; these dietary levels were estimated to be equivalent to doses of 0, 100, 300, and 600 mg/kg bw/day, respectively (JECFA, 1999; FDA, 1993). Sacrifices of 5 rats/sex/group at 6, 12 and 24 months allowed hematological and clinical biochemical tests to be performed. No treatment-related effects were observed in hematological, urinary, or clinical biochemical values at any stage of the study in any dose group up to the highest level tested (1.2% in the diet). There were no differences in growth, food utilization and consumption reported for the first three months, and general appearance or mortality between treated and control groups over the two-year study. From the growth curves, it is apparent that body weight for 1.2% dose level males was decreased compared to controls during the last eight months of the study. Non-neoplastic and neoplastic changes noted during the course of the study were unrelated to the level of stevioside in the diet. In summary, stevioside administration at levels up to 1.2% in the diet was without carcinogenic effect in the rat and a no-observed-effect-level (NOAEL) of 1.2% in the diet was reported.

In a more recent and more robust study, Toyoda *et al.* (1997) administered stevioside (95.6% purity) *ad libitum* in the diet of 50 male and 50 female F344 rats at levels of 0, 2.5, and 5.0% for two years. These levels were equivalent to doses of 0, 970, and 1997 mg/kg bw/day in males and 0, 1120, and 2387 mg/kg/day in females, respectively. Surviving rats were necropsied at week 108 following a 4-week period during which all groups were administered basal diet. Clinical signs, mortality, body weights and food consumption were monitored throughout the study. Following terminal sacrifice, hematology, gross lesions and organ weights were evaluated and tissues were collected for microscopic study of neoplastic and non-neoplastic lesions. At the high-dose level, male rats had a decreased survival rate (60% vs. 78% in controls). Body weight gain was significantly decreased in both sexes (4.4% in males and 9.2% in females for the high-dose groups), as was absolute kidney weight in male and female rats at the high-dose level as compared to the respective control groups. Food consumption parameters were not affected by treatment. A decreased incidence of mammary adenomas in females and a reduced severity of chronic nephropathy in males were observed. However, the non-neoplastic and neoplastic changes noted during the course of the study were not considered to provide significant evidence of effects and the authors concluded that the effects were unrelated to the level of stevioside in the diet. It was concluded by the authors that stevioside is not carcinogenic in F344 rats and a NOAEL of 2.5% in the diet (970 and 1120 mg/kg bw/day for males and females, respectively) was established.

In a study designed to look specifically at the possible promotion of carcinogenicity by stevioside, bladder cancer was initiated in 25 male rats by a four week treatment with N-nitrosobutyl-N-(4-hydroxybutyl)amine (BBN) in drinking water. The rats were then treated with stevioside at 5000 mg/kg bw/day in the diet for 32 weeks. All rats were sacrificed at 36 weeks. No difference in the incidence of papillary or nodular hyperplasia was seen between control and stevioside-treated rats. No preneoplastic or neoplastic lesions were observed in the urinary bladders of control rats treated with stevioside alone (Hagiwara *et al.,* 1984; Ito *et al.,* 1984). The authors concluded that stevioside did not promote bladder carcinogenesis.

Although both stevioside chronic toxicity/carcinogenicity studies have been conducted in the rat, the low systemic exposure potential, the lack of any effects in the extensive and complete subchronic database for Reb A and stevioside, and lack of positive *in vitro* or *in vivo* genotoxicity results for Reb A and stevioside confirm the lack of any potential long-term effects for Reb A.

Table V.-6: Chronic Toxicity/Carcinogenicity Studies with Stevioside

Species	Compound	Route and Duration	NOAEL and Endpoint	Reference
Wistar Rat	Stevioside (Purity 85%)	Oral (diet) 2 years	NOAEL=1.2% (600 mg/kg bw/day) No treatment-related changes.	Xili *et al.,* 1992; JECFA, 1999
F344 Rat	Stevioside (Purity 95.6%)	Oral (diet) 2 years	NOAEL=2.5% (970 mg/kg bw/day) Not carcinogenic. Body weight gain of treated animals was slightly decreased in relation to dose. Final survival rate of males at 5% significantly decreased. Absolute kidney weights decreased in male and female animals at the high dose.	Toyoda *et al.,* 1997
Male F344 Rat	Stevioside (Purity not reported)	Oral (diet) 32 weeks	NOAEL=5000 mg/kg bw/day Does not promote bladder carcinogenesis following 4-week initiation and 32-week dietary administration.	Hagiwara *et al.,* 1984; Ito *et al.,* 1984

<u>Steviol</u>

No published chronic toxicity/carcinogenicity studies of steviol in animals are available. Reb A use as a sweetener will only result in steviol exposure as a result of Reb A hydrolysis in the gut (see ADME Section V.A.1). Therefore, indirect exposures to steviol as a result of Reb A hydrolysis are automatically included in all of the *in vivo* testing with Reb A or stevioside as the test material.

In summary, chronic toxicity/carcinogenicity studies have not been conducted with Reb A; however, no effects were reported in two 2-year oral studies of stevioside in rats. In the first study (Xili *et al.,* 1992), stevioside administration in the diet was without carcinogenic effect in the rat and a NOAEL of 1.2% (600 mg/kg bw/day) in the diet was reported. In a more recent and more robust study (Toyoda *et al.,* 1997) stevioside administered in the diet was not carcinogenic in rats and a NOAEL of 2.5% (970 mg/kg bw/day in males) was established. These findings are in agreement with the results of subchronic toxicity studies in rats and dogs that also demonstrated no adverse signs of toxicity at high doses. In addition all genetic toxicity studies for Reb A and stevioside are negative.

As discussed in this Safety Evaluation (see Section V.A.2.b), decreases in food efficiency data, body weight and/or body weight gain compared to controls are observed at the highest dietary dose levels of Reb A or stevioside tested in rats. These changes are reported for other high intensity sweeteners when the diet contains such large amounts of a non-nutritive sweetener (Flamm *et al*., 2003), and are not an appropriate basis for assigning a no-observed-adverse-effect level (NOAEL).

Reb A

In vitro bacterial reverse mutation assay

Rebaudioside A (purity 99.5%) was tested in the bacterial reverse mutation assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in two phases using the plate incorporation method. The first phase, the initial toxicity-mutation assay, was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. The second phase, the confirmatory mutagenicity assay, was used to evaluate and confirm the mutagenic potential of the test article (Wagner and Van Dyke, 2006 [Unpublished]).

Sterile, distilled water was selected as the solvent based on solubility of the test article and compatibility with the target cells. The test article formed a soluble and clear solution in water at approximately 50 mg/mL, the highest concentration tested.

In the initial toxicity-mutation assay, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 50 mg/mL and 100 µL plating aliquot. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate. In the initial toxicity-mutation assay, no positive mutagenic response was observed. Neither precipitate nor appreciable toxicity were observed. Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, no positive mutagenic response was observed. The dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor appreciable toxicity was observed.

Under the conditions of this study, Reb A was negative in the Bacterial Reverse Mutation Assay.

In vitro forward mutation assay

The mutagenicity of Reb A (purity not specified) was examined in a forward mutation assay (test concentrations not reported) with *Salmonella typhimurium* TM677. No significant mutagenic or bactericidal activity was observed during the assay either in the presence or absence of a metabolic activating system obtained from Aroclor 1254 pretreated rats (Medon *et al*., 1982).

The mutagenicity of pure (purity not specified) Reb A was examined in a forward mutation assay (maximum concentration 10.0 mg/mL) with *Salmonella typhimurium* TM677. No significant mutagenic or bactericidal activity was observed during the assay either in the presence or absence of a metabolic activating system obtained from Aroclor 1254 pretreated rats (Pezzuto *et al*., 1985a).

In vitro mouse lymphoma assay

Reb A (purity 99.5%) was tested in the L5178Y/TK$^{+/-}$ mouse lymphoma mutagenesis assay in the absence and presence of Aroclor-induced rat liver S9. The preliminary toxicity assay was used to establish the concentration range for the mutagenesis assays. The initial (4-hour) and extended (24-hour) treatment mutagenesis assays were used to evaluate the mutagenic potential of the test article. The mutagenic potential of the test article was measured by its ability to induce TK^{+}→ TK^{-} mutations (Clarke, 2006 [Unpublished]).

Sterile, distilled water was selected as the solvent based on solubility of the test article and compatibility with the target cells. The test article was soluble in sterile, distilled water at 50 mg/mL, the maximum concentration tested.

In the preliminary toxicity assay, the maximum concentration of Reb A in the treatment medium was 5000 µg/mL; this dose was achieved using a concentration of 50 mg/mL and 100 µL plating aliquot. No visible precipitate was present at any concentration in treatment medium. Selection of concentrations for the mutation assay was based on reduction of suspension growth relative to the solvent control. Substantial toxicity, i.e., suspension growth of ≤ 50% of the solvent control, was observed only at 5000 µg/mL without activation with a 24-hour exposure.

Based on the results of the preliminary toxicity assay, the concentrations tested in the initial mutagenesis assay ranged from 100 to 5000 µg/mL for both non-activated and S9-activated cultures with a 4-hour exposure. No visible precipitate was present at any concentration in treatment medium. The concentrations chosen for cloning were 1000, 2000, 3000, 4000 and 5000 µg/mL. No cloned cultures exhibited mutant frequencies ≥ 55 mutants per 10^6 clonable cells over that of the solvent control. There was no concentration-related increase in mutant frequency.

Based on the results of the preliminary toxicity assay, the concentrations tested in the extended treatment assay ranged from 100 to 5000 µg/mL for non-activated cultures with a 24-hour exposure. No visible precipitate was present at any concentration in treatment medium. The concentrations chosen for cloning were 500, 1000, 2000, 3000, 4000 and 5000 µg/mL. No cloned cultures exhibited mutant frequencies ≥ 55 mutants per 10^6 clonable cells over that of the solvent control. There was no concentration-related increase in mutant frequency.

The trifluorothymidine-resistant colonies for the positive and solvent control cultures from both assays were sized according to diameter over a range of approximately 0.2 to 1.1 mm. The colony sizing for the methyl methanesulfonate positive control yielded the expected

increase in small colonies (verifying the adequacy of the methods used to detect small colony mutants) and large colonies.

Under the conditions of this study, Reb A was concluded to be negative in the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

In vivo mouse micronucleus assay

Reb A (purity 99.5%) was tested in the mouse micronucleus assay. The assay was performed in two phases. The first phase, the confirmatory toxicity study, was designed to assess toxicity of the test article and set dose levels for the definitive micronucleus study. The second phase, the definitive micronucleus study, was designed to evaluate the potential of the test article to increase the incidence of micronucleated polychromatic erythrocytes in bone marrow of male and female ICR mice (Krsmanovic and Huston, 2006 [Unpublished]).

In the definitive phase of the study, sterile water was used as the vehicle (negative) control article and cyclophosphamide monohydrate (CP), at a dose of 50 mg/kg, as the positive control article. In both phases of the study, a single dose of the test or control article was administered at a dose volume of 20 mL/kg body weight by oral gavage.

The confirmatory toxicity study was performed using 5 male and 5 female ICR mice at a dose of 2000 mg/kg. No mortality occurred during the course of the confirmatory toxicity study. All mice were normal in appearance and behavior following dose administration and no reductions in mean body weights were observed. In the absence of mortality in the confirmatory toxicity study, the high dose for the definitive micronucleus study was set at 2000 mg/kg.

The definitive micronucleus study consisted of seven groups, each containing 5 male and 5 female ICR mice. Mice in five of these groups were treated either with the controls (vehicle or positive) or with Reb A at a dose of 500, 1000 or 2000 mg/kg and were euthanized 24 hours after treatment. Mice in the other two groups were treated either

with the vehicle control article or Reb A at a dose of 2000 mg/kg and were euthanized 48 hours after treatment. No mortality occurred during the course of the definitive micronucleus study. All animals treated with the test or control articles were normal in appearance and behavior following dose administration.

Bone marrow cells [polychromatic erythrocytes and normochromatic erythrocytes], collected 24 and 48 hours after treatment were examined microscopically for the presence of micronuclei. No reduction in the ratio of polychromatic erythrocytes to total erythrocytes was observed in the male test article-treated groups relative to the vehicle control groups. Reductions (up to 11%) in the ratio of polychromatic erythrocytes to total erythrocytes were observed in the female test article treated groups relative to the respective vehicle control groups. No significant increases in micronucleated polychromatic erythrocytes in test article-treated groups relative to the respective vehicle control groups were observed in males or females at 24 or 48 hours after dose administration. All criteria for a valid test were met and the vehicle and positive controls were consistent with historical control data.

The results of the assay indicate that under the conditions of this study, a single oral dose of Reb A at doses up to and including 2000 mg/kg did not induce a significant increase in the incidence of micronucleated polychromatic erythrocytes in either male or female ICR mice. Reb A was concluded to be negative in the mouse micronucleus assay**.**

Table V.-7: Genotoxicity Studies – *In vitro* and *In vivo* Studies with Reb A

Assay & Test System	Purity	Endpoint & Maximum Test Concentration/Dose	Reference
In vitro Bacterial reverse mutation; *S. typhimurium* TA98, TA100, TA1535 and TA1537 and *E. coli* WP2 *uvr*A	99.5%	Not mutagenic with and without metabolic activation; Maximum concentration 5000 µg/plate	Wagner and Van Dyke, 2006
In vitro Bacterial forward mutation; *S. typhimurium* TM677	Not reported	Not mutagenic with and without metabolic activation; Maximum concentration 10 mg/mL	Pezzuto *et al*., 1985a
In vitro Bacterial forward mutation; *S. typhimurium* TM677	Not reported	Not mutagenic with and without metabolic activation; Test concentrations not reported	Medon *et al*., 1982
In vitro Gene mutation; Mouse lymphoma L5178Y/TK$^{+/-}$	99.5%	Not mutagenic with and without metabolic activation; Maximum concentration 5000 µg/mL	Clarke, 2006
In vivo Mouse micronucleus in bone marrow; ICR mice	99.5%	Negative; Maximum dose 2000 mg/kg	Krsmanovic and Huston, 2006

Stevioside

Several *in vitro* and one *in vivo* experiments have examined the genotoxicity of stevioside using *in vitro* bacterial and mammalian cell mutation, chromosomal aberration, sister chromatid exchange, and micronucleus assays, and an *in vivo* mouse micronucleus assay in bone marrow and hepatocytes. The doses and purity of the compound varied between studies (see Table V.-8), but these studies demonstrated that stevioside is not genotoxic (Matsui *et al*., 1996a; Suttajit *et al*., 1993; Klongpanichpak *et al*., 1997; Medon *et al*., 1982; Pezzuto *et al*., 1985a,b; Oh *et al*., 1999; Ishidate *et al*., 1984; Höhn and Zankl, 1990). Stevioside has also been evaluated using the *in vitro* comet assay (Nunes *et al*., 2007), however, these data are not considered to be relevant to the safety evaluation of Reb A because of the opinions of experts on the inadequacy of this study (Williams, 2007; Geuns, 2007).

Table V.-8: Genotoxicity Studies – *In vitro* and *In vivo* Studies with Stevioside

Assay & Test System	Purity	Endpoint & Maximum Test Concentration/Dose	Reference
In vitro Bacterial reverse mutation; *S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, TA1537 and *E.coli* WP2 *uvrA/*pkM101	83.2%	Not mutagenic with and without metabolic activation; Maximum dose 1 or 5 mg/plate with and without metabolic activation, respectively	Matsui *et al*., 1996a
In vitro Bacterial reverse mutation; *S. typhimurium* TA98, TA100	99%	Not mutagenic with and without metabolic activation; Maximum concentration 50 mg/plate[1]	Suttajit *et al*., 1993
In vitro Bacterial reverse mutation; *S. typhimurium* TA98, TA100	96%	Not mutagenic with and without metabolic activation (rat, mouse, hamster and guinea pig); Maximum concentration 50 mg/plate	Klongpanichpak *et al*., 1997
In vitro Bacterial forward mutation; *S. typhimurium* TM677	83.2%	Not mutagenic with and without metabolic activation; Maximum concentration 10 mg/mL	Matsui *et al*., 1996a
In vitro Bacterial forward mutation; *S. typhimurium* TM677	Not reported	Not mutagenic with and without metabolic activation; Test concentrations not reported	Medon *et al*., 1982
In vitro Bacterial forward mutation; *S. typhimurium* TM677	Not reported	Not mutagenic with and without metabolic activation; Maximum concentration 10 mg/mL	Pezzuto *et al*., 1985a
In vitro umu Gene mutation; *S. typhimurium* TA1535/pSK1002	83.2%	Not mutagenic with and without metabolic activation; Maximum concentration 5 mg/mL	Matsui *et al*., 1996a
In vitro rec Gene mutation; *B. subtilis* H17 rec+, M45 rec-	83.2%	Not mutagenic with and without metabolic activation; Maximum concentration 10 mg/disc	Matsui *et al*., 1996a
In vitro Gene mutation; Mouse lymphoma L5178Y/TK$^{+/-}$	Not reported	Not mutagenic; Maximum concentration 5 mg/mL	Oh *et al*., 1999

[1]A weakly positive response towards TA98 was seen without metabolic activation at 50 mg/plate but not at lower concentrations up to 25 mg/plate.

Assay & Test System	Purity	Endpoint & Maximum Test Concentration/Dose	Reference
In vitro Chromosomal aberration; Chinese hamster lung fibroblasts	83.2%	Not mutagenic with and without metabolic activation; Maximum concentration 12 and 8 mg/mL with and without metabolic activation, respectively	Matsui *et al*., 1996a
In vitro Chromosomal aberration; Chinese hamster lung fibroblasts	85%	Not mutagenic without metabolic activation; Maximum concentration 12 mg/mL	Ishidate *et al*., 1984
In vitro Chromosomal aberration; Human lymphocytes	99%	Not mutagenic; Maximum concentration 10 mg/mL	Suttajit *et al*., 1993
In vitro Chromosomal aberration, sister chromatid, and micronucleus; Human lymphocytes	Not reported	No effect on chromosome breaks or sister chromatid exchanges, but micronucleus rate increased; Maximum concentration 10 µM	Höhn and Zankl, 1990
In vitro Human micronucleus; Buccal mucosa cells	Not reported (Stevia sweet)	Micronucleus rate increased in exfoliated cells 3-4 days after treatment (volunteers rinsed several times with Stevia sweet); Test concentrations not reported	Höhn and Zankl, 1990
In vivo Mouse micronucleus in bone marrow and hepatocytes; ddY mice	Not reported	Negative; no increase in micronucleus rate; Maximum dose 250 mg/kg	Oh *et al*., 1999

Steviol

A number of *in vitro* and *in vivo* experiments have examined the genotoxicity of steviol using *in vitro* bacterial and mammalian cell mutation (Matsui *et al*., 1996a,b; Klongpanichpak *et al*., 1997; Pezzuto *et al*., 1985a,b; Pezzuto *et al*., 1986; Terai *et al*., 2002; Compadre *et al*., 1988; Procinska *et al*., 1991; Oh *et al*., 1999), chromosomal aberration (Matsui *et al*., 1996a; Suttajit *et al*., 1993), and *in vivo* micronucleus assays (Oh *et al*., 1999; Matsui *et al*., 1996a; Temcharoen *et al.,* 2000). The doses and purity of the compound varied between studies (see Table V.-9). In the absence of metabolic activation *in vitro*, steviol was not genotoxic. However, in the presence of a metabolic activating system, steviol causes mutations in the Ames assay and chromosomal

aberrations in Chinese hamster lung fibroblasts. In the *in vivo* mouse micronucleus assay, chromosomal damage was not observed, although cytotoxicity and increased lethality were noted.

JECFA (1999) noted that steviol was clearly genotoxic after metabolic activation *in vitro* and concluded at that time that the mutagenic potential of steviol had been tested sufficiently only *in vitro*. In the more recent JECFA monograph (2006), after review of new studies of DNA damage and micronucleus formation in rats, mice and hamsters *in vivo*, the Committee concluded that genotoxicity of steviol is not expressed at doses of up to 8000 mg/kg bw.

Table V.-9: Genotoxicity Studies – *In vitro* and *In vivo* Studies with Steviol and Steviol Metabolites

Assay & Test System	Purity	Endpoint & Maximum Test Concentration/Dose	Reference
In vitro Bacterial reverse mutation; *S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535 and TA1537 and *E. coli* WP2 *uvrA*/pkM101	99%	Not mutagenic with and without metabolic activation; Maximum concentration 5 mg/plate	Matsui *et al*., 1996a,b
In vitro Bacterial reverse mutation; *S. typhimurium* TA98 and TA100	96%	Not mutagenic with and without metabolic activation (rat, mouse, hamster and guinea pig); Maximum concentration 2 mg/plate	Klongpanichpak *et al*., 1997
In vitro Bacterial reverse mutation; *S. typhimurium* TA98 and TA100	From hydrolysis of 99% stevioside	Not mutagenic with and without metabolic activation; Maximum concentration 20 mg/plate	Suttajit *et al*., 1993
In vitro Bacterial forward mutation; *S. typhimurium* TM677	99%	Mutagenic with metabolic activation, not mutagenic without metabolic activation; Maximum concentration 10 mg/mL	Matsui *et al*., 1996a,b
In vitro Bacterial forward mutation; *S. typhimurium* TM677	"Pure" (purity not specified)	Mutagenic with metabolic activation; not mutagenic without metabolic activation; Maximum concentration 10 mg/mL	Pezzuto *et al*., 1985a,b
In vitro Bacterial forward mutation; *S. typhimurium* TM677	Not reported	Mutagenic with metabolic activation; Maximum concentration 10 mg/mL	Pezzuto *et al*., 1986

Assay & Test System	Purity	Endpoint & Maximum Test Concentration/Dose	Reference
In vitro Bacterial forward mutation; *S. typhimurium* strain TM677	"Pure" (purity not specified)	Mutagenic with metabolic activation, not mutagenic without metabolic activation; Maximum concentration 2 mg/mL	Terai *et al*., 2002
In vitro Bacterial forward mutation; *S. typhimurium* strain TM677	Not reported[1]	Not mutagenic with metabolic activation; Maximum concentration 7.5 mg/mL	Compadre *et al*., 1988
In vitro Bacterial forward mutation; *S. typhimurium* strain TM677	Not reported[2]	Mutagenic without metabolic activation; Maximum concentration 0.20 mg/mL	Compadre *et al*., 1988
In vitro Bacterial forward mutation; *S. typhimurium* TM677	Not reported[2]	Not mutagenic without metabolic activation; Maximum concentration 1.22 mg/mL	Procinska *et al*., 1991
In vitro Bacterial forward mutation; *S. typhimurium* TM677	Not reported[3]	Mutagenic with metabolic activation; Maximum concentration 15 mg/mL	Pezzuto *et al*., 1986
In vitro umu Gene mutation; *S. typhimurium* TA1535/pSK1002	99%	Weakly mutagenic with and without metabolic activation; Maximum concentration 2.5 mg/mL	Matsui *et al*., 1996a
In vitro rec Gene mutation; *B. subtilis* H17 rec+, M45 rec	99%	Not mutagenic with and without metabolic activation; Maximum concentration 10 mg/disc	Matsui *et al*., 1996a
In vitro Gene mutation; *pSV2*-gpt *plasmids with* xgprt	Not reported	Mutagenic with metabolic activation; Test concentrations not reported	Matsui *et al*., 1989
In vitro Gene mutation; Mouse lymphoma L5178Y/TK$^{+/-}$	Not reported	Not mutagenic; Maximum concentration 340 µg/mL	Oh *et al*., 1999
In vitro Gene mutation; Chinese hamster lung fibroblasts	99%	Mutagenic with metabolic activation, not tested without metabolic activation; Maximum concentration 400 µg/mL	Matsui *et al*., 1996a,b

[1] 15α-Hydroxysteviol

[2] 15-Oxosteviol

[3] 19- O-ß-D-Glucopyranosyl steviol

Assay & Test System	Purity	Endpoint & Maximum Test Concentration/Dose	Reference
In vitro Chromosomal aberration; Chinese hamster lung fibroblasts	99%	Positive with metabolic activation, negative without metabolic activation; Maximum concentration 1.5 mg/mL	Matsui *et al*., 1996a
In vitro Chromosomal aberration; Human lymphocytes	From hydrolysis of 99% stevioside	Negative; Maximum concentration 0.2 mg/mL	Suttajit *et al*., 1993
In vivo Mouse micronucleus in bone marrow and hepatocytes; ddY mice	Not reported	Negative; Maximum dose 200 mg/kg	Oh *et al*., 1999
In vivo Mouse micronucleus in bone marrow; MS/Ae mice	99%	Negative; Maximum dose 1000 mg/kg	Matsui *et al*., 1996a
In vivo micronucleus in bone marrow; mouse, rat and hamster	90%	Negative; Maximum doses 4000 mg/kg (hamster) and 8000 mg/kg (mouse and rat)	Temcharoen *et al.,* 2000

In summary, Reb A was not mutagenic with and without metabolic activation when tested *in vitro* in bacterial reverse mutation, bacterial forward mutation, and mammalian cell gene mutation assays. In addition, Reb A was negative in an *in vivo* mouse micronucleus assay in bone marrow. Stevioside was not mutagenic with and without metabolic activation in several experiments using *in vitro* bacterial and mammalian cell mutation, and negative in chromosomal aberration, sister chromatid exchange, and micronucleus assays. Also, stevioside did not increase the micronucleus rate in an *in vivo* mouse micronucleus test in bone marrow and hepatocytes. Steviol was not mutagenic *in vitro* without metabolic activation in bacterial cell mutation, mammalian gene cell mutation, and chromosomal aberration studies, but with metabolic activation steviol was positive in some of these assays. However, in three *in vivo* mouse, one hamster and one rat micronucleus assays, chromosomal damage was not observed at dose levels as high as 8000 mg/kg bw.

Reb A

Two-generation dietary reproduction toxicity study in rats

An oral (dietary) two generation reproduction toxicity study was conducted in rats using Chrysanta® 99-P (95.7% Reb A) as the test article. The effects of Reb A on male and female reproductive processes, including gonadal function, estrus cyclicity, mating behavior, conception, gestation, parturition, lactation and weaning and on growth and development of the offspring were determined (Sloter, 2008a [Unpublished]).

Four groups of male and female Crl:CD(SD) rats (30/sex/group) were offered either basal diet or the test article, Reb A, at doses of 0, 500, 1000 and 2000 mg/kg/day for 70 consecutive days prior to mating. F_0 animals were approximately 7 weeks of age at the initiation of test diet exposure. The test diet was offered to the F_1 generation following weaning (beginning on postnatal day [PND] 21). The F_0 and F_1 males continued to receive the test article throughout mating and continuing through the day of euthanasia. The F_0 and F_1 females continued to receive the test article throughout mating, gestation and lactation, and through the day of euthanasia. For both generations (F_1 and F_2), 8 pups per litter (4 per sex, when possible) were selected on PND 4 to reduce the variability among the litters. Offspring (30/sex/group) from the pairing of the F_0 animals were selected on PND 21 to constitute the F_1 generation; an additional 1 pup/sex/litter was selected from 25 litters/test article-treated group and 4 litters in the control group on PND 21 for evaluation of toxicokinetic parameters (plasma, fecal and urine sample analysis). F_0 and F_1 males and females constituting the respective generations were each offered the test diet for 127 to 130 consecutive days; the F_1 animals in the toxicokinetic phase were offered the test diet until euthanasia following the final blood collection at 6 or 12 weeks of age.

Vaginal lavages were performed daily for determination of estrus cycles beginning 21 days prior to pairing. All F_0 and F_1 females were allowed to deliver and rear their pups until weaning on lactation day 21. Clinical observations, body weights and sexes for F_1 and F_2 pups were recorded at appropriate intervals. Developmental landmarks (balanopreputial separation and vaginal patency) were evaluated for the selected F_1 rats. Nonselected F_1 and F_2 pups were necropsied on PND 21; selected organs from 1 pup/sex/litter were weighed. Each surviving F_0 and F_1 parental animal received a complete detailed gross necropsy following the completion of weaning of the F_1 and F_2 pups, respectively; selected organs were weighed. Spermatogenic endpoints (sperm motility, morphology and numbers) were recorded for all F_0 and F_1 males, and ovarian primordial follicle counts and corpora lutea counts were recorded for 10 F_0 and F_1 females in the control and high-exposure groups. Designated tissues from 10 F_0 and F_1 parental animals/sex/group were examined microscopically.

Additional details of the study methodology and the average quantities of test article consumed during the F_0 and F_1 generations are provided in the complete robust summary found in Appendix V.-4 (entitled Two-Generation Dietary Reproduction Toxicity Study in Rats).

F_0 and F_1 parental survival was unaffected by test diet administration at all exposure levels. No remarkable clinical findings were noted for F_0 or F_1 males and females in the 500, 1000 and 2000 mg/kg/day groups.

In the 2000 mg/kg/day group, mean body weight gains were statistically significantly lower for F_0 males during the pre-mating period (study weeks 0-10) and immediately following breeding (study week 12-13), and for F_1 males when the entire generation (study weeks 18-36) was evaluated. As a result, mean body weights in the F_0 and F_1 males were slightly lower than the control group during study weeks 7-18 and 28-36, respectively. These lower mean body weights were generally statistically significant in the F_0 males but not significant in the F_1 males. The authors concluded that the slight reduction in male body weight at 2000 mg/kg/day was due to the proportion of the basal

diet containing the non-nutritive test article; therefore, the lower male body weights were considered test article-related, but not adverse. Mean body weights, body weight gains and cumulative body weight gains in F_0 and F_1 males in the 500 and 1000 mg/kg/day groups and in F_0 and F_1 females in the 500, 1000 and 2000 mg/kg/day groups were unaffected by test diet exposure during the entire generation (males) and during the pre-mating, gestation and lactation periods (females).

Due to the non-nutritive nature of Reb A, mean food efficiency in the F_0 males was generally lower than the control group during the pre-mating period in the 1000 and 2000 mg/kg/day groups, and resulted in higher mean food consumption (primarily on g/kg/day basis). Mean food consumption and food efficiency in the F_0 males in the 500 mg/kg/day group were unaffected by test article administration prior to mating. Following the mating period, mean food consumption and food efficiency in F_0 males were similar to the control group in the 500, 1000 and 2000 mg/kg/day groups. In the F_1 males, mean food consumption and food efficiency in the 500, 1000 and 2000 mg/kg/day groups were generally similar to the control group. Therefore, the transient increase in food consumption affecting only the F_0 males was not considered to be toxicologically significant.

Mean food consumption and food efficiency in the F_0 and F_1 females were comparable to the control group during the pre-mating period. However, as these females entered gestation and lactation, food consumption in the 500, 1000 and/or 2000 mg/kg/day groups was higher than the control group values. These increases in mean food consumption were considered test article-related, but not adverse due to the non-nutritive nature of Reb A and increased demand for calories during pregnancy and nursing. Food efficiency in F_0 females in all test article-exposed groups and F_1 females in the 1000 and 2000 mg/kg/day groups was generally lower than the control group during gestation; the lower food efficiency was attributed to the lower caloric intake in these groups. Food efficiency during lactation was unaffected by test diet exposure in both generations.

As discussed in this Safety Evaluation (see Section V.A.2.b), decreases in food efficiency data, body weight and/or body weight gain compared to controls are observed in other studies at the highest dietary dose levels of Reb A or stevioside tested in rats. These changes are reported for other high intensity sweeteners when the diet contains such large amounts of a non-nutritive sweetener (Flamm *et al*., 2003).

No test article-related effects were observed on F_0 and F_1 reproductive performance (estrus cycles, mating, fertility, copulation or conception indices, the mean number of days between pairing and coitus and the mean length of gestation), parturition or the mean numbers of implantation sites and unaccounted-for sites. Spermatogenic endpoints (mean testicular and epididymal sperm numbers, sperm production rate, motility and the percentage of morphologically normal sperm) were also unaffected by test diet exposure in both generations.

There were no test article-related macroscopic findings or changes in mean organ weights in the F_0 or F_1 males and females in the 500, 1000 and 2000 mg/kg/day groups. Microscopic evaluation of the F_0 and F_1 males and females revealed no test article-related histopathological lesions. No test article-related effects on primordial follicle or corpora lutea counts were observed in the F_0 and F_1 females in the 2000 mg/kg/day group.

No test article-related effects were observed on the mean numbers of F_1 or F_2 pups born, the pup sex ratio, pup survival, or the general physical condition of the pups during the pre-weaning period. Mean body weight gains in F_1 male and female pups in the 1000 and 2000 mg/kg/day groups and F_2 male and female pups in the 2000 mg/kg/day group were lower than the control group at the end of the postnatal period (PND 14-21). The authors concluded that these reductions in mean body weight gains were the result of the pups replacing their consumption of milk from nursing with the non-nutritive Reb A containing test diet just prior to weaning.

No test article-related macroscopic findings were observed in F_1 or F_2 pups. There were no test article-related effects on F_1 or F_2 pup organ weights on PND 21. Decreases in

spleen weights noted in the F_1 and F_2 pups at 2000 mg/kg/day were not considered adverse as decreases were not observed in similarly exposed F_0 or F_1 adults, there were no histopathological correlates observed, and pup spleen weights were comparable to control values in the laboratory's historical control database. The mean day of acquisition of balanopreputial separation or vaginal patency, and mean body weights on the day of acquisition in the F_1 test article-exposed pups were not affected by test article exposure.

There were no effects on reproduction (estrus cycles, mating, fertility, conception or copulation indices, number of days between pairing and coitus, gestation length, and spermatogenic endpoints). The only indications of parental toxicity, slight effects on mean body weights and body weight gains in F_0 and F_1 males at 2000 mg/kg/day and on mean food consumption and food efficiency in F_0 males at all exposure levels, were not considered by the study authors to be adverse due to the magnitude of change and may have been the result of the amount of basal diet that was replaced with the non-nutritive test article rather than a direct action of the test article itself. As the offspring of the F_0 and F_1 generations began to consume less milk from nursing and more of the test diet containing the non-nutritive Reb A, reductions in mean body weight gains (PND 14-21) were noted in male and female F_1 pups at 1000 and 2000 mg/kg/day and in male and female F_2 pups at 2000 mg/kg/day. As noted for the parental animals, and as noted for the 90-day study of Reb A with rats (Nikiforov and Eapen, 2008), these body weight changes were not considered to be adverse or appropriate for setting a NOAEL (see Section V.A.2.b).

Therefore, an exposure level of at least 2000 mg/kg/day was considered to be the no-observed-adverse-effect level (NOAEL) for parental systemic and reproductive toxicity, and an exposure level of at least 2000 mg/kg/day was considered to be the NOAEL for neonatal toxicity of Reb A when administered continuously in the diet to Crl:CD(SD) rats.

Oral (gavage) embryo/fetal developmental toxicity study in rats

An oral (gavage) embryo/fetal developmental toxicity study was conducted in rats using Chrysanta® 99-P (95.7% Reb A) as the test article to determine Reb A's impact on developmental toxicity after maternal exposure during organogenesis, to characterize maternal toxicity at the exposure levels tested and to determine a no-observed-adverse-effect level (NOAEL) for developmental toxicity (Sloter, 2008b [Unpublished]).

Reb A was administered orally by gavage to 3 groups of 25 bred female Crl:CD(SD) rats from gestation days 0 through 20. The animals were dosed twice daily (approximately 4 hours apart). Total daily dosage levels were 500, 1000 and 2000 mg/kg/day. Each of the 2 daily doses was administered at a dosage volume of 10 mL/kg. Clinical observations, body weights and food consumption were recorded daily. On gestation day 21, a laparohysterectomy was performed on each female. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations. Additional details of the study are provided in the complete robust summary found in Appendix V.-5 (entitled Oral (Gavage) Embryo/Fetal Developmental Toxicity Study in Rats).

All females in the control, 500, 1000 and 2000 mg/kg/day groups survived to the scheduled necropsy on gestation day 21. White discoloration of the feces was noted on at least 1 occasion for 2 females in the 1000 mg/kg/day group and the majority of the females in the 2000 mg/kg/day group during gestation days 7-21; this finding was attributed to the test article but was not considered adverse by the study authors. No test article-related clinical findings were noted in the 500 mg/kg/day group. No test article-related macroscopic findings were noted at any dosage level. The only internal finding, dark red uterine contents, was noted for 1 female in the 2000 mg/kg/day group and was not attributed by the study authors to the test article.

Mean food consumption, evaluated as g/kg/day, was slightly higher than the control group during gestation days 6-9, 9-12 and 12-13 in the 1000 and 2000 mg/kg/day groups and gestation days 3-6 and 0-21 in the 2000 mg/kg/day group with most of these differences achieving statistical significance at $p<0.05$ or $p<0.01$. No corresponding effects on body weight were observed. These increases in food consumption were small in magnitude and did not occur in a dose-related pattern; therefore, the differences in food consumption were not considered by the study authors to be test article-related. Mean body weights, body weight gains, net body weights, net body weight gain and mean gravid uterine weight were unaffected by test article administration at all dosage levels.

Intrauterine growth and survival were unaffected by test article administration at any dose level. There were no test article-related fetal malformations or developmental variations at any dosage level.

In the absence of maternal or developmental toxicity in this study, a total daily dosage level of at least 2000 mg/kg/day, the highest dosage level evaluated, was determined by the study authors to be the NOAEL for maternal and embryo/fetal developmental toxicity when Reb A was administered orally, twice daily by gavage, to pregnant Crl:CD(SD) rats.

Stevioside

Yodyingyuad and Bunyawong (1991) conducted a three-generation reproduction study in golden hamsters. Stevioside (90% purity) was orally administered by gavage to groups of 10 male and 10 female hamsters at doses of 0, 500, 1000, and 2500 mg/kg/day. Subsequently, during late gestation and the lactation period, stevioside was administered in the drinking water. Each female was mated and allowed to produce three litters over the course of the study. F_1 and F_2 generation hamsters also received stevioside by oral administration after weaning. No treatment-related effects on the duration of gestation, number of fetuses, or litter size were noted. F_1 and F_2 generation animals also exhibited normal growth and development, although statistically significant transient decreases in

body weights were observed in both sexes. Histological examination of reproductive organs and tissues did not reveal treatment-related abnormalities. In summary, no treatment-related adverse effects were noted in growth or reproduction with doses of stevioside as high as 2500 mg/kg/day.

Mori *et al.* (1981) treated male rats for 60 days before and during mating, and female rats for 14 days before mating and 7 days during gestation, with doses of 0.15, 0.75 and 3.0% stevioside (purity 96%) in the diet, equivalent to 0, 150, 750, and 3000 mg/kg/day. There were no statistically significant effects noted at any treatment level in mating performance or fertility, and no developmental malformations noted in fetuses. However, males and females at the 3% dose level showed slight decreases of body weight gain during the early period of dose administration.

In a developmental toxicity study, pregnant Wistar rats were administered stevioside (purity 95.6%) dissolved in distilled water via oral gavage during days 6-15 of gestation, at doses of 0, 250, 500, or 1000 mg/kg/day. Endpoints examined were maternal and fetal body weights, litter size, litter sex distribution, number of resorptions or dead fetuses, and fetal malformations. Results showed that stevioside was not a teratogen at any level of exposure and that stevioside was not a hazard to the rat conceptus (Takanaka *et al.,* 1991, as cited in JECFA, 1999; Usami *et al.,* 1995).

Steviol

Pregnant golden hamsters were treated by oral gavage with steviol (purity 90%) in corn oil at doses of 0, 250, 500, 750, and 1000 mg/kg body weight on gestation days 6–10. A significant decrease in weight gain of both the dam and fetus, an increase in mortality of the dam, and a decrease in the number of live fetuses per litter was seen at the three highest doses. There was no effect noted on corpora lutea and implantations in any treatment group. Histopathological examination of the dams revealed a dose-dependent effect on the kidneys (i.e., dilation and desquamation of the epithelial cells of the proximal and distal convoluted tubules). Given the maternal and fetal toxicity noted at 500 mg/kg/day and above, NOAELs of 250 mg/kg/day for maternal toxicity and 1000 mg/kg/day for developmental toxicity were established. However, no teratogenic

effects were seen and the results indicate general renal toxicity as the critical endpoint in hamsters rather than developmental toxicity (Wasuntarawat *et al.,* 1998). In contrast, a developmental toxicity study conducted with Reb A in rats did not indicate maternal renal toxicity (Sloter, 2008b [Unpublished]). Additionally, since the amount of steviol absorbed from the hydrolysis of orally administered Reb A at 2000 mg/kg bw/day is extremely low (see ADME, Section V.A.1), the levels of steviol causing renal toxicity in the hamster study could not be achieved following Reb A dosing via oral administration.

Three reproductive toxicity studies conducted with aqueous extracts of *S. rebaudiana* are not considered relevant to the safety evaluation of Reb A due to the undefined nature of the test materials and inadequate study designs (Mazzei Planas and Kuc, 1968; Oliveira-Filho *et al.,* 1989; Melis, 1999).

Table V.-10: Reproductive/Developmental Toxicity Studies

Species	Compound	Route	NOAEL and Endpoint	Reference
Studies on Reb A				
Crl:CD(SD) rats (30/sex/group)	Reb A (Purity: 95.7%)	Oral (diet)	NOAEL 2-Generation reproductive toxicity: ▪ Parental systemic ≥ 2000 mg/kg/day ▪ Parental reproductive ≥ 2000 mg/kg/day ▪ Neonatal toxicity ≥ 2000 mg/kg/day	Sloter, 2008a [Unpublished]
Crl:CD(SD) rats (25/group; pregnant)	Reb A (Purity: 95.7%)	Oral (gavage)	NOAEL Developmental toxicity (dosed GD 0–20): ▪ Maternal toxicity ≥ 2000 mg/kg/day ▪ Developmental toxicity ≥ 2000 mg/kg/day	Sloter, 2008b [Unpublished]
Studies on Stevioside				
Golden hamster (10/sex/group)	Stevioside (Purity: 90%)	Oral (gavage and drinking water)	NOAEL 3-Generation reproductive toxicity ≥ 2500 mg/kg/day Neither growth nor reproduction in hamsters was affected by test article administration.	Yodyingyuad and Bunyawong, 1991
Wistar rat (11/sex/group)	Stevioside (Purity: 96%)	Oral (diet)	NOAEL Reproductive toxicity ≥ 3000 mg/kg/day (3%) Stevioside had no adverse effect on fertility or on the development of fetuses.	Mori *et al.,* 1981
Wistar rat (25-26/group; pregnant)	Stevioside (Purity: 95.6%)	Oral (gavage)	NOAEL Developmental toxicity (dosed GD 6-15): ▪ Maternal toxicity ≥ 1000 mg/kg/day ▪ Developmental toxicity ≥ 1000 mg/kg/day Orally administered stevioside is not teratogenic in rats.	Takanaka *et al.,* 1991, as cited in JECFA, 1999; Usami *et al.,* 1995

Species	Compound	Route	NOAEL and Endpoint	Reference
Chicken embryos (55/group)	Stevioside (Purity: >96%)	Injection into egg yolk	NOAEL = 4 mg/egg (0.3 mM) Stevioside is not toxic to chicken embryos	Geuns *et al.,* 2003b
Studies on Steviol				
Golden hamster (12-20/group; pregnant)	Steviol (Purity: 90%)	Oral (gavage)	NOAEL Developmental toxicity (dosed GD 6-10): ▪ Maternal toxicity NOAEL=250 mg/kg/day ▪ Developmental toxicity NOAEL ≥ 1000 mg/kg/day Maternal nephrotoxicity was evident; however, no dose-dependent teratogenic effects were observed.	Wasuntarawat *et al.,* 1998;
Chicken embryos (55/group)	Steviol (Purity: >98%)	Injection into egg yolk	NOAEL = 1.25 mg/egg (0.25 mM) The authors concluded that steviol is not toxic to chicken embryos	Geuns *et al.,* 2003b
Studies on *S. rebaudiana* Extracts				
Dispert albino rat (14 females/ group)	Decoction of 5 g dry *S. rebaudiana* in 100 mL water	Oral (water decoction)	NOAEL not applicable. Fertility was reduced to 21% of that of control rats and remained reduced (28-43% of controls) after a 50- to 60-day recovery period. Test material not characterized.	Mazzei Planas and Kuc, 1968
Wistar rat (20 males)	Aqueous *S. rebaudiana* extracts (0.67 g/mL dried leaves)	Oral (gastric tubing)	NOAEL not applicable. Seminal vesicle weight fell by approximately 60%. Authors concluded that if the Stevia extract can decrease fertility in rats, the effect is almost certainly not exerted on males. Test material not characterized.	Oliveira-Filho *et al.,* 1989

Species	Compound	Route	NOAEL and Endpoint	Reference
Wistar rat (20 males)	Aqueous *S. rebaudiana* extract (0.67 g/mL dried leaves)	Oral (gastric tubing)	NOAEL not applicable. Significantly decreased relative testis, cauda epididymidis and seminal vesicle weight. Significantly decreased spermatozoa concentration and testosterone levels. Test material not characterized.	Melis, 1999

In summary, Reb A and stevioside were not reproductive toxicants in multigeneration studies with rats or golden hamsters nor were they developmental toxicants in studies with rats. Steviol was not a developmental toxicant in golden hamsters, although it was maternally toxic in golden hamsters.

As discussed in this Safety Evaluation (see Section V.A.2.b), decreases in food efficiency data, body weight and/or body weight gain compared to controls are observed in other studies at the highest dietary dose levels of Reb A or stevioside tested in rats. These changes are reported for other high intensity sweeteners when the diet contains such large amounts of a non-nutritive sweetener (Flamm *et al*., 2003), and are not considered an appropriate basis for assigning a no-observed-adverse-effect level (NOAEL).

Effects on Blood Pressure

Reb A

Two groups of diabetic Goto-Kakizaki male rats (12/group) were orally administered Reb A (97.8% purity) at 25 mg/kg bw/day in the diet for eight weeks. Systolic blood pressure was measured weekly by an automated system with the animals placed in a restrainer. Animals were acclimated to the restrainer prior to study initiation. Systolic blood pressure decreased in both groups during the eight week period, however there was no difference in blood pressure in the treated group as compared to controls (Dyrskog *et al.,* 2005a).

Stevioside

Oral administration of stevioside (91% stevioside, 4% Reb A, 5% other glycosides) in the obese type 2 diabetic Zucker fatty rat model at 30 mg/kg bw/day significantly ($P<0.01$) reduced systolic blood pressure during eight weeks of treatment, resulting in a mean blood pressure 14 ± 3 mm Hg lower than blood pressure measured before treatment (Dyrskog *et al.,* 2005b).

Jeppesen *et al.* (2003) studied the effects of stevioside on nonhypertensive, diabetic Goto-Kakizaki rats. Stevioside (>99.6% purity) was administered orally at a dosage of 25 mg/kg/day for 6 weeks and blood pressure was measured weekly. A significant decrease in blood pressure was seen in the treated group (systolic/diastolic: 153 ± 5/83 ± 1 mm Hg in the control group vs. 135 ± 2/74 ± 1 mm Hg in the stevioside group ($P < 0.001$). Stevioside caused a progressive lowering of blood pressure from week 1 and onward.

Liu *et al.* (2003) studied the antihypertensive effect of stevioside in anesthetized dogs. A single nasogastric or intravenous dose of 200 mg/kg or 50 mg/kg of stevioside, respectively, was administered to anesthetized mongrel dogs and blood pressure was

measured. Both routes of administration produced a reduction in blood pressure; however, only the nasogastric route is discussed because of its relevance to oral intake. In anesthetized dogs receiving the nasogastric administration of stevioside, systolic blood pressure, diastolic blood pressure and mean arterial pressure were monitored prior to administration and every 30 minutes for 180 minutes following administration. All three measures of blood pressure were reduced significantly ($P<0.05$ or $P<0.01$; mean values 13-16 mm Hg lower than baseline blood pressure) at 60, 90 and 120 minutes but had returned to baseline levels by 180 minutes.

Effects on Renal Function

Studies conducted with aqueous extracts of *S. rebaudiana* are not considered relevant to the safety evaluation of Reb A due to the undefined nature of the test materials and inadequate study designs; however, two studies of the effects on renal function conducted with stevia extracts are briefly summarized below since other data for steviol glycosides are not available for this endpoint.

Stevia extracts

Wistar rats were treated with extracts of dried Stevia rebaudiana leaves (Melis, 1995). Three groups of 10 rats each were treated with the extract by gavage for either 20, 40 or 60 days. Each rat received the extract of 1.334 g of dried leaves twice daily. Additionally, three groups of 10 rats received saline on the same schedule. At the end of each testing period, glomerular filtration rate and renal plasma flow were assessed. No significant differences in mean arterial pressure or renal function parameters were seen between control and treated groups after 20 days. No significant differences were seen in glomerular filtration rate or renal plasma flow; however, urine flow as a function of glomerular filtration rate and fractional urinary sodium excretion were both significantly increased in the treated group. Similarly, at 60 days, urine flow as a function of glomerular filtration rate and fractional urinary sodium excretion were both significantly increased in the treated group. Renal plasma flow was also significantly increased in the treated group; no significant changes were seen in glomerular filtration rate.

In a similar study, normal Wistar rats and Wistar rats with experimentally induced renal hypertension were treated with extracts of dried Stevia rebaudiana leaves (Melis, 1996). Experimental hypertension was induced by placing a silver clip on the left renal artery. There were four experimental groups of 10 rats each: normal untreated (control N) rats, hypertensive untreated (control H) rats, normal treated (stevia N) rats and hypertensive treated (stevia H) rats. For 30 days, animals were given either saline (control group) or the extract of 1.334 g of dried leaves (stevia group) twice daily via gavage. In both treated groups, renal plasma flow was significantly increased. However, glomerular filtration rate was only significantly increased in hypertensive treated rats. Both treated groups showed significant increases in urine flow as a function of glomerular filtration rate, fractional urinary sodium excretion and fractional urinary potassium excretion.

Effects on Blood Glucose Concentrations

Reb A

In an *in vivo* study, oral administration of Reb A at 25 mg/kg bw/day in the Goto-Kakizaki rat (an established animal model for type 2 diabetes) did not affect plasma glucose, insulin, and glucagon levels during or after eight weeks of treatment (Dyrskog *et al.,* 2005a). In an *in vitro* study, it was reported that Reb A increased insulin secretion from mouse islets in a concentration-dependent fashion with the effects of Reb A on insulin secretion glucose-dependent at glucose concentrations >6.6 mmol/L. Subsequent to this study report, additional research has been conducted that has confirmed that steviol glycosides must be hydrolyzed by microflora in the gut before only steviol is absorbed (see Section V.A.1. ADME). Therefore, because Reb A is not systemically available following oral administration, the results of the *in vitro* study are not considered relevant.

Stevioside and steviol

A number of studies have been performed to investigate if stevioside and steviol had effects on blood glucose parameters in normal mice and diabetic rats. Lailerd *et al.* (2004) examined the effect of a single dose of oral stevioside (200 mg/kg or 500 mg/kg) on blood glucose in lean (insulin-sensitive) and obese (insulin-resistant) Zucker rats.

Rats were restricted to 4 g of chow after 6 p.m. on the night prior to the test, treated with stevioside via gavage at 8 a.m. the following morning, and two hours later administered a 1 g/kg glucose load by gavage. Blood was drawn at 0, 15, 30, 60 and 120 minutes after glucose administration. No changes were seen in the low dose group either during fasting or during the oral glucose tolerance test. In the high dose group, no significant differences were seen at fasting levels of plasma glucose, insulin or free fatty acids. During the oral glucose tolerance test, plasma insulin values were significantly reduced (compared to controls) at 15 and 30 minutes in the lean Zucker rats; plasma glucose was not significantly changed. In the obese Zucker rats, insulin levels were not affected but plasma glucose was reduced at 30 minutes, returning to background at 120 minutes.

Suanarunsawat and Chaiyabutr (1997) studied the effect of oral and intravenous administration of stevioside on glucose metabolism in non-diabetic rats. The plasma glucose level in animals fed stevioside (500-600 mg twice over 1.5 hours) was not different from that of controls. An increase in plasma glucose level was only noted in intravenously treated animals; however, this route of administration is not relevant to the safety evaluation of Reb A. Additionally, it is clear that stevioside is not systemically available after oral intake as it would be following intravenous administration, which explains these differing results.

Jeppesen *et al.* (2002) studied the effects of stevioside on nonhypertensive, diabetic Goto-Kakizaki rats. Stevioside (>99.6% purity) was administered orally at a dosage of 25 mg/kg/day for 6 weeks. At week 5 animals were catheterized so that blood samples could be drawn. At week six, blood samples were taken from 15 minutes prior to 180 minutes post bolus injection of glucose. Prior to glucose injection, there was no significant difference in plasma glucose or insulin levels in treated and control groups. After glucose injection, plasma glucose was decreased in the treated group relative to controls (IAUC: 985 ± 20 *vs.* 1,575 ± 21 mmol/L × 180 minutes, treated *vs*. control, $P < 0.05$). Plasma insulin (first phase) was also increased in the treated group relative to controls (IAUC: 343 ± 33 [stevioside] *vs.* 136 ± 24 [control] μU/mL insulin × 30 min, $P < 0.05$). A concomitant suppression of glucagon was also seen in the treated group.

Xili *et al.* (1992) administered stevioside (85% purity) in the diet of 45 male and 45 female Wistar rats at levels of 0, 0.2, 0.6, or 1.2% for two years. These levels were equivalent to doses of 0, 129, 368, and 749 mg/kg/day in males and 0, 146, 416, and 839 mg/kg/day in females, respectively. No treatment-related effects were observed in glucose levels, hematological, urinary, or clinical biochemical values at any stage of the study at any dose up to the highest level tested (1.2% in the diet).

In *in vitro* studies in islets and pancreatic β-cells, stevioside and steviol increased insulin secretion in the presence of glucose (Abudula *et al.,* 2004; Jeppesen *et al.,* 2000; Jeppesen *et al.,* 2002). Research has been conducted that has confirmed that steviol glycosides must be hydrolyzed by microflora in the gut before only steviol is absorbed (see Section V.A.1. ADME). Therefore, because stevioside is not systemically available following oral administration, the results reported for stevioside of are not considered relevant.

In summary, results for the 8-week study in diabetic Goto-Kakizaki rats with orally administered Reb A indicates that systolic blood pressure, plasma glucose, insulin, and glucagon levels were unaffected during and after treatment. In addition, the rat 90-day toxicity study with Reb A at dose levels up to 2000 mg/kg bw/day, plasma glucose levels were unaffected (Nikiforov and Eapen, 2008).

Orally administered stevioside may have effects on blood pressure. No consistent effects on plasma glucose and insulin levels were apparent based on differences in study designs (i.e. compound administered, test species, dose, time period of administration, time period of assay, disease state of animal and route of administration).

As previously discussed, the studies conducted with stevia extracts are not considered relevant to the safety evaluation of Reb A.

The effects of steviol glycosides on blood pressure and blood glucose have been studied in humans and are presented in Section V.B.

V. Safety Evaluation

B. Human Studies

Reb A alone has not been used in clinical studies evaluating blood pressure and glucose control. However, steviol glycosides have been studied in human volunteers following a variety of different protocols. Several studies have demonstrated that steviol glycosides are hydrolyzed in the gut to steviol by sequential removal of glucose units in animals and humans (Koyoma *et al*., 2003; Gardana *et al*., 2003; and Wingard *et al*., 1980). Therefore, the data for any steviol glycoside administered by the oral route is relevant to the safety evaluation of Reb A. However, it should be noted that some reported effects may be due to other substances in the crude extracts of stevia leaf that were used in some studies (for additional information see the ADME section of this document; V.A.1.). The type of test material is noted in each study summarized below.

In its review of steviol glycosides, JECFA (2006) specifically requested additional information on two endpoints: blood pressure and blood glucose. Therefore, those endpoints are specifically discussed in the sections below.

Most of the studies evaluated potential treatment-related effects on both blood pressure and blood glucose as well as other parameters. In order to facilitate comparisons of the results of all of the studies, the effects on blood pressure are discussed first, followed by a discussion of the effects on blood glucose. For each endpoint, the studies are organized by the duration of exposure to the test material. Each study is described under blood pressure effects and briefly re-summarized under glucose effects, where appropriate.

Effects of Reb A/Stevioside on Blood Pressure

The following studies examined the effect of stevioside treatment on the blood pressure of normal, hypertensive or mildly hypertensive subjects.

Single dose exposure:

Gregersen *et al.* (2004) administered commercial stevioside (91% of stevioside, 4% of Reb A, and 5% of other steviol glycosides) to twelve type 2 diabetic patients in an acute, paired crossover study design. Each subject received a test meal supplemented with one gram of stevioside or maize starch (control). Blood samples were drawn at regular intervals before and after test meal consumption and blood pressure was measured during the test meal. Systolic and diastolic blood pressures were not altered by an acute stevioside administration.

Haebisch (1992) evaluated the effect of oral dosing with crude stevia extract. The extract was 13.85% stevioside; however, other components were not identified or quantitated. Two oral doses were administered (equivalent to 28 or 111 mg stevioside) in an acute, placebo-controlled, double-blind study in 60 normal, healthy volunteers. Fifteen volunteers received the placebo; 25 individuals received the low dose and 25 the high dose regimen. The extract was given in capsule form at approximately 8-hour intervals. On the first day of the study all subjects had individual control tests; on the second day measurements of blood pressure and blood samples were taken in the fasting (catabolic) state (i.e. after an overnight fast) and approximately 1 hour after a meal (in the anabolic state). The authors concluded that there was a small antihypertensive effect but that the effects were within the normal physiological limits. The systolic and diastolic blood pressure was measured in both a supine and upright position. In the supine position, fasted (catabolic) phase systolic blood pressure was 109.3 $\pm$ 3.3 mm Hg in the placebo group, and 106.0 $\pm$ 3.0 mm Hg in the high dose group. The diastolic blood pressure was 77.0 $\pm$ 3.7 versus 73.2 $\pm$ 1.6 mm Hg in the placebo and high dose groups, respectively. Similar differences were seen in the upright position. Although statistically significant, the observed changes are small and not clinically significant. Similarly, after a meal, heart rate was slightly but significantly reduced in the upright but not supine position and systolic pressure was reduced in the supine and upright positions. The study authors concluded that there was no dose-response effect for any parameter tested in either position.

Short-term exposure:

Geuns *et al.* (2007) studied the metabolism of stevioside in 10 healthy subjects following oral administration of 250 mg stevioside (> 97% purity), 3x/day for 3 days. Systolic and diastolic blood pressure was measured at various time points before and after treatment on the third day of stevioside administration. No statistically significant difference in blood pressure was noted following stevioside treatment in comparison to control values.

Long-term exposure:

Two studies have evaluated the effect of orally administered stevioside (either 250 or 500 mg, 3x/day for 1 or 2 years) in hypertensive (antihypertensive medications were not taken during the study) or mildly hypertensive subjects and reported an antihypertensive effect. Both were multicenter, randomized double-blind, placebo-controlled trials with 100–200 subjects each conducted in China using Chinese subjects. The purity of the administered stevioside was not reported for either study. Both studies reported a reduction in systolic and diastolic blood pressure of 10–15 mm Hg. In the first study, the mean systolic blood pressure measurements were 166.5 $\pm$ 7.4 mm Hg pre-treatment and 152.6 $\pm$ 6.8 mm Hg post-treatment, respectively. The mean diastolic blood pressure measurements were 102.1 $\pm$ 4.0 mm Hg pre-treatment and 90.3 $\pm$ 3.6 mm Hg post-treatment (Chan *et al*., 2000). In the second study, the mean systolic blood pressure measurements were 150 $\pm$ 7.3 mm Hg pre-treatment and 140 $\pm$ 6.8 mm Hg post-treatment. The mean diastolic blood pressure measurements were 95 $\pm$ 4.2 mm Hg pre-treatment and 89 $\pm$ 3.2 mm Hg post-treatment (Hsieh *et al*., 2003). Neither study reported any significant treatment-related adverse effects (Chan *et al.,* 2000; Hsieh *et al.,* 2003). Although the differences are statistically significant the small magnitude of the changes are not likely to be clinically important. In addition, it should be noted that in hypertensive individuals this slight decrease in blood pressure would not be adverse.

Ferri *et al.* (2006) administered either a placebo or increasing doses of crude stevioside (mixture of stevioside and Reb A, concentrations not specified) to mildly hypertensive subjects. Baseline systolic blood pressures were 140 $\pm$ 13 mm Hg for the stevioside treatment group and 133 $\pm$ 12 mm Hg for the placebo group; baseline diastolic blood

pressure was 94 ± 8 mm Hg for both the placebo and treatment groups. Seven subjects received placebo and seven subjects received stevioside as follows: 3.75 mg/kg/day for 7 weeks followed by 7.5 mg/kg/day for 11 weeks followed by 15.0 mg/kg/day for 6 weeks for a total of 24 weeks of stevioside administration. Twice daily doses were administered in capsules. Blood pressure was measured biweekly. Systolic and diastolic blood pressure decreased ($p<0.05$) in both placebo and stevioside-treated subjects over the course of 24 weeks. The mean systolic blood pressure post-treatment was 124 ± 6 mm Hg for the placebo group and 123 ± 12 mm Hg for the stevioside treatment group; the mean diastolic blood pressure post-treatment was 82 ± 4 mm Hg for the placebo group and 84 ± 8 mm Hg for the treatment group. However, the decreases in blood pressure were not significantly different when the stevioside-treated and placebo groups were compared.

Jeppesen *et al.* (2006) conducted a randomized, placebo-controlled, double-blind, parallel long-term trial in which placebo or stevioside was administered at 500 mg, 3x/day, to fifty-five type 2 diabetic subjects on antihypertensive treatment for a period of 3 months. No treatment-related antihypertensive effect was noted in these subjects.

Barriocanal *et al.* (2008) conducted a 3-month, randomized, double-blind, placebo-controlled, parallel-design study employing type 2 diabetics (N=30), type 1 diabetics (N=16), and non-diabetics with normal to low normal blood pressure (N=30). A dose of 250 mg stevioside (>92% purity) was administered 3x/day for 3 months to half the subjects of each treatment group. The other half of the subjects received the placebo. Blood pressure was measured every two weeks during the study period. The results are presented in Tables V.-11, V.-12, and V.-13 below. There were differences in the baseline blood pressure values between the placebo and treatment group. Fortunately, each individual served as his own control with baseline and post-treatment measurements. Thus, the individuals in the stevioside treatment group's pre and post-treatment values are compared rather than placebo and treatment group. No clinically significant differences between groups were observed in systolic or diastolic blood pressure measurements during the treatment period or after 3 months of treatment. In

addition, within the group of subjects receiving stevioside, post-treatment systolic blood pressure and diastolic blood pressure measurements were not significantly different from baseline measurements.

Table V.-11: Characteristics of the Steviol Glycosides and Placebo Groups at Baseline and Post-treatment. Group 1 (Type 1 Diabetes). Values are Mean (±SD)

	Steviol glycosides (n = 8)		Placebo (n = 8)	
	Baseline	Post-treatment	Baseline	Post-treatment
BMI (kg/m^2)	23.2 (3.3)	23.1 (3.1)	22.4 (1.0)	22.4 (1.0)
24-h SBP (mmHg)	117.1 (6.6)[1]	115.9 (8.6)	108.3 (3.0)	105.7 (2.8)[2]
24-h DPB (mmHg)	72.6 (6.9)	68.9 (7.2)	70.7 (4.4)	69.7 (3.3)
Laboratory				
Glucose (mg/dl)	144.9 (95.1)	155.3 (78.3)	219.3 (74.1)	298.3 (58.8)[2]
HbA_{1c} (%)	7.1 (1.6)	7.3 (1.1)	8.2 (1.4)	8.3 (1.6)

SD = standard deviation
BMI = body mass index; 24-h SBP = 24-hour systolic blood pressure; 24-h DPB = 24-hour diastolic blood pressure; HbA_{1c} = glycated hemoglobin
[1] $p < 0.05$ versus placebo baseline
[2] $p < 0.05$ Post-treatment versus baseline (within treatment group)
Barriocanal *et al.* (2008)

Table V.-12: Characteristics of the Steviol Glycosides and Placebo Groups at Baseline and Post-treatment. Group 2 (Type 2 diabetes). Values are Mean (±SD)

	Steviol glycosides (n = 15)		Placebo (n = 15)	
	Baseline	Post-treatment	Baseline	Post-treatment
BMI (kg/m^2)	28.7 (3.4)	29.2 (2.9)	30.1 (3.3)	30.2 (3.5)
24-h SBP (mmHg)	127.3 (15.1)	124.3 (13.5)	127.9 (13.7)	124.9 (13.3)
24-h DPB (mmHg)	77.3 (9.1)	74.7 (8.3)	76.7 (5.6)	77.4 (9.5)
Laboratory				
Glucose (mg/dl)	151.2 (54)	133.8 (34.5)	131.3 (46.7)	118.9 (34.0)
Insulin (µIU/mL)	13.3 (15.3)	11.6 (11.1)	14.7 (10.2)	15.3 (9.6)
HbA_{1c} (%)	6.8 (1.2)	6.6 (1.1)	6.8 (1.6)	6.8 (1.0)

SD = standard deviation
BMI = body mass index; 24-h SBP = 24-hour systolic blood pressure; 24-h DPB = 24-hour diastolic blood pressure; HbA_{1c} = glycated hemoglobin
Barriocanal *et al.* (2008)

Table V.-13: Characteristics of the Steviol Glycosides and Placebo Groups at Baseline and Post-treatment. Group 3 (Non-diabetics with Normal/Low-normal BP). Values are Mean (±SD)

	Steviol glycosides (n = 13)		Placebo (n = 17)	
	Baseline	Post-treatment	Baseline	Post-treatment
BMI (kg/m^2)	22.9 (2.7)	23.2 (2.7)	24.4 (3.8)	24.5 (3.5)
24-h SBP (mmHg)	111.0 (8.9)	113.3 (10.8)	111.7 (10.4)	112.2 (11.9)
24-h DPB (mmHg)	69.9 (7.2)	69.8 (7.1)	68.8 (5.5)	69.9 (8.1)
Laboratory				
Glucose (mg/dl)	82.5 (6.6)	82.9 (7.8)	82.9 (10.2)	83.9 (7.0)
Insulin (µIU/mL)	4.2 (1.8)	4.9 (2.4)	8.4 (8.1)	8.7 (8.1)
HbA_{1c} (%)	5.3 (0.4)	5.6 (0.6)	5.3 (0.6)	5.4 (0.7)

SD = standard deviation
BMI = body mass index; 24-h SBP = 24-hour systolic blood pressure; 24-h DPB = 24-hour diastolic blood pressure; HbA_{1c}= glycated hemoglobin
Barriocanal *et al.* (2008)

In summary, the effects of stevioside on blood pressure appear to be somewhat inconsistent; perhaps because of differences in study design, purity of the test materials, dosing regimens, measurement technology, or duration of exposure or possibly differences in responses by different study populations (Asian versus Europeans and Latin Americans). The recent results in the study by Barriocanal *et al*. (2008) indicate that there is no evidence that stevioside administered to normotensive or hypotensive individuals would lower blood pressure to a degree that would be considered an adverse effect (FSANZ, 2007).

Effects of Reb A/Stevioside on Glucose Concentrations

The following studies examined the effect of stevioside treatment on plasma glucose and/or insulin levels of diabetic and non-diabetic subjects.

Single dose exposure:

Haebisch (1992) evaluated the effect of oral doses of crude extracts of stevia. The extracts were 13.85% stevioside (28 or 111 mg stevioside) as described above in the section on blood pressure effects. The study was an acute, placebo-controlled, double-blind study in normal, healthy volunteers. The authors reported decreased ($p<0.05$) plasma glucose in the high dose group. Although statistically significant, the observed changes are small and not clinically significant, i.e. 71.4 ± 2.0 for placebo, 75.1 ± 2.2 for low-dose and 67.8 ± 2.9 for high-dose (catabolic phase) and 71.8 ± 2.6 for placebo, 73.1 ± 3.8 for low-dose and 68.3 ± 3.0 for high-dose (anabolic phase).

Short-term exposure:

Geuns *et al.* (2007) studied the metabolism of stevioside in 10 healthy subjects following oral administration of 250 mg stevioside, 3x/day for 3 days. Plasma glucose and insulin levels were measured at various time points before stevioside administration and up to 7 hours following treatment. No statistically significant difference in fasting plasma glucose or insulin levels was noted following control or stevioside treatment.

Curi *et al.* (1986) evaluated the effect of orally administered stevia extracts on glucose concentrations in 16 non-diabetic individuals. Subjects were given aqueous extracts of approximately 5 grams of *S. rebaudiana* leaves (uncharacterized) at 6-hour intervals for 3 days. Glucose tolerance tests were performed before and after administration (a dose had been administered 2 hours before the glucose tolerance test). The extract increased glucose tolerance and significantly decreased plasma glucose concentrations during the test and after overnight fasting (Curi *et al.,* 1986).

The effects of stevioside on type 2 diabetic subjects were evaluated in a randomized, crossover study, 12 diabetic subjects were given either a single dose of 1 g of stevioside (91% stevioside, 4% Reb A) or control with a meal on the first test day, and the other treatment on the second test day one week later. Measurements were made from 30 minutes prior to dosing to 240 minutes post-dosing. The authors found an average 18% decrease in postprandial blood glucose response concentrations as compared to placebo

over the 4 hours of testing post-dosing, a 40% increase in the insulinogenic index and a 20% reduction in serum glucagon concentrations in treated subjects (Gregersen *et al*., 2004). These effects are unlikely to be related to stevioside or Reb A exposure since neither substance would be hydrolyzed or absorbed as steviol in this short time period.

Long-term exposure:

Ferri *et al.* (2006) administered either a placebo or increasing doses of stevioside to mildly hypertensive subjects for a period of 24 weeks. As described in greater detail in the blood pressure effects section, the subjects received either placebo or stevioside orally as follows: 3.75 mg/kg/day for 7 weeks followed by 7.5 mg/kg/day for 11 weeks followed by 15.0 mg/kg/day for 6 weeks for a total of 24 weeks of stevioside administration. Blood samples were collected after each phase of treatment and plasma glucose and insulin levels determined. Plasma glucose and insulin levels decreased ($p<0.05$) in both placebo and stevioside-treated mildly hypertensive subjects after the final phase of the study (week 24).

Two studies evaluated the effect of oral stevioside (either 250 or 500 mg, 3x/day for 1 or 2 years) in hypertensive or mildly hypertensive, non-diabetic subjects and reported no effect on plasma glucose levels. Both were multicenter, randomized double-blind, placebo-controlled trials with 100–200 subjects each. Neither study characterized the purity of the administered stevioside. Blood samples were collected at each monthly visit during the course of the study. Neither study found a significant change in fasting plasma glucose levels during the course of the study (Chan *et al.,* 2000; Hsieh *et al.,* 2003).

Barriocanal *et al.* (2008) conducted a 3-month, randomized, double-blind, placebo-controlled, parallel-design study employing type 2 diabetics (n=30), type 1 diabetics (N=16), and non-diabetics with normal to low normal blood pressure (n=30). A dose of 250 mg stevioside ((>92% purity) was administered 3x/day for 3 months to half the subjects of each treatment group. The other half of the subjects received the placebo. Plasma glucose and insulin levels were measured before and after the three-month study period. As noted above, there were differences in the baseline values between the placebo

and treatment group. Fortunately, each individual served as his own control with baseline and post-treatment measurements. Thus, the individuals in the stevioside treatment group's pre and post-treatment values are summarized. The results are summarized in Tables V.-12, V.-13 and V.-14 above. No clinically significant differences were observed in the plasma glucose or insulin levels of type 1 and type 2 diabetics or in non-diabetic subjects.

Jeppesen *et al.* (2006) conducted a randomized, placebo-controlled, double-blind, parallel long-term trial in which they administered placebo or stevioside, 500 mg, 3x/day, to fifty-five type 2 diabetic subjects on antihypertensive treatment for a period of 3 months. Blood glucose and insulin levels were determined before and after the 3-month study period. Fasting blood glucose and insulin levels were not affected by stevioside administration

Food Standards Australia New Zealand (FSANZ) reviewed the same studies discussed above and concluded:

> "…that there is a relatively low risk of hypoglycaemia in normal healthy human subjects, particularly at concentrations that may be encountered in the diet. […] In summary, the weight-of-evidence indicates that stevioside would be unlikely to produce hypoglycaemia in humans at concentrations encountered in the diet. (FSANZ, 2007)"

In conclusion, no treatment related effects of steviol glycosides (including Reb A and stevioside) on blood glucose are anticipated at intake levels from Reb A use as a sweetener.

V. Safety Evaluation

C. Possible Reb A Degradation Products

Introduction

The pH range for the beverages for which Reb A is foreseen as a sweetener is from pH 2.8 to 3.2 and recommended storage temperature is ambient. Therefore, the results from experiments conducted using those conditions (see III. Stability) are most pertinent to the intended condition of use for these beverage products. At pH 3.0 and ambient temperature (i.e. 70°F) or refrigeration (i.e. 40°F) over the 25 week period of the stability study, Reb A degradation is insignificant (Stetson, 2008c [Unpublished]). However, under extreme environmental storage conditions, the following degradation products of Reb A may be formed: iso-rebaudioside A, iso-rebaudioside B, iso-stevioside, iso-steviolbioside, isosteviol, and isosteviol II (see III. Stability).

Safety Evaluation of Possible Reb A Degradation Products

Rebaudioside B, stevioside, steviolbioside and steviol, are normal constituents of Reb A products present at low concentrations. There is no further discussion of these substances in this section, since it has been demonstrated that these substances do not accumulate even under low pH and extreme temperature storage conditions, their safety evaluation is encompassed in the context of the safety evaluation of Reb A, and/or their toxicology profiles are the same as all of the steviol glycosides discussed throughout this document.

Comprehensive literature searches were performed for the two possible main (iso-rebaudioside A and iso-rebaudioside B) and four possible minor (iso-stevioside, iso-steviolbioside, isosteviol and isosteviol II) degradants identified during the stability studies. Although published literature was found only for isosteviol, consideration of low exposure potential and structure-activity relationships with other steviol glycosides allows appropriate safety evaluation to be performed for the other main and minor degradants that possibly may be formed under extreme temperature storage conditions. Safety evaluation of these possible degradants is presented below.

In the storage stability study with Reb A, iso-rebaudioside A and iso-rebaudioside B were determined to be the substances most likely to accumulate under the most severe pH and temperature test conditions (see III. Stability). Because of the very slight change of the structure of Reb A that occurs in the conversion to iso-rebaudioside A (see Figure V.-9 above), the toxicology and toxicokinetic profile of iso-rebaudioside A is not expected to differ significantly from that of Reb A or other steviol glycosides. Since iso-rebaudioside B is the immediate hydrolysis product of iso-rebaudioside A and its structure is very slightly changed compared to rebaudioside B, the toxicology and toxicokinetic profile of iso-rebaudioside B is not expected to differ significantly from that of Reb A or other steviol glycosides. As a consequence of the very slight structural difference in the aglycone for iso-rebaudioside A and iso- rebaudioside B as compared to Reb A, the two possible main degradants are considered to have the same safety profile as Reb A and other steviol glycosides, and not expected to be absorbed any more efficiently.

The highest concentration for three of the possible minor degradants (i.e. iso-stevioside, iso-steviolbioside and isosteviol II) under the test conditions of pH 2.0 (concentration data is not available at pH 3.0) and ambient temperature (i.e. 70°F) in the stability study, approximating intended condition of use for beverage products, was ≈ 1.55 µg/mL for iso-stevioside in comparison to the initial nominal concentration of 600 µg/mL for Reb A. Concentrations were one and two orders of magnitude lower than iso-stevioside for iso-steviolbioside and isosteviol II, respectively. The extremely low concentrations of these three possible minor degradants derived from the hydrolysis of iso-rebaudioside A suggests that they do not present a significant exposure potential. In addition, since iso-stevioside, iso-steviolbioside and isosteviol II are hydrolysis products of iso-rebaudioside A and their structures are very slightly changed compared to Reb A (analogous to Figure V.-9 above), the toxicology profiles of iso-stevioside, iso-steviolbioside and isosteviol II are not expected to differ significantly from that of Reb A, other steviol glycosides and/or steviol. As a consequence of the very slight structural difference in the aglycone for iso-stevioside, iso-steviolbioside and isosteviol II as compared to Reb A, the three possible

minor degradants are considered to have the same safety profile as Reb A, other steviol glycosides and/or steviol.

The highest concentration of the other possible minor degradant, isosteviol, under the test conditions of pH 2.0 (concentration data is not available at pH 3.0) and ambient temperature (i.e. 70°F) in the stability study, approximating intended condition of use for beverage products, was ≤ 0.047 µg/mL in comparison to the initial nominal concentration of 600 µg/mL for Reb A. The extremely low concentration of this possible minor degradant, which may be derived from the conversion of steviol under low pH and high temperature conditions (see Figure V.-10 above), suggests that it does not present a significant exposure potential.

Although the extremely low concentration of isosteviol determined under test conditions approximating intended condition of use for beverage products allows one to conclude that exposure potential is insignificant, published literature were found for isosteviol and the pertinent data are presented below.

The oral LD_{Lo} for isosteviol in mice and rats is 5060 and 3160 mg/kg, respectively (Xu *et al*., 1994). The mutagenicity of pure isosteviol (purity not specified) was examined in a forward mutation assay (maximum concentration 10.0 mg/mL) with *Salmonella typhimurium* TM677; no significant mutagenic or bactericidal activity was observed during the assay either in the presence or absence of a metabolic activating system obtained from Aroclor 1254 pretreated rats (Pezzuto *et al*., 1985a). In a preliminary pharmacokinetic evaluation of isosteviol in rats, the terminal half-life ($t_{½}$) was 406 ± 31.7 minutes and clearance (CL) was 2.9 ± 0.3 mL/min/kg (Bazargan *et al*., 2007).

Similarly to stevioside and steviol, isosteviol has been investigated for potential effects on blood glucose control. In Zucker diabetic rats administered isosteviol by infusion at 5 and 10 mg/kg, a significant reduction in AUC of glucose was determined during the i.v. glucose tolerance test; however, isosteviol did not increase plasma insulin concentrations. On the other hand, for isosteviol administered by infusion at 10 mg/kg in non-diabetic

Wistar rats there was not a significant effect on plasma glucose or insulin levels during the i.v. glucose tolerance test (Ma *et al*., 2007). In diabetic KKAy mice administered isosteviol at 20 mg/kg bw/day for 9 weeks, improved glucose homeostasis, increased insulin sensitivity, lowered plasma triglycerides, and lowered body weights were observed (Nordentoft *et al*., 2007). In addition, cardiovascular function and cardioprotective effects of isosteviol have been investigated. Studies have suggested that isosteviol may reduce systemic blood pressure (Liu *et al*., 2001) and may relax smooth muscle in blood vessels (Wong *et al*., 2004). In the isolated Langendorff perfused guinea pig heart model, the ischemia-reperfusion injury was partially alleviated by infusion pretreatment (50, 250 or 500 nmol) with isosteviol (Xu *et al*., 2007a). The same investigators demonstrated a cardioprotective effect of i.v. pretreatment with isosteviol (0.5, 1.0 and 2.0 mg/kg) on rats with heart ischemia-reperfusion injury; hemodynamic parameters were improved, and the myocardial infarct size, the activities of serum enzymes, and the incidences of ventricular tachycardia and ventricular fibrillation were decreased compared to the control group (Xu *et al*., 2007b).

In summary, the only main degradants formed under severe pH and temperature test conditions were iso-rebaudioside A and iso-rebaudioside B. As a consequence of the very slight structural difference in the aglycone for iso-rebaudioside A and iso-rebaudioside B as compared to Reb A, the two possible main degradants are considered to have the same safety profile as Reb A and other steviol glycosides as discussed throughout this document. The concentrations of the minor degradant products (i.e. iso-stevioside, iso-steviolbioside, isosteviol, and isosteviol II) of Reb A that may be formed under severe pH and temperature test conditions were extremely low under test conditions approximating the intended condition of use for beverage products, indicating that they do not present a significant exposure potential. As a consequence of the very slight structural difference in the aglycone for iso-stevioside, iso-steviolbioside and isosteviol II as compared to Reb A, these three possible minor degradants are considered to have the same safety profile as Reb A, other steviol glycosides and/or steviol. The available data and the similarity of their structures suggest that the toxicology, toxicokinetic and pharmacological profile of isosteviol would be similar to that of steviol.

The pH range for the beverages for which Reb A is foreseen as a sweetener is from pH 2.8 to 3.2 and recommended storage temperature is ambient. At pH 3.0 and ambient temperature (i.e. 70°F) or refrigeration (i.e. 40°F) over the 25 week period of the stability study (approximating intended condition of use for beverage products), Reb A degradation is insignificant. Since the toxicology profiles of all possible Reb A degradation products are expected to be similar to Reb A, other steviol glycosides and/or steviol because of structure-activity relationships, and they are expected to be poorly absorbed, the potential exposure to degradation products is toxicologically insignificant.

Absorption, Distribution, Metabolism and Excretion (ADME)

The ADME of Reb A was investigated in a study in rats who received Reb A via the diet at dose levels up to 2000 mg/kg bw/day (Sloter, 2008a [Unpublished]). The findings of the study support the results obtained with other steviol glycosides and include:

- Mean plasma concentrations (C_{avg}) of Reb A and total steviol (as *Reb A Equivalents*) are extremely low (≈ 0.6 and 12 µg/mL for high-dose, respectively);
- Mean plasma concentrations of other measured Reb A hydrolysis products (as *Reb A Equivalents*) are below the limits of the analytical methods or are insignificant (< 0.06 µg/mL for high-dose);
- Mean plasma concentrations of Reb A and steviol (as *Reb A Equivalents*) for the 2000 mg/kg/day dose group are less than dose-proportional in comparison to mean plasma concentrations at 500 and 1000 mg/kg/day, suggesting that dose levels higher than 2000 mg/kg/day would not result in significantly higher mean plasma concentrations;
- Mean urinary Reb A and measured hydrolysis products expressed as *Total Reb A Equivalents* are low (≈ 0.285 mg for high-dose) and accounted for almost exclusively by free and conjugated steviol;
- Mean urinary Reb A and *Total Reb A Equivalents* amounts in comparison to the daily administered dose results in estimates of dose absorbed of ≈ 0.02% and ≈ 0.08%, respectively;
- Mean fecal Reb A and measured hydrolysis products expressed as *Total Reb A Equivalents* are high (≈ 344 mg for high-dose);
- Mean fecal Reb A and measured hydrolysis products expressed as *Total Reb A Equivalents* compared to daily administered dose results in an estimate of percent of dose recovered ≈ 84%; and
- Mean fecal levels of rebaudioside B (expressed as *Reb A Equivalents*) are relatively high and stevioside is very low leading to the conclusion that hydrolysis of Reb A goes predominantly *via* a rebaudioside B pathway rather than stevioside and/or hydrolysis of stevioside to steviolbioside occurs rapidly.

These data corroborate the findings in numerous other studies of the ADME of steviol glycosides.

Key features of the ADME of steviol glycosides including Reb A are:

- Steviol glycosides are not metabolized or transformed in the stomach.

No absorption or structural modification of steviol glycosides in the stomach as was found in stevioside-dosed rats (Nakayama *et al.,* 1986). Stevioside incubated in human stomach juice for 6 hours was not altered (Geuns *et al*., 2007).

- The principal steviol glycosides, Reb A and stevioside, are metabolized in experimental animals and humans by intestinal microflora by sequential hydrolysis of glucose sugar moieties to free steviol which is excreted into feces (the primary pathway) or absorbed into plasma via the hepatic portal vein.

The human fecal microflora were found to completely hydrolyze stevioside and Reb A to their common aglycone, steviol, in 10 and 24 h, respectively, but the microflora did not degrade steviol. The incubation of stevioside or Reb A with human intestinal microflora from different volunteers did not confirm the presence of steviol epoxide derivatives. The lack of stevioside in the feces indicates that the bacterial flora degraded all the stevioside into steviol, which itself was not further metabolized, as was also shown in pigs *in vivo* (Geuns *et al.,* 2003a) and in pig and human feces in vitro under anaerobic conditions (Geuns *et al*., 2003a, Koyoma *et al.,* 2003b, Gardana *et al.,* 2003).

- Steviol glycosides do not influence the intestinal microflora composition.

Stevioside and Reb A did not influence significantly the human intestinal microflora composition; however, stevioside weakly inhibited anerobic bacteria, whereas Reb A weakly inhibited activity on aerobic bacteria particularly coliforms (Gardana *et al*., 2003).

- Blood primarily contains the conjugated form of steviol; the conjugated form is excreted in urine.

Besides steviol glucuronide and extremely small amounts of test material or free steviol, none of the possible steviol metabolites have been detected in blood or urine. Geuns *et al.* (2007) reported that between 13 and 40 mg free steviol could be detected in a 24-hr feces sample collected following 3 doses/day of 250 mg stevioside/dose.
Geuns *et al.* (2007) did not detect free steviol in the peripheral blood of the study subjects. Reb A, free steviol and steviol glucuronide were detected in peripheral blood of rats but at extremely low levels representing less than 0.08% of the administered dose, which suggests that free steviol that is absorbed from the intestines is transported to the liver via the portal vein is converted into steviol glucuronide by the liver (Sloter, 2008a [Unpublished]; Geuns *et al.,* 2007; Simonetti *et al.,* 2004), which is excreted in the urine.

Therefore, any study in which the test material was a characterized steviol glycoside is relevant in evaluating safety. However, some studies used uncharacterized extracts and those may have effects due to other non-glycosidic substances that are present. The studies in which the test material was characterized are thus more likely to be reliable and are used in this evaluation to confirm the safety of Reb A.

The toxicology results are summarized below by study type. Human test results have been grouped together to facilitate the evaluation of those studies.

Acute Toxicity

No toxicity could be demonstrated in mice with crude Reb A administered by oral gavage at 2000 mg/kg body weight. Two weeks after dose administration, no significant differences were noted at necropsy in body or organ (heart, spleen, lung, pancreas, brain, kidney, and liver) weights in comparison to the control group (Medon *et al.*, 1982).

Stevioside (purity >96%) was not acutely toxic to mice, rats, or hamsters at doses as high as 15,000 mg/kg bw/d (Toskulkao *et al*. 1997; Medon *et al*. 1982).

Similarly, steviol (purity 90%) is not acutely toxic to rats and mice at doses as high as 14,000 mg/kg bw (Toskulkao *et al*. 1997; JECFA 1999). However, signs of acute toxicity, including death (1/15 male and female for rats and mice) were observed in rats and mice at a dose of 15,000 mg/kg. No morphological or histopathological changes were noted.

Subchronic Toxicity

Subchronic studies are available for Reb A, and stevioside. Dietary administration of Reb A to rats and dogs for 90-days and 6-months, respectively, at doses as high as 2000 mg/kg bw/day resulted in no adverse systemic toxicity.

A 90-day oral (dietary) toxicity study was conducted with treatment doses of 500, 1000 and 2000 mg/kg/day. There were no test-article-related effects on clinical observations, food consumption or functional observational battery or locomotor activity parameters. There were no test article-related macroscopic, organ weight or microscopic findings. Lower body weight gains were noted in the 2000 mg/kg bw/day group males throughout the study. These lower mean body weight gains resulted in lower cumulative body weight gains and a body weight difference that was 9.1% lower compared to the control group at the end of the dosing period (study week 13). The lower body weights were not considered to be adverse due to the small magnitude of difference from the control group value and were most likely due to the large proportion of the diet represented by the test material. (Eapen, 2007 [Unpublished]; Nikiforov and Eapen, 2008). The no-observed-adverse-effect level (NOAEL) was ≥2000 mg/kg bw/day.

A 6-month oral (dietary) toxicity study in dogs was conducted to evaluate the potential toxic effects of Reb at dosage levels of 0, 500, 1000 or 2000 mg/kg bw/day. All groups consisted of 4 males and 4 females. There were no unscheduled deaths during the course of the study. No test-article-related clinical observations were noted. Home cage, open field observations and functional observations and measurements were unaffected by test article administration. No test-article-related hematology findings, serum chemistry findings, or urinalysis findings were noted. No test-article-related gross necropsy

observations, alterations in final body weight, alterations in organ weights, or histologic changes were noted at the scheduled necropsy. Based on the results of this study, no systemic toxicity of Reb A was observed at dosage levels up to 2000 mg/kg bw/day and the NOAEL ≥2000 mg/kg bw/day (Eapen, 2008 [Unpublished]).

Stevioside administered to rats in the diet at concentrations up to 2500 mg/kg bw/day for 13 weeks resulted in a few statistically significant changes at some doses for some biochemical parameters. However, the lack of a dose-response relationship resulted in the authors' conclusion that the effects were nonspecific and not treatment related (Aze *et al.,* 1991).

As discussed throughout this Safety Evaluation (see Section V.A.2.b), decreases in food efficiency data, body weight and/or body weight gain compared to controls are observed at the highest dietary dose levels of Reb A and stevioside in rat subchronic toxicity (Eapen, 2007; Nikiforov and Eapen, 2008; Aze *et al*., 1991), rat chronic toxicity/carcinogenicity (Xili *et al*., 1992; Toyoda *et al*., 1997) and rat or hamster reproduction toxicity (Sloter, 2008a; Yodyingyuad and Bungawong, 1991; Mori *et al*., 1981) studies. It may be concluded that decreases in body weight and/or body weight gain in these studies are due to the extremely high levels of Reb A or stevioside administered via the dietary route which provide no caloric value in the diet, resulting in a decrease in total calorie consumption. Effects on body weight, body weight gain, and/or feed efficiency have been reported for other high intensity sweeteners when they are administered in the diet to test animals at similarly high dietary inclusion rates. For example, the reductions in body weight gain that have been reported for neotame, sucralose, and saccharin ranged from 3.7 to >20% in comparison to controls (Flamm *et al*., 2003).

In the absence of toxicity, JECFA has concluded that changes in body weight gain are not appropriate for establishing NOAELs when they are associated with lower food consumption or food efficiency. It is the normal practice of JECFA to recognize when body weight is affected by reduced palatability of food containing high concentrations of

test material. For example, JECFA noted that lower body weight gain for sucralose at the high dose in the long-term rat study (1500 mg/kg bw/day) was due to poor palatability of the diet and did not consider this finding adverse when setting the NOAEL and an ADI of 0-15 mg/k bw/day. Similarly, the highest dose of acesulfame-potassium, 1500 mg/kg bw/day was likewise associated with lower body weight gain. JECFA did not consider this an adverse finding when establishing an ADI for acesulfame-potassium of 0-15 mg/kg bw/day (WHO, 1983 as summarized in Flamm, *et al*., 2003).

Chronic Toxicity

Two chronic toxicity/carcinogenicity studies have been conducted with stevioside. These studies are relevant to Reb A since both compounds are converted to steviol in the gut prior to absorption. Both studies were 2-year studies that examined the effect of oral stevioside in rats. In the first study (Xili *et al*., 1992), stevioside administration in the diet showed no carcinogenic effects in the rat, and a no-observed adverse effect level (NOAEL) of 1.2% (600 mg/kg bw/day) in the diet was reported. In a more recent and more robust study (Toyoda *et al*., 1997) stevioside administered in the diet was not carcinogenic in rats, and a NOAEL of 2.5% (970 mg/kg bw/day in males) was established.

Genotoxicity Studies

Reb A was not mutagenic with and without metabolic activation when tested *in vitro* in bacterial reverse mutation, bacterial forward mutation, and mammalian cell gene mutation assays. In addition, Reb A was negative in an *in vivo* mouse micronucleus assay in bone marrow. Stevioside was not mutagenic with and without metabolic activation in several experiments using *in vitro* bacterial and mammalian cell mutation, and negative in chromosomal aberration, sister chromatid exchange, and micronucleus assays. Also, stevioside did not increase the micronucleus rate in an *in vivo* mouse micronucleus test in bone marrow and hepatocytes. Steviol was not mutagenic *in vitro* without metabolic activation in bacterial cell mutation, mammalian gene cell mutation, and chromosomal aberration studies, but with metabolic activation steviol was positive in some of these assays. However, in three *in vivo* mouse, one hamster and one rat

micronucleus assays, chromosomal damage was not observed at dose levels as high as 8000 mg/kg bw.

Reproductive/Developmental Toxicity Studies

Eleven studies have been reported in the literature in which reproductive/developmental toxicity was evaluated. Studies have been conducted using Reb A, stevioside and steviol as the test material in hamsters and rats at doses up to 3000 mg/kg bw/day.

Reb A was tested in a two-generation dietary reproduction toxicity study in rats (Sloter, 2008a [Unpublished]). There were no effects on reproduction (estrus cycles, mating, fertility, conception or copulation indices, number of days between pairing and coitus, gestation length, and spermatogenic endpoints). A dose level ≥2000 mg/kg bw/day (highest dose administered) was considered to be the NOAEL for parental systemic and reproductive toxicity, and an exposure level ≥2000 mg/kg bw/day was considered to be the NOAEL for neonatal toxicity of Reb A in rats.

Reb A was tested in an oral (gavage) embryo/fetal development study in rats (Sloter, 2008b [Unpublished]). Intrauterine growth and survival were unaffected by test article administration 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 when Reb A was administered by oral gavage to pregnant rats.

Stevioside had no effect on reproduction or development in a 3-generation hamster study with doses up to 2500 mg/kg bw/day (Yodyingyuad & Bunyawong, 1991). No effects were seen in a rat fertility study with doses up to 3000 mg/kg bw/day (Mori *et al.*, 1981) or in a developmental study in pregnant rats with doses up to1000 mg/kg bw/day (Takanaka *et al.*, 1991; Usami *et al.*, 1995).
Steviol was not teratogenic in hamsters (Wasuntarawat *et al.*, 1998). Decreased weight gain and increased mortality in dams and fetuses (LOAEL = 500 mg/kg bw/day) and a

dose-dependent effect on kidneys in dams were observed. The effects with steviol but not Reb A and stevioside are likely due to the rapid absorption of steviol, whereas Reb A and stevioside must first be hydrolyzed in the gut before absorption of steviol is possible.

Human Studies

Several studies have evaluated the oral administration of stevioside in humans. These studies indicate that steviol glycosides are unlikely to cause any effects on blood pressure or blood glucose in normal healthy humans (Barriocanal, 2008). If any effect is to be found, it would point to a slightly reduced blood pressure in hypertensive individuals; an effect that would not be adverse (Chan *et al*., 2000; Hseigh *et al.,* 2003). FSANZ also reached these conclusions in their risk assessment (FSANZ, 2007).

Likewise, there are unlikely to be any effects of steviol glycosides (including Reb A and stevioside) on blood glucose at doses that could result from its use as a sweetener (Barriocanal *et al*., 2008).

Food Standards Australia New Zealand (FSANZ, 2007) has reviewed the studies investigating control of glucose and concluded the following:

> "…there is a relatively low risk of hypoglycaemia in normal healthy human subjects, particularly at concentrations that may be encountered in the diet. […] In summary, the weight-of-evidence indicates that stevioside would be unlikely to produce hypoglycaemia in humans at concentrations encountered in the diet."

Summary

In summary, there is an extensive database on steviol glycosides including specific studies for which Reb A was the test material. All steviol glycosides are metabolized by gut microflora via the same intermediates and same hydrolysis pathways; therefore, the safety data for all characterized steviol glycosides are relevant to the safety evaluation of Reb A. However, data for other steviol glycosides provide a conservative safety evaluation for Reb A since they all hydrolyze to steviol more rapidly than Reb A. There are no outstanding toxicology issues, and the metabolism and pharmacokinetic data for humans are well understood.

The published literature and unpublished study results clearly demonstrate the lack of toxicity of Reb A and other steviol glycosides in animals and humans from oral ingestion at levels that are well above those anticipated from Reb A use as a sweetener in tabletop, beverages, and cereals.

VI. Acceptable Daily Intake (ADI)

Background

Based on the NOAEL for stevioside of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol) in a 2-year rat study, JECFA (2006) established a temporary ADI for steviol glycosides of 2 mg/kg bw/day (expressed as steviol). JECFA used a safety factor of 200 (100 for inter- and intra-species differences and additional factor of 2 because JECFA required additional information on pharmacological effects in humans). More recently, Food Standards Australia New Zealand (FSANZ, 2007) established an ADI for steviol glycosides of 4 mg/kg bw/day (expressed as steviol). FSANZ used a safety factor of 100 for inter- and intra-species differences. FSANZ provided the following rationale for applying a safety factor of 100:

> 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 [applied by JECFA] for uncertainty related to effects in normotensive or diabetic individuals is no longer required.

Totality-of-Evidence

An extensive safety database exists for Reb A, steviol glycosides and steviol to support safety in the proposed food categories. A NOAEL ≥2000 mg/kg bw/day (expressed as Reb A) has been established based on rat 90-day and dog 6-month subchronic, rat embryo/fetal developmental and rat 2-generation reproduction toxicity studies conducted with Reb A. Toxicokinetics and excretion data for Reb A (discussed in the "Absorption, Distribution, Metabolism and Excretion", Section V.A.1) are consistent with published literature on other steviol glycosides. These data indicate that Reb A and other steviol glycosides are hydrolyzed via the same pathways by gut microflora and systemic absorption is insignificant. Therefore, long-term toxicology studies conducted with stevioside are relevant to the safety evaluation of Reb A. In addition, human metabolism of Reb A and other steviol glycosides is well understood. Clinical data for steviol glycosides, in particular stevioside, show no 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.

This information is summarized in Table IV.-1 and the ADI is expressed as both steviol and Reb A equivalents.

Table VI.-1 Acceptable Daily Intake from Totality of Evidence

Basis of ADI (Safety Factor)	**Expressed as Steviol (mg/kg bw/day)**	**Expressed as Reb A[1] (mg/kg bw/day)**
JECFA temporary (200)	2	6
FSANZ (100)	4	12
Corroborating Reb A studies (100)	7	20
Conclusion for Reb A	**4-7**	**12-20**

[1] Calculated using a conversion factor of 3.04 based on the molecular weights of 318.45 and 967.03 for steviol and Reb A, respectively.

Summary

Consideration of the totality-of-evidence related to the safety evaluation of Reb A leads to the conclusion that an ADI of 12-20 mg/kg bw/day (expressed as Reb A) is appropriate.

VII. Basis for the GRAS Determination

Regulatory Framework

The regulatory framework for determining whether a substance can be considered generally recognized as safe (GRAS) in accordance with section 201(s) (21 U.S.C. § 321(s)) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 et. Seq.) ("The Act"), is set forth at 21 CFR 170.30, which states:

> General recognition of safety may be based only on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. 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 based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies which may be corroborated by unpublished studies and other data and information.

These criteria are applied below in an analysis of whether the use of Reb A as a sweetener for selected foods (tabletop sweeteners, sweetened ready-to-drink teas, diet carbonated soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal and sweetened cold cereals) is GRAS based upon scientific procedures.

Authoritative Bodies

Steviol glycosides have been reviewed by other authoritative bodies including the Food and Agriculture Organization/World Health Organization Joint Expert Committee on Food Additives (JECFA) and the Food Standards Australia New Zealand (FSANZ).

The JECFA and FSANZ reviews have included a detailed analysis of the literature, proposed acceptable daily intakes and provided estimates of consumer intakes. Therefore, the conclusions of these reviews are summarized below and in the safety section of this dossier.

JECFA Reviews of Steviol Glycosides

In 2006, JECFA established a temporary ADI for steviol glycosides of 2 mg/kg bw/day (expressed as steviol) based on the NOAEL for stevioside of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol or 1165 mg/kg bw/day Reb A) in a 2-year rat study. The corresponding ADI for Reb A (adjusting for the difference in molecular weight between steviol and Reb A) is 6 mg/kg bw/day. JECFA used a safety factor of 200 in calculating its ADI (100 for inter- and intra-species differences and an additional factor of 2 because at that time JECFA required additional information on pharmacological effects in humans). Data have now been published that address the data gap for pharmacological effects in humans. These data are scheduled for review by JECFA at its next meeting and have been included in this determination of the GRAS status of Reb A.

FSANZ Review Of Steviol Glycosides

FSANZ, in responding to a petition for authorization of steviol glycosides conducted an in-depth safety evaluation and concluded that the proposed uses could be authorized (the document has been available for review for about a year and a final publication is anticipated in mid 2008).

FSANZ (2007) published an ADI for steviol glycosides of 4 mg/kg bw/day (expressed as steviol). Adjusting for molecular weight, the equivalent Reb A ADI is 12 mg/kg bw/day. FSANZ used a safety factor of 100 for inter- and intra-species differences. FSANZ provided the following rationale for applying a safety factor of 100:

> 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 [applied by JECFA] for uncertainty related to effects in normotensive or diabetic individuals is no longer required.

Stevia was authorized in Japan in 1970 and is used in more than 100 different food products. Argentina approved stevioside under Resolution 101 of the Argentine Food Code in 1993. Mercosur is a trade agreement among several countries in Latin America that is similar to the North American Free Trade Agreement (NAFTA). Stevioside is

approved for use in countries within the Mercosur group. Stevioside is listed in the Mercosur Harmonized List of permitted food additives (Lista General Armonizada De Aditivos Mercosur) under Res GMCN°055/94. Columbia, Paraguay, Uruguay and Venezuela all rely on the Mercosur listing and therefore stevioside is allowed to be used as a sweetener in those countries. Brazil also follows the Mercosur harmonized listing but in addition has adopted its own regulations for stevioside and is currently considering adopting the JECFA specification for stevioside. Steviol glycosides are authorized in a number of other countries including Taiwan, Russia, Korea and China. Taiwan has approved stevia extracts for use in foods (Food Additives List, Section 11, 038-041, 043, 046, as amended). Russia has approved stevioside (at least 75% stevioside) for GMP use as a sweetener in their regulations (SanPiN 2.3.21078 - 01, Annex 7, List of Permitted Additives). Thailand has approved stevioside for use in products specifically marketed for weight loss but not for general use in foods. Korea has approved products that are at least 98% steviol glycosides in its regulations (KFDA, Korea Food Additives Code #174) (http://fa.kfda.go.kr). The Chinese Ministry of Health approved stevioside to be used in drinks, candies and cakes in 1984, and then expanded its use to French fries, seasoning, preserved fruits and melon seeds.

Other countries, including the European Union, are currently considering petitions for the use of steviol glycosides. At the present time steviol glycosides are not permitted in member countries of the European Union. A revised safety assessment by the European Food Safety Authority is anticipated later in 2008 or early 2009.

Comparison of the Estimated Daily Intake to the Acceptable Daily Intake

Consideration of the totality-of-evidence related to the safety evaluation of Reb A (including acute/subchronic/chronic toxicity, genotoxicity, reproductive/developmental toxicity, and carcinogenicity, as well as absorption, distribution, metabolism and excretion (ADME) data, leads to the conclusion that an ADI of 12-20 mg/kg bw/day (expressed as Reb A) is appropriate.

The EDI of Reb A was calculated using the most recent food consumption survey results from the National Health and Nutrition Examination Survey (NHANES). Reb A intake was determined by multiplying each NHANES respondents' 2-day average food intake by the maximum proposed use level for each category. The total EDI from all proposed uses is approximately 2 mg/kg bw/day at the mean and 5 mg/kg bw/day at the 90th percentile of intake among users. A user is defined as having consumed a food that would contain Reb A during the 2 days of the NHANES survey. Consideration of only 2 days of food consumption data results in a conservative upper estimate of potential Reb A intake.

In conclusion, even using conservative upper estimates of intake (5 mg/kg bw/day) and the lowest potential ADI (12 mg/kg bw/day), consumer intakes of Reb A from the proposed uses would not exceed the ADI.

Discussion of Information Inconsistent With GRAS Determination

The authors of this notification are not aware of information that would be inconsistent with a finding that the proposed use of Reb A as a natural sweetener in tabletop sweeteners, sweetened ready-to-drink iced teas, diet carbonated soft drinks, fruit juice drinks, energy drinks, flavored waters, cereal bars, oatmeal, and sweetened cold cereals is generally recognized as safe.

The regulatory framework for determining whether a substance is generally recognized as safe (GRAS) is in 21 CFR 170.30, which states that GRAS status through scientific procedures shall ordinarily be based upon published studies, which may be corroborated by unpublished studies and other data and information. These criteria have been applied to the existing data for Reb A in this notification.

The issues that have been raised by FDA in the past, which largely arose over concerns about stevia extracts, have been addressed. Specifically, this notification is for chemically defined pure Rebaudioside A (Reb A). The pathways for absorption, distribution, metabolism and excretion (ADME) have now been fully defined. Because

of the common hydrolysis pathways and toxicokinetics of steviol glycosides and steviol, any study in which the test material was a steviol glycoside is relevant in evaluating safety. Additional toxicology data are now available that further confirm the safety in animals and humans. Potential effects on blood pressure and blood glucose in normal, hypotensive, and diabetic subjects have been evaluated.

Several studies have evaluated the oral administration of stevioside in humans for durations of one day to two years. These studies indicate that steviol glycosides are unlikely to cause any effects on blood pressure or blood glucose in normal healthy humans. Steviol glycosides may decrease blood pressure slightly in hypertensive individuals; however, this effect is not considered to be adverse. FSANZ reached this conclusion in their risk assessment, which was published in 2007.

Conclusion: GRAS Determination

The safety of Reb A has been evaluated using a wide variety of published toxicology and other safety-related studies available for Reb A, other steviol glycosides and steviol. The absorption, distribution, metabolism, and excretion (ADME) of steviol glycosides is well understood for laboratory animals and humans, and has been described in publicly available studies. Understanding the ADME for steviol glycosides has made it possible to bring a wide array of data and information to bear upon the evaluation of the safety of Reb A. The safety-related studies on Reb A, other steviol glycosides and steviol include acute, subchronic, chronic/carcinogenicity and reproduction and developmental toxicity studies in laboratory animal models as well as genotoxicity studies. Clinical studies have evaluated the potential for effects of steviol glycosides on blood pressure and on blood glucose levels.

The totality-of-evidence, both that which is published and that which is unpublished but corroborative, provides a basis upon which to conclude that the uses of Reb A described in this GRAS Notice satisfy the safety standard of Reasonable Certainty of No Harm and also conclude that an ADI of 12-20 mg/kg bw/day (expressed as Reb A) is supported by the data and is appropriate for use in assessing the safety of Reb A for use in food.

Finally, these data and information are known and accepted by a consensus of qualified experts in the general scientific community. Thus, this information base not only assures that the intended uses of Reb A described in this notice are safe, but also comprises common knowledge that Reb A is also generally recognized as safe under its intended conditions of use.

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Appendices

to

Safety Evaluation Dossier for Expert Panel Determination of the GRAS Status of Rebaudioside A (Reb A)

Appendix I: Identification

Appendix I.-1: Structures of Steviol Glycosides Found in *Stevia rebaudiana* (Bertoni) Leaf

Appendix I.-2: Reb A NMR, FTIR, Mass Spectra, and HPLC

Appendix I.-3: JECFA Monograph (2007) for Steviol Glycosides

Appendix I.-4: Reb A Lot Analyses and Certificates of Analysis

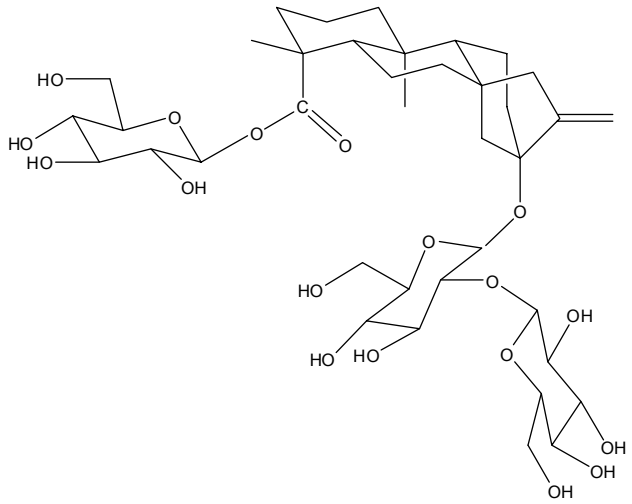
Appendix I.-5: Test Methods

Appendix I.-6: Pesticide Declaration and Analyses

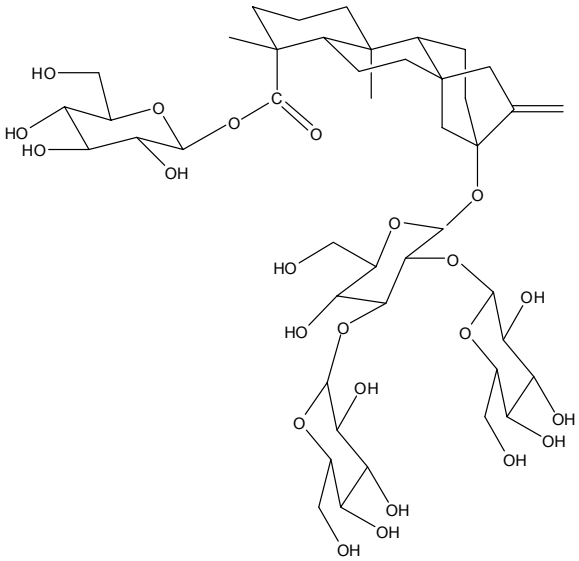
Appendix I.-1: Structures of Steviol Glycosides Found in *Stevia rebaudiana* (Bertoni) Leaf

Appendix Figure I.-1: Major Steviol Glycosides

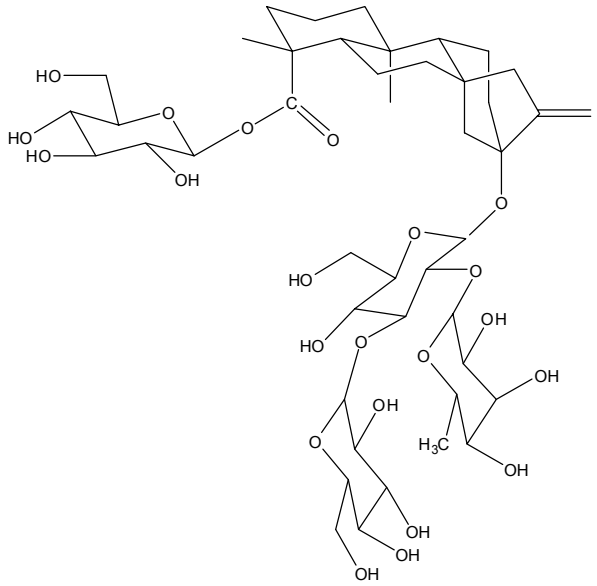
Stevioside



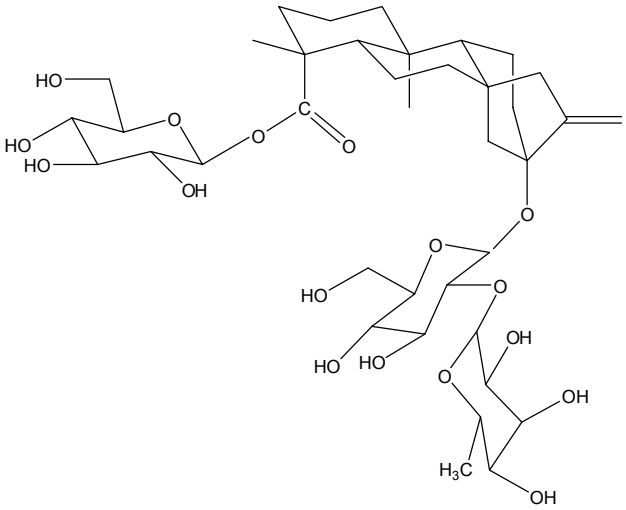
Rebaudioside A



Rebaudioside C

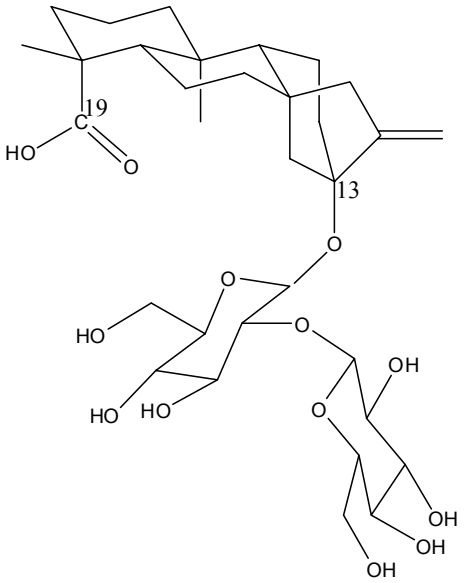


Dulcoside A

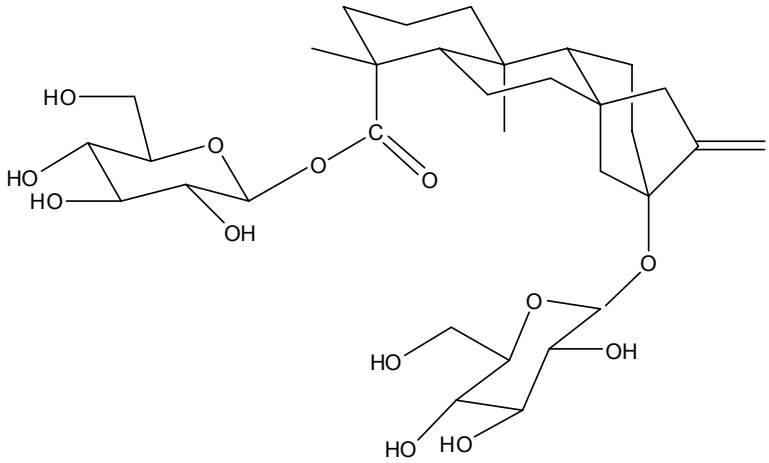


Appendix Figure I.-2: Minor Steviol Glycosides

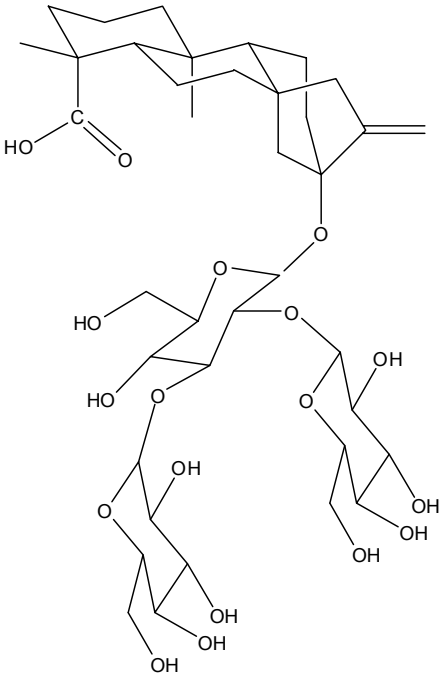
Steviolbioside



Rubusoside

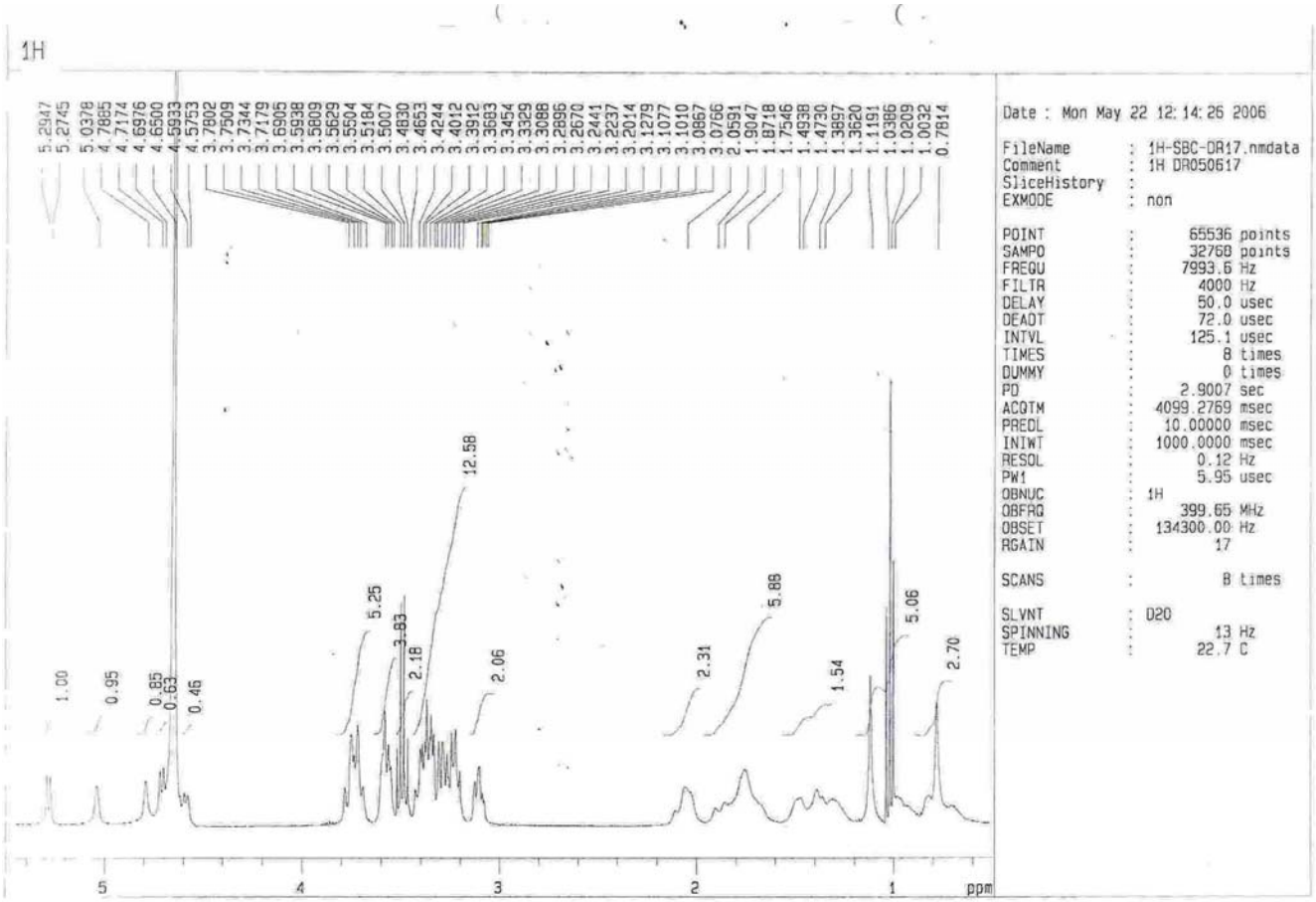


Rebaudioside B



Appendix I.-2: Reb A NMR, FTIR, Mass Spectra, and HPLC

Appendix Figure I.-3: Reb A NMR Spectra



Appendix Figure I.-4: Reb A FTIR Spectra

D0907041.SP Peak Table 5 / 10 / 2007

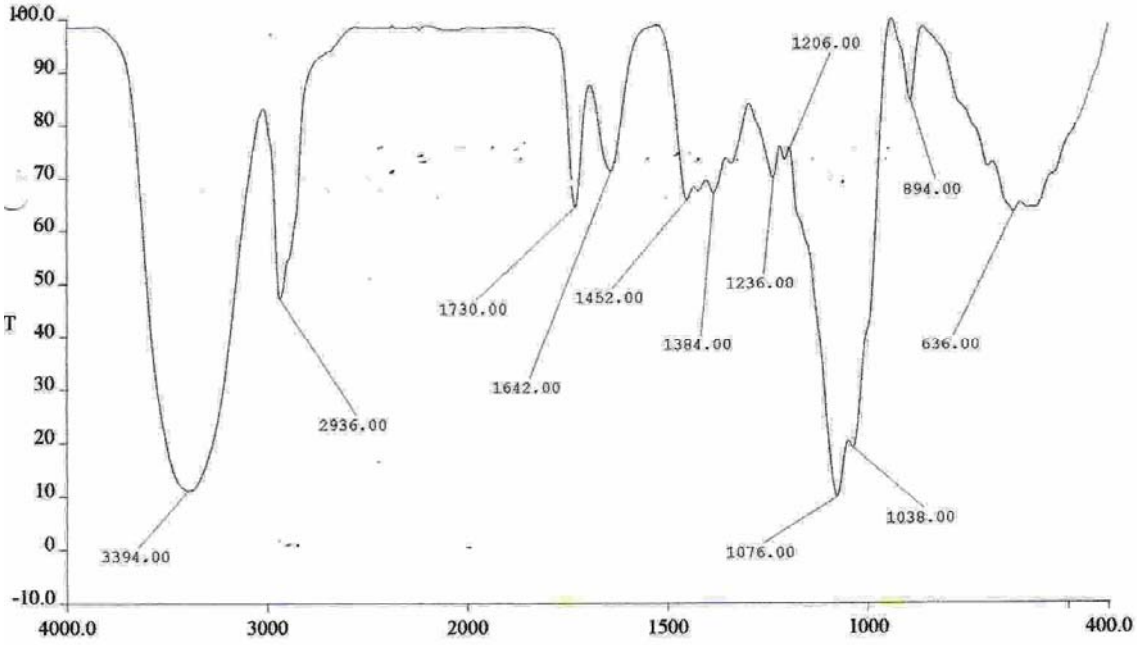
d0907041.sp 1801 4000.00 400.00 10.00 100.00 4.00 %T 64 2.00
/ Stevian Biotechnology
REF: 4000 98.49 2000 98.52 250
3394.00 11.08 2936.00 47.26 1730.00 64.59 1642.00 71.41 1452.00 65.90
1384.00 67.23 1236.00 70.04 1206.00 73.46 1076.00 10.00 1038.00 19.11
894.00 84.78 636.00 63.73

END 12 Peaks found

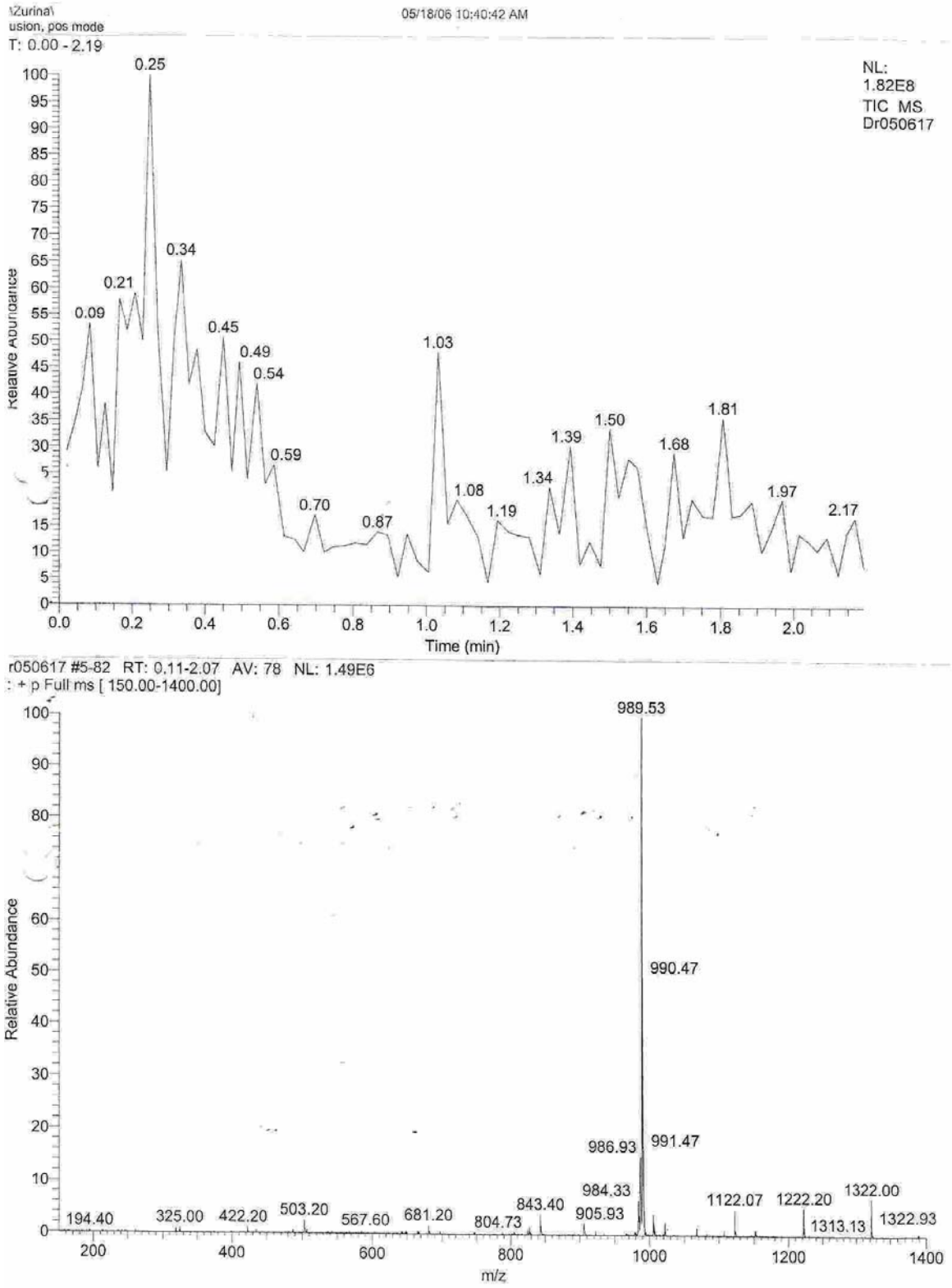
D0907041.SP Possible Structural Units

201 ALKYL GROUP - GENERAL
223 ALKYL GROUP - HYDROXY OR POSSIBLY AMINO SUBSTITUENT
402 HYDROXY OR AMINO COMPOUND - GENERAL
502 ALIPHATIC ALCOHOL - PRIMARY OR SECONDARY HYDROXY GROUP
511 ALIPHATIC ALCOHOL - PRIMARY OR SECONDARY OR POSSIBLY CYCLIC HYDROXY

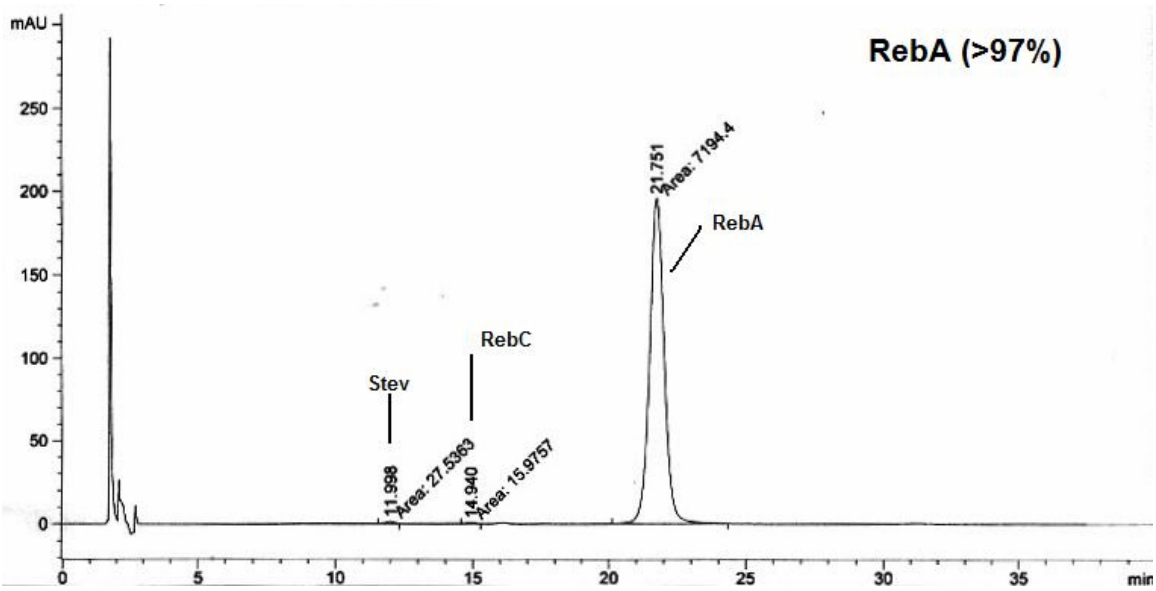
PSUs above may be subject to interference



Appendix Figure I.-5: Reb A Mass Spectra



Appendix Figure I.-6: Reb A HPLC



Appendix I.-3: JECFA Monograph (2007) for Steviol Glycosides

STEVIOL GLYCOSIDES

Prepared at the 68th JECFA (2007) and published in FAO JECFA Monographs 4 (2007), superseding tentative specifications prepared at the 63rd JECFA (2004), in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). A temporary ADI of 0-2 mg/kg bw (expressed as steviol) was established at the 63rd JECFA (2004).

SYNONYMS INS no. 960

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

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

Chemical name

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

Rebaudioside A: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-6-en-8-oic acid, β-D-glucopyranosyl ester

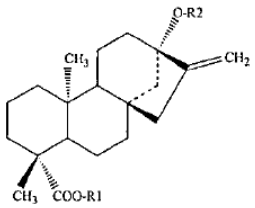
C.A.S. number

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

Chemical formula

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

Structural formula The seven named steviol glycosides:



Compound name	*R1*	*R2*
Stevioside	β-Glc	β-Glc-β-Glc(2→1)
Rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside C	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
Dulcoside A	β-Glc	β-Glc-α-Rha(2→1)
Rubusoside	β-Glc	β-Glc
Steviolbioside	H	β-Glc-β-Glc(2→1)
Rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)

Steviol (R1 = R2 = H) is the aglycone of the steviol glycosides.
Glc and Rha represent, respectively, glucose and rhamnose sugar moieties.

Formula weight

Stevioside: 804.88
Rebaudioside A: 967.03

Assay

Not less than 95% of the total of the seven named steviol glycosides, on the dried basis.

DESCRIPTION

White to light yellow powder, odourless or having a slight characteristic odour. About 200 - 300 times sweeter than sucrose.

FUNCTIONAL USES

Sweetener

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Freely soluble in water and in ethanol

Stevioside and rebaudioside A

The main peak in the chromatogram obtained by following the procedure in Method of Assay corresponds to either stevioside or rebaudioside A.

pH (Vol. 4)

Between 4.5 and 7.0 (1 in 100 solution)

PURITY

Total ash (Vol. 4)

Not more than 1%

Loss on drying (Vol. 4)

Not more than 6% (105°, 2h)

Residual solvents (Vol. 4)

Not more than 200 mg/kg methanol
(Method I in Vol. 4, General Methods, Organic Components, Residual Solvents)

Arsenic (Vol. 4) — Not more than 1 mg/kg
Determine by the atomic absorption hydride technique (Use Method II to prepare the test (sample) solution)

Lead (Vol. 4) — Not more than 1 mg/kg
Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under "General Methods, Metallic Impurities).

METHOD OF ASSAY Determine the percentages of the individual steviol glycosides by high pressure liquid chromatography (Volume 4).

Standards
Stevioside, >99.0% purity and rebaudioside A, >97% purity (available from Wako pure Chemical Industries, Ltd. Japan).

Mobile phase
Mix HPLC-grade acetonitrile and water (80:20). Adjust the pH to 3.0 with phosphoric acid (85% reagent grade). Filter through 0.22 µm Millipore filter or equivalent.

Standard solutions
(a) Accurately weigh 50 mg of dried (105°, 2 h) stevioside standard into a 100-ml volumetric flask. Dissolve with mobile phase and dilute to volume with mobile phase.
(b) Repeat with previously dried rebaudioside A standard.

Sample solution
Accurately weigh 60-120 mg of dried (105°, 2 h) sample into a 100-ml volumetric flask. Dissolve with mobile phase and dilute to volume with the mobile phase.

Chromatography Conditions

Column: Supelcosil LC-NH2 or equivalent (length: 15-30 cm; inner diameter: 3.9-4.6 mm)

Mobile phase: A 80:20 mixture of acetonitrile and water (see above)

Flow rate: Adjust so that the retention time of rebaudioside A is about 21 min.

Injection volume: 5-10 µl

Detector: UV at 210 nm

Column temperature: 40°

Procedure
Equilibrate the instrument by pumping mobile phase through it until a drift-free baseline is obtained. Record the chromatograms of the sample solution and of the standard solutions.

The retention times relative to rebaudioside A (1.00) are:

0.45-0.48 for stevioside	0.12-0.16 for rubusoside
0.25-0.30 for dulcoside A	0.35-0.41 for steviolbioside
0.63-0.69 for rebaudioside C	0.73-0.79 for rebaudioside B

Measure the peak areas for the seven steviol glycosides from the sample solution (the minor components might not be detected). Measure the peak area for stevioside for the standard solution.

Calculate the percentage of each of the seven steviol glycosides, *X*, in the sample from the formula:

$$\%X = [W_S/W] \times [f_X A_X/A_S] \times 100$$

where

W_S is the amount (mg) of stevioside in the standard solution

W is the amount (mg) of sample in the sample solution

A_S is the peak area for stevioside from the standard solution

A_X is the peak area of *X* for the sample solution

f_X is the ratio of the formula weight of *X* to the formula weight of stevioside: 1.00 (stevioside), 0.98 (dulcoside A), 1.20 (rebaudioside A), 1.18 (rebaudioside C), 0.80 (rubusoside), 0.80 (steviolbioside), and 1.00 (rebaudioside B).

Calculate the percentage of total steviol glycosides (sum the seven percentages).

Appendix I.-4: Reb A Lot Analyses and Certificates of Analysis

Test	Acceptable Target/Range	Test Results
Purity Reb A (wt/wt %)	≥ 95% Reb A (on dry basis)	97.61% (lot 071201) 96.11% (lot 071202) 97.27% (lot 071203)
Total Steviol Glycosides (wt/wt %)	≥ 95% (on dry basis)	100.71% (lot 071201) 99.13% (lot 071202) 100.24% (lot 071203)
Stevioside (wt/wt %)	2% (on dry basis) maximum	1.49% (lot 071201) 0.76% (lot 071202) 0.76% (lot 071203)
Steviol (wt/wt %)	< 0.005% (on dry basis)	Pass (X621301) Pass (DR610608) Pass (DR060627b)
Moisture Content (%) by Loss on Drying	5.0% maximum	1.91% (lot 071201) 2.43% (lot 071202) 2.65% (lot 071203)
Optical Rotation	-29º to -37º	-30º (lot Stevian Reb A 95) -30.1º (lot 0707J006-2-1) -31.9º (lot Q070731-2)
Arsenic (as As)	1.0 mg/kg maximum	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Lead	1.0 mg/kg maximum	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Cadmium	1.0 mg/kg maximum	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Total Aerobic Plate Count	1000 cfu/g maximum	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Total Aerobic Mold Count	100 mold cfu/g maximum	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Total Aerobic Yeast Count	100 yeast cfu/g maximum	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Heat Resistant Mold	< 1 cfu/50 g	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Coliform/E. coli	< 0 cfu/g	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)

Test	Acceptable Target/Range	Test Results
E. coli	< 3 MPN/g	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Salmonella ssp	Negative in 25g	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Staphylococcus aureus	< 10 cfu/g	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Alicyclobacillus acidoterrestris	< 1 cfu/50g	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Residual Solvents	Methanol < 300 ppm Ethanol < 1000 ppm	Pass (lot TL111407A) Pass (lot TL111407B) Pass (lot TL111407C)
Pesticide Residues	None Detectable	N.D. (lot TL111407A) N.D. (lot TL111407B) N.D. (lot TL111407C)

STEVIAN BIOTECHNOLOGY CORPORATION SDN BHD (578803-K)

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CERTIFICATE OF ANALYSIS

NAME OF PRODUCT: **Rebaudioside A**
BATCH #: **DR0807036**
MANUFACTURING DATE: **4th AUGUST 2007**
EXPIRY DATE: **2 Years from Manufacturing Date**
STORAGE CONDITIONS: **Store in cool, dry and ventilated area.**

PARAMETER	METHODS	SPECIFICATION	RESULT
Appearance	--	White Powder	Approved
Rebaudioside A, % (wt/wt) on anhydrous basis	HPLC	>97.0	99.19
Other Steviol Glycosides, % (wt/wt)	HPLC	<3.0	< 3.0
Ash	AOAC 945.46	<0.2	< 0.1
Specific Rotation, on anhydrous basis	Polarimeter	-29° to -33°	-30.3°
Lead (as Pb), ppm	ICP-MS	<1.0	0.009
Cadmium (as Cd), ppm	ICP-MS	<0.1	< 0.005
Arsenic (as As), ppm	ICP-MS	<0.1	< 0.005
Mercury (as Hg), ppm	ICP-MS	<0.1	< 0.005
Faecal Coliforms, MPN/g	Chapter 4, FDA	<3	Not Detected
Total Coliforms, MPN/g	Chapter 4, FDA	<3	Not Detected
Escherichia coli count, CFU/g	AOAC 991.14	<10	Not Detected
Listeria	Tecra Unique	Negative/11g	Absent
Salmonella sp.	Tecra Unique / AOAC 2000.07	Negative/25g	Absent
Standard Plate Count, CFU/g	AOAC 990.12	<1000	Not Detected
Staphylococcus aureus, CFU/g	Chapter 12, FDA	<10	Not Detected
Yeast, CFU/g	AOAC 997.02	<100	Not Detected
Mold, CFU/g	AOAC 997.02	<100	10

Conclusion: Qualified for sales and human consumption

Prepared by:
Choung Chia Mei
Quality Assurance Department
Date: 21st August 2007

Reviewed by:
Mr. Lai Chee Seng
Quality Assurance Department
Date: 21st August 2007

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CERTIFICATE OF ANALYSIS

NAME OF PRODUCT: **Rebaudioside A**
BATCH #: **DR0807038**
MANUFACTURING DATE: **20th AUGUST 2007**
EXPIRY DATE: **2 Years from Manufacturing Date**
STORAGE CONDITIONS: **Store in cool, dry and ventilated area.**

PARAMETER	METHODS	SPECIFICATION	RESULT
Appearance	--	White Powder	Approved
Rebaudioside A, % (wt/wt) on anhydrous basis	HPLC	>97.0	97.74
Other Steviol Glycosides, % (wt/wt)	HPLC	<3.0	< 3.0
Ash	AOAC 945.46	<0.2	< 0.1
Specific Rotation, on anhydrous basis	Polarimeter	-29° to -33°	-30.5°
Lead (as Pb), ppm	ICP-MS	<1.0	0.029
Cadmium (as Cd), ppm	ICP-MS	<0.1	< 0.005
Arsenic (as As), ppm	ICP-MS	<0.1	0.007
Mercury (as Hg), ppm	ICP-MS	<0.1	< 0.005
Faecal Coliforms, MPN/g	Chapter 4, FDA	<3	Not Detected
Total Coliforms, MPN/g	Chapter 4, FDA	<3	Not Detected
Escherichia coli count, CFU/g	AOAC 991.14	<10	Not Detected
Listeria	Tecra Unique	Negative/11g	Absent
Salmonella sp.	Tecra Unique / AOAC 2000.07	Negative/25g	Absent
Standard Plate Count, CFU/g	AOAC 990.12	<1000	Not Detected
Staphylococcus aureus, CFU/g	Chapter 12, FDA	<10	Not Detected
Yeast, CFU/g	AOAC 997.02	<100	Not Detected
Mold, CFU/g	AOAC 997.02	<100	Not Detected

Conclusion: Qualified for sales and human consumption

Prepared by:
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CERTIFICATE OF ANALYSIS

NAME OF PRODUCT: **Rebaudioside A**
BATCH #: **DR0907040**
MANUFACTURING DATE: **5th September 2007**
EXPIRY DATE: **2 Years from Manufacturing Date**
STORAGE CONDITIONS: **Store in cool, dry and ventilated area.**

PARAMETER	METHODS	SPECIFICATION	RESULT
Appearance	--	White Powder	Approved
Rebaudioside A, % (wt/wt) on anhydrous basis	HPLC	>97.0	97.67
Other Steviol Glycosides, % (wt/wt)	HPLC	<3.0	< 3.0
Ash	AOAC 945.46	<0.2	< 0.1
Specific Rotation, on anhydrous basis	Polarimeter	-29° to -33°	- 30.9°
Lead (as Pb), ppm	ICP-MS	<1.0	0.019
Cadmium (as Cd), ppm	ICP-MS	<0.1	<0.005
Arsenic (as As), ppm	ICP-MS	<0.1	0.005
Mercury (as Hg), ppm	ICP-MS	<0.1	<0.005
Faecal Coliforms, MPN/g	Chapter 4, FDA	<3	Not Detected
Total Coliforms, MPN/g	Chapter 4, FDA	<3	Not Detected
Escherichia coli count, CFU/g	AOAC 991.14	<10	Not Detected
Listeria	Tecra Unique	Negative/11g	Absent
Salmonella sp.	Tecra Unique / AOAC 2000.07	Negative/25g	Absent
Standard Plate Count, CFU/g	AOAC 990.12	<1000	Not Detected
Staphylococcus aureus, CFU/g	Chapter 12, FDA	<10	Not Detected
Yeast, CFU/g	AOAC 997.02	<100	Not Detected
Mold, CFU/g	AOAC 997.02	<100	Not Detected

Conclusion: Qualified for sales and human consumption

Prepared by: _____
Choung Chia Mei
Quality Assurance Department
Date: 27th September 2007

Reviewed by: _____
Mr. Lai Chee Seng
Quality Assurance Department
Date: 27th September 2007

STEVIAN BIOTECHNOLOGY CORPORATION SDN BHD (578803-K)

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71760 Bandar ENSTEK
Negeri Sembilan, Malaysia
Tel: +606-791 2222 • Fax: +606-791 3333
E-mail: office@sbc.com.my

CERTIFICATE OF ANALYSIS

NAME OF PRODUCT: **Rebaudioside A**
BATCH #: **DR0907042**
MANUFACTURING DATE: **26th SEPTEMBER 2007**
EXPIRY DATE: **2 Years from Manufacturing Date**
STORAGE CONDITIONS: **Store in cool, dry and ventilated area.**

PARAMETER	METHODS	SPECIFICATION	RESULT
Appearance	--	White Powder	Approved
Rebaudioside A, % (wt/wt) on anhydrous basis	HPLC	>97.0	97.97
Other Steviol Glycosides, % (wt/wt)	HPLC	<3.0	<3.0
Ash	AOAC 945.46	<0.2	<0.1
Specific Rotation, on anhydrous basis	Polarimeter	-29° to -33°	-30.9°
Lead (as Pb), ppm	ICP-MS	<1.0	0.019
Cadmium (as Cd), ppm	ICP-MS	<0.1	<0.005
Arsenic (as As), ppm	ICP-MS	<0.1	<0.005
Mercury (as Hg), ppm	ICP-MS	<0.1	<0.005
Faecal Coliforms, MPN/g	Chapter 4, FDA	<3	Not Detected
Total Coliforms, MPN/g	Chapter 4, FDA	<3	Not Detected
Escherichia coli count, CFU/g	AOAC 991.14	<10	Not Detected
Listeria	Tecra Unique	Negative/11g	Absent
Salmonella sp.	Tecra Unique / AOAC 2000.07	Negative/25g	Absent
Standard Plate Count, CFU/g	AOAC 990.12	<1000	Not Detected
Staphylococcus aureus, CFU/g	Chapter 12, FDA	<10	Not Detected
Yeast, CFU/g	AOAC 997.02	<100	Not Detected
Mold, CFU/g	AOAC 997.02	<100	Not Detected

Conclusion: Qualified for sales and human consumption

Prepared by:
Choung Chia Mei
Quality Assurance Department
Date: 7th November 2007

Reviewed by:
Mr. Lai Chee Seng
Quality Assurance Department
Date: 7th November 2007

Appendix I.-5: Test Methods

Reb A and Steviosides

The method of assay for purity, total steviol glycosides, and stevioside is modified from JECFA (2007). The method has been subjected to an interlaboratory review including 10 samples per laboratory.

Reb A

Laboratory	*Sample*	*Target %*	*Mean %*	*95% Confidence Interval of the Mean*	*Accuracy (% Recovery)*	*Precision (% RSD)*
Lab- A	**R-75**	**75.0**	**77.67**	**76.85 --- 78.49**	**103.56**	**1.48**
Lab- B	**R-75**	**75.0**	**80.90**	**80.67 --- 81.12**	**107.86**	**0.39**
Lab- C	**R-75**	**75.0**	**78.02**	**77.68 --- 78.36**	**104.02**	**0.61**
Lab- D	**R-75**	**75.0**	**81.10**	**80.70 --- 81.50**	**108.14**	**0.68**

Laboratory	*Sample*	*Target %*	*Mean %*	*95% Confidence Interval of the Mean*	*Accuracy (% Recovery)*	*Precision (% RSD)*
Lab- A	**R-95**	**95.0**	**99.92**	**99.16 --- 100.69**	**105.18**	**1.07**
Lab- B	**R-95**	**95.0**	**104.04**	**103.68 --- 104.39**	**109.51**	**0.48**
Lab- C	**R-95**	**95.0**	**99.61**	**99.21 --- 100.01**	**104.85**	**0.57**
Lab- D	**R-95**	**95.0**	**100.07**	**99.70 --- 100.43**	**105.33**	**0.51**

Total Steviol Glycosides

Laboratory	*Sample*	*Target %*	*Mean %*	*95% Confidence Interval of the Mean*	*Accuracy (% Recovery)*	*Precision (% RSD)*
Lab- A	**R-75**	**95.0**	**93.88**	**93.02 --- 94.74**	**98.82**	**1.28**
Lab- B	**R-75**	**95.0**	**94.85**	**94.57 --- 95.13**	**99.84**	**0.41**
Lab- C	**R-75**	**95.0**	**94.03**	**93.69 --- 94.38**	**98.98**	**0.51**
Lab- D	**R-75**	**95.0**	**97.46**	**97.03 --- 97.90**	**102.59**	**0.63**

Laboratory	*Sample*	*Target %*	*Mean %*	*95% Confidence Interval of the Mean*	*Accuracy (% Recovery)*	*Precision (% RSD)*
Lab- A	**R-95**	**95.0**	**100.16**	**98.59 --- 101.74**	**105.43**	**2.20**
Lab- B	**R-95**	**95.0**	**104.56**	**104.17 --- 104.96**	**110.07**	**0.53**
Lab- C	**R-95**	**95.0**	**100.48**	**100.09 --- 100.87**	**105.77**	**0.54**
Lab- D	**R-95**	**95.0**	**100.91**	**100.55 --- 101.28**	**106.23**	**0.51**

Assay Method for Rebaudioside A and other Steviol Glycosides
Determine the percentages of Reb A and other steviol glycosides by high pressure liquid chromatography (HPLC).

Standards
a) Primary standards: Reb A, > 95.0% adjusted purity and Stevioside, > 95% adjusted purity (available from ChromaDex, Inc. Santa Ana, CA 92705 USA)
b) Retention time markers: Steviolbioside, Rebaudioside B, Rebaudioside C, Dulcoside A (all available from ChromaDex, Inc. Santa Ana, CA 92705 USA)
Store all standards in desiccators.

Mobile phase
Prepare acidified HPLC-grade water (pH 3.0) by addition of H_3PO_4 (85% reagent grade). Final solution contains 150 mg/L acid. Filter through 0.22 µm Millipore filter. Using HPLC pumping system, mix HPLC grade Acetonitrile and acidified water (80:20).

Sample Diluent
Mix HPLC grade water and 200-proof ethanol (50:50).

Standard solutions
(a) Reb A Stock Solution: Accurately weigh 50 mg Reb A standard into a 10-mL volumetric flask. Dissolve and dilute to volume with ethanol-water sample diluent. Correct for adjusted purity of Reb A reference standard per COA.
Prepare working dilutions:

Level 1 (~3000 mg/L Reb A): 3.0 mL stock diluted to 5 mL with sample diluent

Level 2 (~4000 mg/L Reb A): 4.0 mL stock diluted to 5 mL with sample diluent

Level 3 (~5000 mg/L Reb A): use Reb A stock solution undiluted.

(b) Stevioside Stock Solution: Accurately weigh 50 mg stevioside standard into a 100-mL volumetric flask. Dissolve and dilute to volume with ethanol-water sample diluent. Correct for adjusted purity of stevioside reference standard per COA.
Prepare working dilutions:

Level 1 (~50 mg/L stevioside): 10.0 mL stock diluted to 100 mL with sample diluent

Level 2 (~100 mg/L stevioside): 10.0 mL stock diluted to 50 mL with sample diluent

Level 3 (~500 mg/L stevioside): use stevioside stock solution undiluted

(c) Steviol Glycoside retention time marker **M6**:
Prepared by dissolving approximate amounts of steviol glycosides in 25 mL ethanol-water diluent:

Reb A 50 mg, Rebaudioside C 5 mg, Steviolbioside 5 mg, Dulcoside A 5 mg, stevioside 25 mg, Rebaudioside B 5 mg

Sample solution
Accurately weigh 500 mg of dried (105°C, 2 h) sample into a 100-mL volumetric flask. Dissolve and dilute to volume with ethanol-water sample diluent.

Chromatography Conditions
Column: Amino-based stationary phase bonded to silica: Supelcosil LC-NH2 (length: 15-30 cm; inner diameter: 3.9-4.6 mm), or equivalent.

Mobile phase: 80:20 mixture of acetonitrile and acidified (pH 3.0) water (see above)
Flow rate: 1.50 mL/min
Injection volume: 10 µL
Detector: UV at 210 nm
Column temperature: 40°C

Procedure
Equilibrate the instrument by pumping mobile phase through column until a drift-free baseline is obtained. Confirm baseline separation of 6 steviol glycosides using retention time marker M6. Before starting analysis, condition instrument by injecting level 1 standard twice, disregarding results.

Calibrate instrument by injecting stevioside standard levels 1-3 in triplicate (total injections = 9).

Level 1 -3 injections
Level 2 -3 injections
Level 3 -3 injections

Repeat calibration procedure for Reb A standards levels 1-3.

Measure Reb A and stevioside peak areas.

Perform linear regression on 9 calibration data points each for stevioside and Reb A:

x = peak area,
y = amount of stevioside or Reb A (mg/L)
by definition, y intercept = 0

For Reb A and Stevioside, response factor (RF) = slope of regression line from respective calibration data:

$y = \mathbf{RF} * x$

Measure peak areas for the seven Steviol Glycosides from sample solution (minor components might not be detected).

Calculate percentage of Reb A in the sample:

$$\% \text{ Reb A} = \frac{[RF_{\text{Reb A}}]}{W} \times (Ax_{\text{Reb A}}) \times 10$$

where

$RF_{\text{Reb A}}$ is the response factor for Reb A

$Ax_{\text{Reb A}}$ is the peak area for Reb A in the sample solution

W is the amount (mg) of sample in the sample solution (100 mL)

Calculate percentage of stevioside and each of the remaining five Steviol Glycosides:

$$\%X = f_x \times \frac{[RF_{\text{stevioside}}]}{W} \times (Ax) \times 10$$

where

$RF_{\text{stevioside}}$ is the response factor for stevioside

Ax is the peak area of X in the sample solution

W is the total amount (mg) of sample in 100 mL sample solution

$\mathbf{f_x}$ is the ratio of the formula weight of X to formula weight of stevioside: 1.00 (Stevioside), 0.98 (Dulcoside A), 1.18 (Rebaudioside C), 0.80 (Rubusoside), 0.80 (Steviolbioside), and 1.00 (Rebaudioside B).

Sum the percentages of all glycosides to calculate total steviol glycosides (TSG)

TSG = % Reb A + % Stevioside + % Rebaudioside B + % Rebaudioside C + % Steviolbioside + % Dulcoside A + % Rubusoside.

Steviol

Steviol is assayed by an LC-MS/MS method similar to the one published in the literature (Bazargan et al., 2007).

Appendix I.-6: Pesticide Declaration and Analyses

FROM : FAX NO. : 2006. 12. 12 20:01 P1

赣州菊隆高科技食品有限公司
Ganzhou Julong High-Tech Food Industry Co., Ltd.

GUARANTEE LETTER

December 07, 2006

To Whom It May Concern:

The Company guarantees, that during the production of Stevia Extract no any Genetically Modified microorganisms or Materials from Genetically Modified plants are used.

No any pesticides are used during the cultivation of Stevia plant and the final product is pesticide-free.

In Stevia Extract none of the following products are included: allergens, such as egg, wheat, milk, buckwheat, peanuts, abalone, cuttlefish, salmon roe, shrimp(lobster), orange, crab, kiwi fruit, cattle meat, walnut, salmon, mackerel, soy bean, chicken, pork, matsutake mushroom, peach, yam (Dioscorea japonica), apple and gelatin.

Yours sincerely

GANZHOU JULONG HIGH-TECH FOOD INDUSTRY COMPANY LTD 赣州菊隆高科技食品有限公司

MR. LI SHAN WANG

地址：江西省赣县祥塘工业区
Add: yangtang Industry Zone,Gan county,Jiangxi Provice
电话 Tel: 86-797-4601621 http://www.gzstevia.com
传真 Fax: 86-797-4601663 E-mail: gzstevia@yahoo.com.cn
邮编 Post: 341100

2007 年　5 月　1 日

Messrs.Stevian Biothechnology Corp. Sdn. Bhd

株式会社キューサイ分析研究所
中央研究所
担当　瀬戸

〒811-3422 福岡県宗像市王丸411－1
TEL 0940－37－3988
FAX 0940－37－3991

書類送付のご案内

拝啓　時下ますますご清栄のこととお慶び申し上げます。
下記の通り、成績書をお送り致しますので、ご査収の上、
宜しくお取り計らい下さいますようお願い申し上げます。
尚、請求書は別途お送りさせていただきます。

敬具

記

検査成績書　　受付番号 2007042311

以上

Analysis report

receipt No. 2007042311-01-1
report date 27-Apr-07

Messrs. Stevian Biothechnology Corp. Sdn. Bhd

Q'SAI Analysis and Research Center Co.Ltd.
441-1 Omaru Munakata-shi Fukuoka Pref.
Tel 81-940-37-3988
Fax 81-940-37-3991

seal	seal	seal
横下	入江	瀬戸

Title

Simultaneous analysis 110 agricultural chemicals

Analysis Results

1.Sample
Swetener Powder DR0307020

2.Analysis item
Simultaneous analysis 110 agricultural chemicals

3.Analysis method
See the attached sheets of paper.

4.Analysis Results
See the attached sheets of paper.

No. 2007042311-01-1 P. 1/3

Table 1 analysis results (ppm)

Agricultural chemical	Result	Limit of detection	Analysis method	Agricultural chemical	Result	Limit of detection	Analysis method	Agricultural chemical	Result	Limit of detection	Analysis method	Agricultural chemical	Result	Limit of detection	Analysis method
BHC	N. D.	0.01	*1	ENDRIN	N. D.	0.01	*1	SILAFLUOFEN	N. D.	0.01	*1	PYRACLOFOS	N. D.	0.01	*1
α-BHC	N. D.	0.01	*1	CADUSAFOS	N. D.	0.01	*1	DIAZINON	N. D.	0.01	*1	PYRAFLUFEN ETHYL	N. D.	0.01	*1
β-BHC	N. D.	0.01	*1	CARBARYL	N. D.	0.01	*1	THIOBENCARB	N. D.	0.01	*1	PYRIDABEN	N. D.	0.01	*1
γ-BHC	N. D.	0.01	*1	QUINALPHOS	N. D.	0.01	*1	THIOMETON	N. D.	0.01	*1	PYRIFENOX	N. D.	0.01	*1
δ-BHC	N. D.	0.01	*1	KRESOXIM-METHYL	N. D.	0.01	*1	DIELDRIN	N. D.	0.01	*1	PYRIPROXYFEN	N. D.	0.01	*1
DDT	N. D.	0.01	*1	CHLORPYRIFOS	N. D.	0.01	*1	THENYLCHLOR	N. D.	0.01	*1	PIRIMICARB	N. D.	0.01	*1
p,p'-DDD	N. D.	0.01	*1	CHLORPHENAPYR	N. D.	0.01	*1	TEBUCONAZOLE	N. D.	0.01	*1	PYRIMIDIFEN	N. D.	0.05	*1
p,p'-DDE	N. D.	0.01	*1	CHLORFENVINPHOS	N. D.	0.01	*1	TEBUFENPYRAD	N. D.	0.01	*1	PIRIMIFOS-METHYL	N. D.	0.01	*1
o,p'-DDT	N. D.	0.01	*1	CHLORPROPHAM	N. D.	0.01	*1	TEFLUTHRIN	N. D.	0.01	*1	PYRIMETHANIL	N. D.	0.01	*1
p,p'-DDT	N. D.	0.01	*1	CHLOROBENZILATE	N. D.	0.01	*1	DELTAMETHRIN	N. D.	0.01	*1	FIPRONIL	N. D.	0.01	*1
EPN	N. D.	0.01	*1	CYANAZINE	N. D.	0.01	*1	TERBUFOS	N. D.	0.01	*1	FENARIMOL	N. D.	0.01	*1
ACRINATHRIN	N. D.	0.01	*1	DIETHOFENCARB	N. D.	0.01	*1	TRIADIMENOL	N. D.	0.01	*1	FENITROTHION	N. D.	0.01	*1
ACEPHATE	N. D.	0.5	*1	DICHLORVOS	N. D.	0.01	*1	TRIAZOPHOS	N. D.	0.01	*1	FENOBUCARB	N. D.	0.01	*1
AZOXYSTROBIN	N. D.	0.01	*1	DICOFOL	N. D.	0.01	*1	TRICHLAMIDE	N. D.	0.01	*1	FENSULFOTHION	N. D.	0.01	*1
ALACHROL	N. D.	0.01	*1	CYHALOTHRIN	N. D.	0.01	*1	TRIFLUMIZOLE	N. D.	0.01	*1	FENTHION	N. D.	0.01	*1
ALDRIN	N. D.	0.01	*1	CYHALOFOP-BUTYL	N. D.	0.01	*1	TRIFLURALIN	N. D.	0.01	*1	FENVALERATE	N. D.	0.01	*1
ISOFENPHOS	N. D.	0.01	*1	DIFENOCONAZOLE	N. D.	0.01	*1	TOLCLOPHOS-METHYL	N. D.	0.01	*1	FENPROPATHRIN	N. D.	0.01	*1
IPRODIONE	N. D.	0.01	*1	CYFLUTHRIN	N. D.	0.01	*1	PACLOBUTRAZOL	N. D.	0.01	*1	BUTAMIFOS	N. D.	0.01	*1
IMAZALIL	N. D.	0.05	*1	DIFLUFENICAN	N. D.	0.01	*1	PARATHION	N. D.	0.01	*1	FLUDIOXONIL	N. D.	0.01	*1
UNICONAZOLE-P	N. D.	0.01	*1	CYPROCONAZOLE	N. D.	0.01	*1	PARATHION-METHYL	N. D.	0.01	*1	FLUCYTHRINATE	N. D.	0.01	*1
ETHIOFENCARB	N. D.	0.01	*1	CYPERMETHRIN	N. D.	0.01	*1	HALFENPROX	N. D.	0.01	*1	FLUSILAZOLE	N. D.	0.01	*1
ETOXAZOLE	N. D.	0.01	*1	DIMETHIPIN	N. D.	0.01	*1	BIORESMETHRIN	N. D.	0.01	*1	FLUTOLANIL	N. D.	0.01	*1
ETHOFENPROX	N. D.	0.01	*1	DIMETHYLVINPHOS	N. D.	0.01	*1	BITERTANOL	N. D.	0.01	*1	FLUVALINATE	N. D.	0.01	*1
ETHOPROPHOS	N. D.	0.01	*1	DIMETHENAMID	N. D.	0.01	*1	BIFENOX	N. D.	0.01	*1	PROCYMIDONE	N. D.	0.01	*1
ETRIMFOS	N. D.	0.01	*1	DIMETHOATE	N. D.	0.01	*1	BIFENTHRIN	N. D.	0.01	*1	PROTHIOFOS	N. D.	0.01	*1

Analysis method : *1 GC/MS

※ N.D. : not detected

No. 200042311-01-1 P. 2/3

Table 1 (continued) analysis results

(ppm)

Agricultural chemical	Result	Limit of detection	Analysis method	Agricultural chemical	Result	Limit of detection	Analysis method	Agricultural chemical	Result	Limit of detection	Analysis method	Agricultural chemical	Result	Limit of detection	Analysis method
PROPAMOCARB	N. D.	0.1	*1												
PROPICONAZOLE	N. D.	0.01	*1												
HEXACONAZOLE	N. D.	0.01	*1												
PERMETHRIN	N. D.	0.01	*1												
PENCONAZOLE	N. D.	0.01	*1												
BENDIOCARB	N. D.	0.01	*1												
PENDIMETHALIN	N. D.	0.01	*1												
PHOSALONE	N. D.	0.01	*1												
FOSTHIAZATE	N. D.	0.01	*1												
MALATHION	N. D.	0.01	*1												
MYCLOBUTANIL	N. D.	0.01	*1												
METHAMIDOPHOS	N. D.	0.05	*1												
METHIOCARB	N. D.	0.01	*1												
METOLACHLOR	N. D.	0.01	*1												
METRIBUZIN	N. D.	0.01	*1												
MEFENACET	N. D.	0.01	*1												
MEPRONIL	N. D.	0.01	*1												
LENACIL	N. D.	0.01	*1												
From here down blank															

Analysis method : *1 GC/MS

※ N.D.:not detected

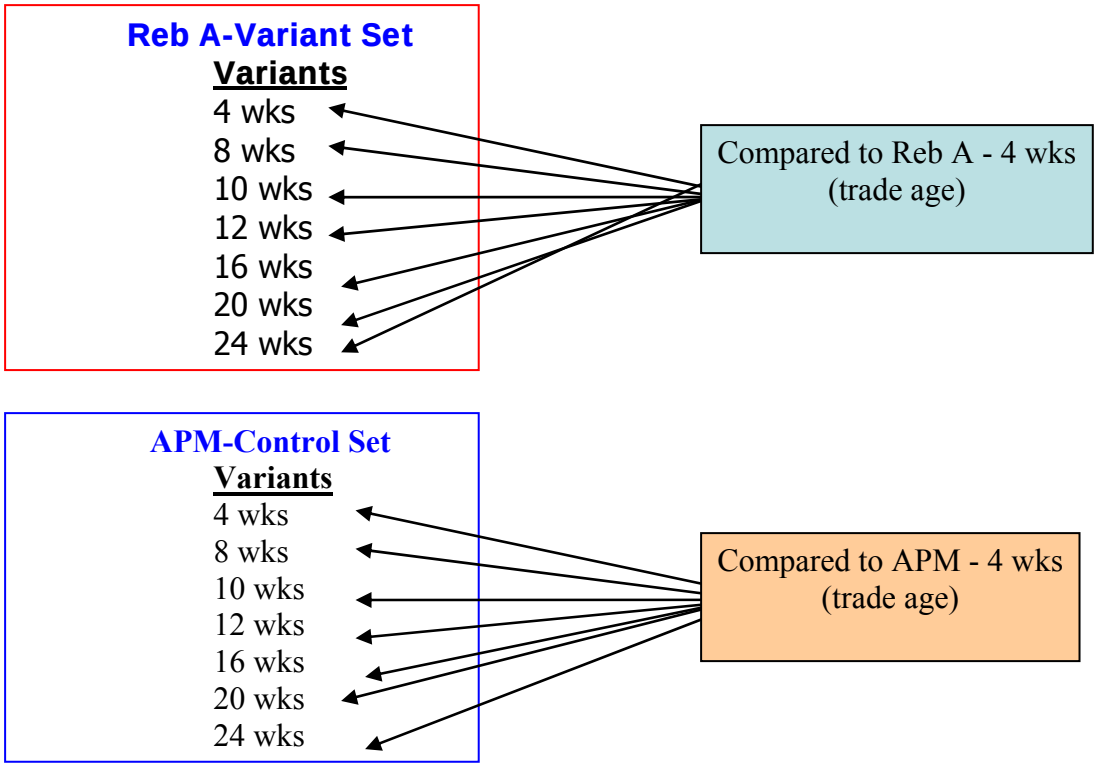
No. 200042311-01-1 P. 3/3

Appendix II: Technical Function and Self-Limiting Use

Appendix II.-1: Reb A Beverage Sensory Study Methodology

Appendix II.-1: Reb A Beverage Sensory Study Methodology

Study Plan



Reb A Beverage Sensory Study – Test Samples

All products were produced on the bench and stored at 70°

SAMPLE DESCRIPTION		AGE
1	Diet cola Control	4 Weeks
2	Diet cola Control	8 Weeks
3	Diet cola Control	10 Weeks
4	Diet cola Control	12 Weeks
5	Diet cola Control	16 Weeks
6	Diet cola Control	20 Weeks
7	Diet cola Control	24 Weeks

SAMPLE DESCRIPTION		AGE
1	Diet cola Reb A	4 Weeks
2	Diet cola Reb A	8 Weeks
3	Diet cola Reb A	10 Weeks
4	Diet cola Reb A	12 Weeks
5	Diet cola Reb A	16 Weeks
6	Diet cola Reb A	20 Weeks
7	Diet cola Reb A	24 Weeks

Reb A Beverage Sensory Methodology

Double Triangle Test

- Randomized complete block design
- # of panelists: 48
- # of evaluations: 2
- Rinsing materials: Unsalted crackers and purified water

Test Protocol

- Panelists were presented with three samples and asked to choose the different sample. This process was then replicated. Replication allows us to show respondent repeatability to correctly choose the odd sample. It also increases statistical power while allowing for smaller sample sizes. Open-ended comments were collected and used for those panelists who correctly identified the "odd" sample.

- Criterion for all significant differences: $p \leq 0.05$

Statistical Analysis

- Beta-Binomial analysis: binomial distribution variance & people variance

Statistical Packages

- Sample randomization was conducted using Compusense 5
- Beta-Binomial analysis was conducted using ART (Advanced Research Tool)

Appendix III: Stability

Appendix III.-1: HPLC Assay for Reb A in Tabletop Products

Appendix III.-2: Forced Degradation of Reb A Powder

Appendix III.-3: Stability of Reb A in Carbonated Beverages and Model Solutions

Appendix III.-4: Stability of Reb A in Aqueous Citric Acid Matrix

Appendix III.–1: HPLC Assay for Reb A in Tabletop Products

Document No: CM121 - NA4444-07

1.0 PURPOSE

To test for Reb A in final products using high performance liquid chromatography (HPLC).

2.0 SCOPE

This method applies to the determination of Reb A using HPLC.

3.0 PRINCIPLE OF METHOD

Reb A assay is determined by reverse phase high performance liquid chromatography.

Samples are prepared in the HPLC mobile phase at a concentration of approximately 0.7 mg / mL and compared to a Reb A standard curve containing samples above and below the same concentration.

4.0 INTERFERENCES

N/A

5.0 SAFETY

All personnel must comply with and adhere to all standard and recognized laboratory and plant safety practices, policies, and procedures.

6.0 ENVIRONMENTAL

All wastes generated in the execution of this test method will be accumulated in a well-labeled container prior to pick-up by an approved hazardous waste acceptor.

7.0 EQUIPMENT

7.1 Analytical Balance accurate to 0.01 mg or equivalent.

7.2 Column: Agilent Zorbax 5µm Spherical NH_2 4.6mm X 250 mm or equivalent.

7.3 Liquid Chromatograph detection equipment including pump, autosampler, detector & integrator.

7.4 Glass Auto-sampler vials

7.5 Volumetric glassware – assorted Class A volumetric glassware and pipettes

7.6 Graduated cylinder

7.7 Vacuum filter apparatus

7.8 0.45µm, 47mm diameter filters or equivalent.

8.0 REAGENTS AND TEST MATERIALS

8.1 Acetonitrile (HPLC Grade)

8.2 Water (HPLC Grade)

8.3 Phosphoric Acid

8.4 Reb A reference material for use in Standard preparation. Available from Chromadex (www.chromadex.com) (1-949-419-0288) as part # 00018226.

9.0 PROCEDURE

9.1 Mobile Phase Preparation

9.1.1 Acetonitrile/Water (80/20) –Prepare a mixture of acetonitrile and water (80/20). Make adjustments if necessary to maintain retention time of peak of interest. Mix well with a stirbar, and transfer to a suitable, labeled, mobile phase reservoir and vacuum degas.

9.1.2 As needed, filter through a 0.45-micron membrane filter, using vacuum filtration apparatus.

9.1.3 Adjust pH to 3.0 using phosphoric acid.

9.2 **Standard Curve Preparation**

9.2.1 Accurately weigh 15.00 mg of the solid Reb A reference standard to the nearest 0.1-mg into a clean dry 10-mL class A volumetric flask.

9.2.2 Add 20% ACN diluent to the 10mL mark and dissolve using sonication if necessary.

9.2.3 The resulting solution should be about 1.5 mg/mL Reb A.

9.2.4 Calculation of exact concentration:

Reb A mg/mL = [Weight Reb A (mg) x decimal % purity]/10 mL

Example: 15.13 mg of a 98.5% Reb A standard diluted to 10 mL total volume would result in a stock solution of 1.4903 mg/mL Reb A. Reb A mg/mL = (15.13 X 0.9850)/10 = 1.4903 mg/mL Reb A.

9.2.5 If cloudy, filter a portion through a 0.45 micron filter prior to injection

9.2.6 Standard solutions may be used for up to 5 days after preparation.

9.2.7 Prepare a dilution series following the table below.

Standard Identification	Amount Reb A	Volumetric Flask	Reb A (mg/mL)
STD A	15mg +/- 1 mg	10 mL	1.5000
STD B	5 mL STD A	10 mL	0.7500
STD C	5 mL STD B	10 mL	0.3750
STD D	5 mL STD C	10 mL	0.1875
STD E	5 mL STD D	10 mL	0.9375
Control A	10 mg +/- 1 mg	10 mL	0.1000
Control B	5 mL Control A	10 mL	0.0500

9.3 **Sample Preparation**

9.3.1 Weigh an appropriate amount of sample to give a ~0.6 mg/mL final concentration of Reb A.

9.3.2 Dissolve in 20/80 ACN/Water by filling to approximately 50% of the flask volume and mixing using a vortex mixer followed by sonication for about
1-2 minutes. Allow solution to cool to ambient temperature and fill the flask to the QS mark with filtered water and vortex to mix.

9.4 **System Preparation**

9.4.1 If the HPLC system flow rate is zero, perform a "wet prime" for the mobile phase.

9.4.2 Set the system to pump 100% from the 80/20 Acetonitrile/ Water reservoir.

9.4.3 Gradually increase the HPLC flow rate up to 2.0 mL/min.

9.4.4 Allow the system to equilibrate for 15 – 20 minutes if the system was not pumping.

9.4.5 HPLC Conditions:

Column: Agilent Zorbax 5µm Spherical NH_2 4.6mm x 250 mm or equivalent
Mobile phase: 80% HPLC Grade Acetonitrile / 20% HPLC Grade Water pH=3.0
Flow rate: 2.0 mL/min.
Detector: Set wavelength to 210
Inj. Vol: 10 µl
Run time: 10 min. (for all samples and standards)

9.5 **System suitability check**:

9.5.1 Create a standard curve using standards A-E immediately preceding analysis of any final samples for release. The correlation coefficient (r^2) must be 0.9990 – 1.0000. Calculate the controls A and B as samples using the standard curve. Compare calculations to know concentration (adjusted for purity). The difference must be no more than 5%. If system suitability check fails to meet the acceptance criteria, contact QA Manager or designee for corrective action.

9.5.2 Inject controls A and B as samples at the beginning of sample set and after every ten-sample injections.

9.6 **Test Procedure**

9.6.1 Inject single 10 ul injections of the sample solution.

9.6.2 Report percent Reb A from chromatography data system printout, or
calculate the percent Reb A in each sample using the calculations in Section 10.

9.7 **Peak Identification**

9.7.1 The retention time of the principal peak in the chromatogram of the assay preparation is approximately 8.1 minutes.

10.0 CALCULATIONS AND INTERPRETATIONS

10.1 Reb A Weight Percent:

$$\text{w/w Reb A \%} = [(A_X - Y) / m] / C_X$$

where A_X = Sample x peak area of interest
Y = Y intercept of standard curve
m = Slope of standard curve
C_X = Concentration of Sample

11.0 REPORTING/CONCLUSIONS

Results should be reported to four significant digits (i .e. 98.04%).

Report identity based on retention time comparison to reference.

Appendix III.-2: Forced Degradation of Reb A Powder

OBJECTIVE

The purpose of this study was to assess the chemical stability of Rebaudioside A (Reb A) when exposed to prolonged high heat conditions.

The study had two goals:

1. Compare the amount of Reb A, Stevioside, Reb C, and Dulcoside A present in samples of Reb A before and after exposing samples to high heat.
2. Determine if any degradation components can be identified after exposing samples to high heat

MATERIALS USED IN EXPERIMENT

Test Article: PureCircle Reb A (Batch #SR1107046)
HPLC Standards:
Rebaudioside A - ChromaDex Inc. Part # 00018226
Stevioside - ChromaDex Inc. Part # 00019351
Rebaudioside C - ChromaDex Inc. Part # 00018228
Dulcoside A - ChromaDex Inc. Part # 00004949

DESIGN OF THE STUDY

Samples were taken from two separate packages (Sample #1 and Sample #2) of the same batch of powdered Reb A material. Approximately 10g of each sample were placed in a laboratory oven maintained at 105°C for 4 days (96 hours). At the end of the four day heating period, the samples were removed from the oven and allowed to cool in a desiccator prior to chemical analyses. Oven samples were designated as "heated", whereas ambient samples were designated as "fresh". Table 1 shows the sample matrix. Two individual samples from each bag / condition were weighed into volumetric flasks for chemical analysis, creating a total of 8 samples (4 fresh / 4 heated).

CHEMICAL METHODOLOGY

Samples were analyzed by HPLC using ultraviolet detection method developed by Merisant Co. The samples were first dissolved in a diluent consisting of 20% acetonitrile and 80% water (v/v), then separated using an Agilent Zorbax 5µm Spherical NH_2 4.6mm

X 250 mm column and analyzed by a Perkin Elmer photodiode array detector at 210 nm. The complete spectral analysis was also collected to analyze peak purity using Perkin Elmer Iris software.

Appendix III.-3: Stability of Reb A in Carbonated Beverages and Model Solutions (Chang and Cook, 1983)

Introduction

Studies were conducted to determine the stability of Reb A in both pure sweetener solutions and carbonated beverages under various storage conditions (Chang and Cook, 1983).

Reb A used in the study was obtained from crude stevioside by preparative column chromatography with subsequent solvent recrystallization; it was then recrystallized twice from methanol to obtain chromatographically pure Reb A. Beverage samples were concentrated 5-fold by flash evaporation at room temperature followed by reconstitution in water.

Stability of Pure Sweetener Solutions

To determine the stability of Reb A in neutral solutions, 2.0-mL samples of aqueous Reb A solution (6.5 mg/mL) were sealed in separate 5-mL glass vials and heated for various time periods at 100ºC. Chemical degradation was assessed by TLC and HPLC analysis.

To determine the stability of Reb A in acidic solutions, Reb A was dissolved in either a citric acid system (0.22% w/v citric acid, 0.0348% w/v sodium citrate, pH 2.6) or a phosphoric acid system (0.04% w/v H_3PO_4, pH 2.4) at 6.5 mg/mL. Samples of the acidic Reb A solutions were prepared as described above and heated to 60 or 100ºC for 137 or 13 hours, respectively.

Stability in Carbonated Beverages

A long-term storage test for Reb A was conducted with carbonated citric acid and phosphoric acid containing beverages (to simulate citrus and cola-type beverages, respectively) prepared with 0.1% of Reb A. Unsweetened carbonated beverages were also prepared and served as the control. Samples of each beverage were stored in tightly

closed glass bottles at 4ºC, room temperature (22ºC) and 37ºC for up to 5 months. Sweetener loss and subsequent degradation products were analyzed by HPLC and TLC. Beverage samples were placed in an oven at 60ºC for 137 hours to determine the effects of short-term exposure to high temperatures. Additional samples were placed outdoors (at 10-25ºC) for approximately one week to evaluate the effect of sunlight (i.e. a reported exposure equal to 3000 langleys of sunlight).

Results for Pure Sweetener Solutions

Results of the HPLC and TLC analyses indicated that prolonged heating of the neutral Reb A solution (6.5 mg/mL) at 100ºC resulted in a decrease in the Reb A concentration (i.e. 68.5 % recovery at 48 hours) and the appearance of rebaudioside B and glucose as the degradation products.

Results for Acidic Solutions

Acidic Reb A solutions heated to 60ºC for up to 137 hours did not demonstrate appreciable degradation of Reb A; however, heating to 100ºC resulted in considerable degradation (i.e. 13.2 and 24.1 % recovery after 13 hours in the phosphoric or citric acid systems, respectively).

Citric acid Reb A solutions heated to 100ºC resulted in one unknown degradation product at 4 hours and two unknown degradation products at 10 and 14 hours. A third unknown degradation product was detected during 4-10 hours of heating in the phosphoric acid system, but not at 13 hours. Rebaudioside B and glucose were present during heating of both acidic solutions.

Results for Carbonated Beverages

Reb A showed no significant degradation during 4 months of storage at 4ºC, 3 months at room temperature (22ºC), or 1 month at 37ºC in carbonated citric or phosphoric acid beverages. Although a decrease in Reb A concentration was observed by HPLC (i.e. 13-25% degradation after 4 months of storage at 37ºC), no degradation products were observed.

Minimal reduction in Reb A concentration was observed after storage at 60ºC for 137 hours (i.e. 3% and 6% degradation in citric and phosphoric acid beverages, respectively); however, substantial degradation did occur when beverage samples were exposed to 3000 langleys of sunlight (i.e. 22 and 18% degradation in the phosphoric and citric acid beverages, respectively). A single unidentified degradation product was detected after storage of the Reb A beverage samples in sunlight for one week.

Summary

Reb A in carbonated citric or phosphoric acid beverages showed no significant degradation during prolonged storage at refrigeration, normal ambient or elevated ambient temperatures. However, minimal reduction in Reb A concentration was observed after storage at extremely high temperature for carbonated citric or phosphoric acid beverages. In model neutral or acidic test solutions, prolonged heating at 100ºC resulted in a decrease in the Reb A concentration, but for prolonged heating at 60ºC degradation of Reb A was minimal.

Appendix III.-4: Stability of Reb A in an Aqueous Citric Acid Matrix (Stetson, 2008a-c [Unpublished])

Introduction

The pH range for the beverages for which Reb A is foreseen as a sweetener is from pH 2.8 to 3.2 and recommended storage temperature is ambient. Therefore, the experiments conducted at pH 3 and 70°F in the following summary are most pertinent to the intended condition of use for these products. However, results also are reported for extreme environmental conditions of temperature ≥ 90ºF and pH < 3. The Reb A commercial products are referred to by their brand names throughout this summary in order to distinguish between the results for each product.

Methods

In an FDA GLP (21 CFR Part 58) compliant study (Stetson, 2008a [Unpublished]), Reb A concentrations in aqueous solutions containing two purified Reb A commercial products (Chrysanta® 99-P or Rebaudioside A-98%) at an initial concentration of 600 µg/mL were determined analytically. A high performance liquid chromatography method using ultraviolet absorbance detection at a wavelength of 212 nm for the determination of Reb A concentration in aqueous solutions ranging in concentration from 200 to 800 µg/mL was validated in a previous study (Stetson, 2008b [Unpublished]). In a separate study (Stetson, 2008c [Unpublished]), the assay validation was extended to include the determination of Reb A concentrations from 25.0 to 800 µg/mL. Also in this study (Stetson, 2008c [Unpublished]), a high performance liquid chromatography tandem mass spectrometry method in the negative electrospray ionization mode for the determination of rebaudioside B, stevioside, steviolbioside, and steviol concentrations in aqueous formulations containing rebaudioside B, stevioside and steviolbioside ranging in concentration from 0.050 to 50.0 µg/mL and steviol ranging in concentration from 0.0050 to 5.0 µg/mL was validated. An internal standard method of quantitation was used in both assays with 4-chlorosalicylic acid as the internal standard.

Purity and Composition of Three Reb A Products

Reb A, rebaudioside B, stevioside, steviolbioside, and steviol concentrations in three purified Reb A commercial products (Chrysanta® 99-P, Rebaudioside A-92% and Rebaudioside A-98%) were identified and quantitated (Stetson, 2008a [Unpublished]). Rebaudioside B and stevioside were the contaminants present at the highest concentrations. Steviol was present in the preparations in only trace amounts. Because there were no other significant chromatographic peaks present in these analyses, the percent of the sum of the Reb A, rebaudioside B, stevioside, steviolbioside and steviol concentrations represented by Reb A is a reliable estimate of purity. These results support the reported test article purity values for Chrysanta® 99-P (Certificate (C of A) purity = 95.7%), Rebaudioside A-98% (C of A purity = 99.5%) and Rebaudioside A-92% (C of A purity = 96.2%). The results are summarized in Table III.-3 below.

Table III.-3: Purity of Analyzed Reb A Commercial Products

	Concentration, µg/mL and (% of total)				
Reb A Commercial Product	**Rebaudioside A**	**Rebaudioside B**	**Stevioside**	**Steviolbioside**	**Steviol**
Chrysanta® 99-P	579 (96.90)	11.3 (1.89)	7.03 (1.18)	0.17 (0.028)	0.00534 (0.00089)
Rebaudioside A-98%	618 (98.70)	6.00 (0.96)	2.08 (0.33)	0.064 (0.010)	*0.00073 (0.00012)*
Rebaudioside A-92%	608 (99.03)	4.78 (0.78)	1.16 (0.19)	*0.019 (0.0031)*	*0.00021 (0.000034)*

Note: Italics indicate extrapolated values < LLOQ (lower limit of quantification).

Stability in an Aqueous Citric Acid Matrix

Solutions of two purified Reb A commercial products (Chrysanta® 99-P and Rebaudioside A-98%), prepared at nominal concentrations of 0.60 mg Reb A/mL in aqueous citric acid solutions (50mM) at various pH values (2.0, 4.0 and 7.0 ± 0.5), were sterile filtered using 0.22 µm filters and stored for up to 15 weeks at approximately 40, 70, 90 or 110 ± 5 ºF. Samples were collected and analyzed at 0, 1, 3, 5, 7, 9, 11, 13 and 15 weeks of incubation (Stetson, 2008a [Unpublished]). In a second study, solutions of Chrysanta® 99-P prepared at nominal concentrations of 0.60 mg Reb A/mL in aqueous

citric acid (50mM) solutions at various pH values (2.5, 3.0 and 3.5 ± 0.5), were sterile filtered using 0.22 µm filters and stored for up to 25 weeks at approximately 40, 70, 90 or 110 ± 5 ºF. Samples were collected and analyzed at 0, 1, 3, 5, 7, 9, 11, 13, 15, 20 and 25 weeks of incubation (Stetson, 2008c [Unpublished]). In both studies the rates of Reb A degradation, as well as the concentrations of rebaudioside B, stevioside, steviolbioside, and steviol contaminants/degradants, were monitored analytically.

During incubation for 15 weeks, no significant Reb A degradation was detected in 1) the Chrysanta® 99-P and Rebaudioside A-98% pH 7 solutions at 40, 70, 90 or 110ºF, 2) the Chrysanta® 99-P and Rebaudioside A-98% pH 4 solutions at 40, 70 or 90ºF and 3) the Chrysanta® 99-P and Rebaudioside A-98% pH 2 solutions at 40ºF. However, solutions prepared at pH 4 and incubated at 110ºF, or at pH 2 and incubated at 70, 90 and 110ºF, resulted in significant Reb A degradation. The expected effect of increasing incubation temperature on Reb A degradation rate was apparent, i.e. the half-life values for Reb A in the Chrysanta® 99-P solutions at pH 2 and stored at 70, 90 and 110ºF were 67.3, 12.3 and 3.2 weeks, respectively. The solution pH also had a significant effect on Reb A degradation. The half-life values for Reb A in the Chrysanta® 99-P solutions at pH 2 and pH 4 and stored at 110ºF were 3.2 and 126 weeks, respectively. There were no significant differences in the stability (or instability) of Reb A observed with solutions of Chrysanta® 99-P or Rebaudioside A-98% (Stetson, 2008a [Unpublished]). In addition, during incubation for 25 weeks, Chrysanta® 99-P solutions prepared at pH = 2.5, 3.0 and 3.5 and incubated at 70, 90 and 110ºF resulted in Reb A degradation (Stetson, 2008c [Unpublished]). Just as in the previous study (Stetson, 2008a [Unpublished]), the expected effect of increasing incubation temperature on Reb A degradation rate occurred, i.e. the half-life values for Reb A in the Chrysanta® 99-P solutions at pH 2.5 and stored at 70, 90 and 110ºF were 128, 21.9 and 4.7 weeks, respectively. As in the previous study, pH had a significant effect on Reb A degradation. The half-life values for Reb A in the Chrysanta® 99-P solutions at pH = 2.5, 3.0 and 3.5 and stored at 110ºF were 4.7, 10.3 and 29.9 weeks, respectively (Stetson, 2008c [Unpublished]). However, the half-life values for Reb A decreased in the Chrysanta® 99-P solutions at pH=2.5, 3.0 and 3.5 stored at 40ºF, which was opposite to the trend demonstrated with all other data. Since

the data collected in the previous study (Stetson, 2008a [Unpublished]) during incubation at 40ºF of Chrysanta® 99-P solutions at pH=2.0, 4.0 and 7.0 resulted in positive degradation constants (no detectable Reb A degradation), it is likely that the trend of the data for the conditions of pH=2.5, 3.0 and 3.5 stored at 40ºF was the result of assay variability, rather than decreasing half-lives. The results are summarized in Table III.-4 below (Stetson, 2008c [Unpublished]).

Table III.-4: Degradation of Analyzed Reb A Commercial Products

		Degradation Constant and ($T_{½}$ in weeks)			
Reb A Commercial Product	**pH**	**40ºF**	**70ºF**	**90ºF**	**110ºF**
Chrysanta® 99-P*	2.0	NSD	-0.0103 (67.3)	-0.0562 (12.3)	-0.2179 (3.18)
Rebaudioside A-98%*	2.0	NSD	-0.0122 (56.8)	-0.0614 (11.3)	-0.2552 (2.72)
Chrysanta® 99-P	2.5	-0.0012 (578)	-0.0054 (128)	-0.0317 (21.9)	-0.1462 (4.74)
Chrysanta® 99-P	3.0	-0.0021 (330)	-0.0047 (147)	-0.0144 (48.1)	-0.0674 (10.3)
Chrysanta® 99-P	3.5	-0.0028 (248)	-0.0002 (347)	-0.0039 (178)	-0.0232 (29.9)
Chrysanta® 99-P*	4.0	NSD	NSD	NSD	-0.0055 (126)
Rebaudioside A-98%*	4.0	NSD	NSD	NSD	-0.0036 (192)
Chrysanta® 99-P*	7.0	NSD	NSD	NSD	NSD
Rebaudioside A-98%*	7.0	NSD	NSD	NSD	NSD

NSD = No significant degradation of Rebaudioside A detected
* = Data from previous study (Stetson, 2008a [Unpublished])

For test article solutions prepared at pH = 2.0, 2.5, 3.0 and 3.5 and incubated at 70, 90 and 110ºF or prepared at pH 4 and incubated at 110ºF, Reb A degradation resulted in two major unidentified degradant peaks in the HPLC/UV chromatograms (Stetson, 2008a,c [Unpublished]). The major degradant peak (i.e. Compound X; identified as iso-Reb A, see p. App-51) eluted within 0.3 minutes following the elution of Reb A. The second degradant peak (i.e. Compound Y; identified as iso-rebaudioside B, see p. App-51) eluted within 0.3 minutes following the elution of rebaudioside B. Initially, Reb A degradation appears to be quantitatively accounted for by the accumulation of Compound X, suggesting that the initial step in Reb A degradation under severe pH and temperature conditions is the formation of Compound X. However, there is a delay in Compound Y appearance and significant accumulation, which appears to be related to the accumulation of Compound X to significant concentration levels. The accumulation of Compound X in solutions at pH 3 during incubation at 110ºF, for example, appears to plateau by weeks 15 to 20. These data would suggest that Compound Y is the direct product of Compound X

degradation. The four following figures (Figures III.-1, 2, 3, and 4) demonstrate the effect of low vs. high temperature at pH 3.0 (Stetson, 2008c [Unpublished]). At pH 3.0 and ambient temperature (i.e. 70°F) or refrigeration (i.e. 40°F) over the 25-week period of the stability study, Reb A degradation is insignificant as demonstrated in the first and second graphs below. It is not until an extreme temperature of 90 or 110°F is sustained for several weeks that significant degradation of Reb A occurs as demonstrated in the third and fourth graphs below.

Figure III.-1: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 3 and 40°F

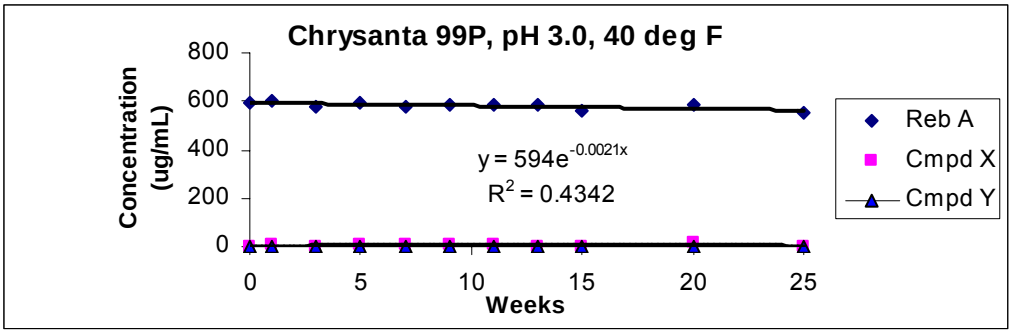


Figure III.-2: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 3 and 70°F

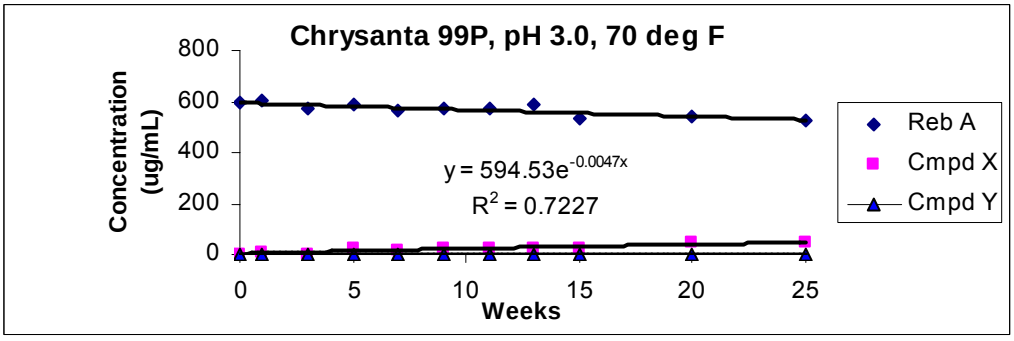


Figure III.-3: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 3 and 90°F

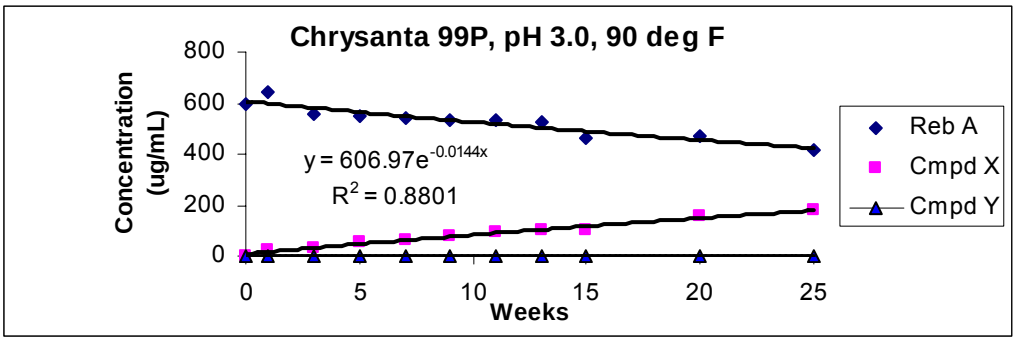
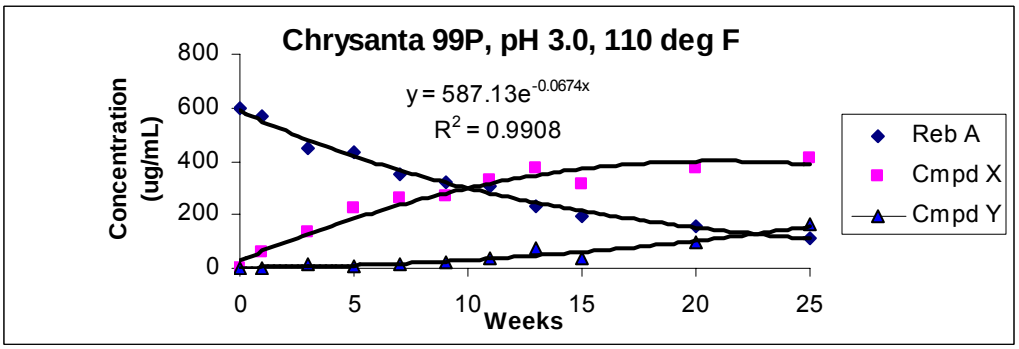
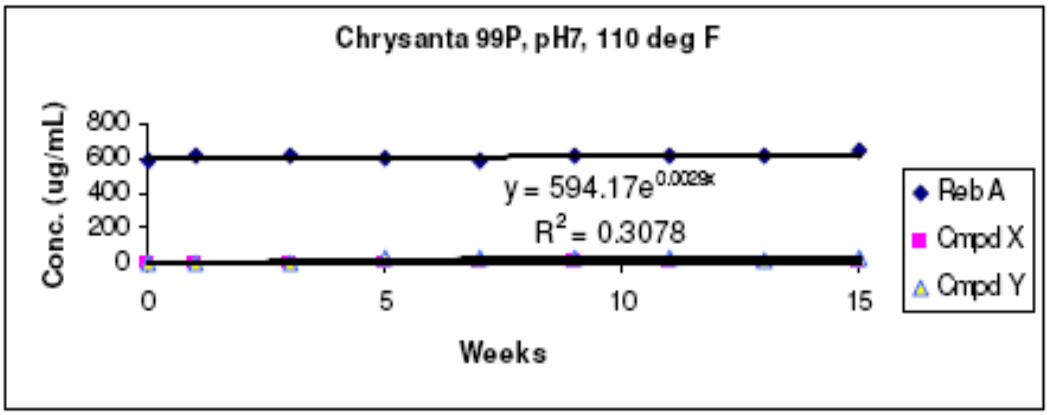


Figure III.-4: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 3 and 110°F



The following figure (Figure III.-5), in comparison to the one directly above, demonstrates that Reb A degradation is more dependent on low pH rather than high temperature (Stetson, 2008a [Unpublished]).

Figure III.-5: Degradation of Reb A Commercial Product (Chrysanta® 99-P) at pH 7 and 110°F



In addition to monitoring Reb A degradation over time, the concentrations of rebaudioside B, stevioside, steviolbioside, and steviol degradants/contaminants were analyzed. The results of these analyses and the range of concentration values (expressed as µg/mL) for rebaudioside B, stevioside, steviolbioside, and steviol generated from samples collected throughout the 15-week incubation period are summarized in Table III.-5 below (Stetson, 2008a [Unpublished]).

Table III.-5: Concentration of Degradants in Analyzed Reb A Commercial Products

			Concentration Range (µg/mL)			
Test Article	**pH**	**T (ºF)**	**Reb B**	**SS**	**SBS**	**Steviol**
Chrysanta® 99-P	2.0	40	9.45-16.8	6.73-11.1	0.173-0.351	*0.0046*-0.0093
		70	11.0-20.7	6.53-11.3	0-1.23	0.0059-0.0133
		90	11.6-23.6	4.13-10.6	0.198-0.526	0.0058-0.0496
		110	11.6-28.4	*0*-4.12	0.190-0.518	0.0061-0.177
Chrysanta® 99-P	4.0	40	10.1-15.2	7.24-9.69	0.191-0.393	*0.0045*-0.0089
		70	10.4-14.6	7.65-9.92	0.191-0.380	0.0058-0.0102
		90	11.0-17.5	6.54-11.7	0.205-0.386	0.0066-0.0117
		110	10.4-27.3	6.09-9.85	0.208-0.556	0.0061-0.0168
Chrysanta® 99-P	7.0	40	11.3-14.0	6.93-10.2	0.190-0.344	0.0067-0.0124
		70	9.26-14.8	6.46-10.7	0.193-0.370	0.0063-0.0134
		90	9.40-17.4	6.03-10.7	0.193-0.367	0.0068-0.0212
		110	12.0-28.5	7.01-10.7	0.203-0.516	0.0067-0.0151
Rebaudioside A-98%	2.0	40	5.34-7.39	2.25-3.30	0.074-0.127	*0*-0.0054
		70	5.80-16.8	1.78-3.42	0.067-0.156	*0.00094*-0.0078
		90	6.45-20.9	1.27-3.20	0.077-0.232	*0.0012*-0.0522
		110	6.55-21.7	0.320-3.50	0.066-0.290	*0.00093*-0.214
Rebaudioside A-98%	4.0	40	4.86-7.91	1.76-3.49	0.068-0.129	*0-0.0041*
		70	5.34-8.00	1.98-3.20	0.067-0.129	*0*-0.0063
		90	5.65-9.95	1.90-3.20	0.066-0.135	*0.00085*-0.0092
		110	6.90-22.2	2.02-3.35	0.083-0.204	*0.00141*-0.0125
Rebaudioside A-98%	7.0	40	4.79-7.35	2.18-3.45	0.0716-0.121	*0.00086*-0.0051
		70	5.23-8.00	2.20-3.25	0.076-0.126	*0.00081-0.0043*
		90	5.15-10.4	2.33-4.10	0.069-0.128	*0.00051*-0.0054
		110	7.35-23.7	1.98-3.35	0.079-0.223	*0.00098*-0.0093

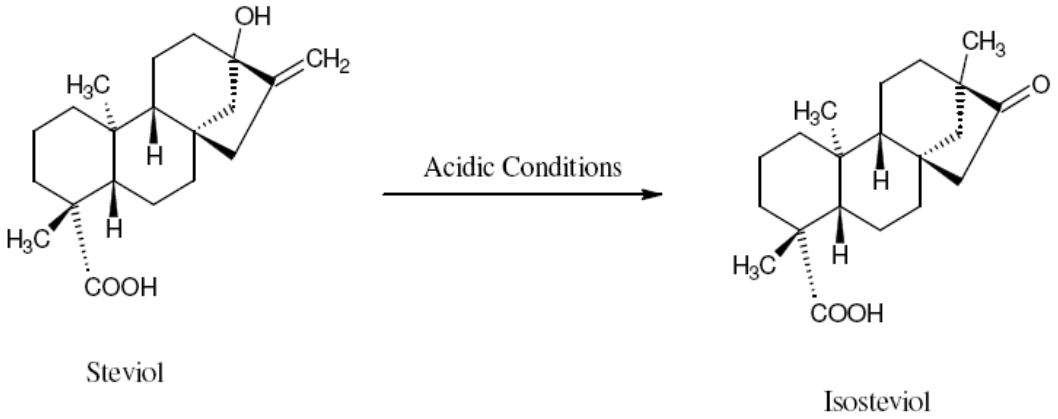
Note: Italics indicate extrapolated values < LLOQ (lower limit of quantification).
Reb B = Rebaudioside B
SS = Stevioside
SBS = Steviolbioside

These data indicate that rebaudioside B, stevioside, steviolbioside and steviol, initially present in both Chrysanta® 99-P and Rebaudioside A-98% in trace amounts, failed to accumulate to significant levels even during incubation of pH 2 test article solutions at 110ºF, suggesting that the degradation of Reb A, which occurs under acidic conditions at elevated temperatures, proceeds primarily via the Compound X, Compound Y pathway (Stetson, 2008a [Unpublished]).

Four unidentified and minor (in apparent concentration) degradant peaks appeared in the LC/MS/MS chromatograms (Stetson, 2008a [Unpublished]). Similar to what was

observed with Reb A and Compound X, and with rebaudioside B and Compound Y, three of the four unknown peaks chromatographically eluted immediately following the chromatographic peak of one of the steviol glycosides (or steviol). In these cases, the unknown peaks eluted immediately following the stevioside, steviolbioside and steviol chromatographic peaks and were detected with the same m/z mass ion as the compound eluting immediately before. This consistent chromatographic retention time and mass spectrometric ionization relationship between the known steviol glycosides and the unknown Reb A degradation products suggests that the initial step in Reb A degradation is the formation of a structural isomer probably within the steviol core of the molecule, and that further degradation occurs as the successive loss of glucosyl residues. Since Compound X is an isomer of Reb A (i.e. iso-Reb A) and Compound Y is an isomer of rebaudioside B (i.e. iso-rebaudioside B), then the minor degradant peaks may be designated as iso-stevioside, iso-steviolbioside and isosteviol II. The fourth unknown peak was detected at $(m/z)^- = 317.3$, the $(M-1)^-$ mass ion of steviol and isosteviol II, and eluted about 2 minutes after steviol. This unknown peak has been designated isosteviol, and is presumed to be the isomer previously described in the published literature as the product of the acid-catalyzed Wagner-Meerwein rearrangement of steviol (Hanson and De Oliveira, 1993), as follows.

Figure III.-6: Degradation of Steviol to Isosteviol under acidic conditions



At test conditions of pH 2.0 and 70°F, the most relevant test conditions for which concentration data are available for the minor degradants, they were detected at the

following concentrations over the 15-week stability study (Stetson, 2008a [Unpublished]): iso-stevioside [≈0 – 1.55 µg/mL]; iso-steviolbioside [≈0 – 0.150 µg/mL]; isosteviol II [≈0 – 0.0173 µg/mL]; and isosteviol [≈0 – 0.0470 µg/mL] in comparison to the initial nominal concentration of 600 ug/mL for Reb A. The concentrations for the minor degradants peaks are approximate (≈) because they are estimated based on the peaks of the known Reb A components immediately preceding them, rather than reference standards.

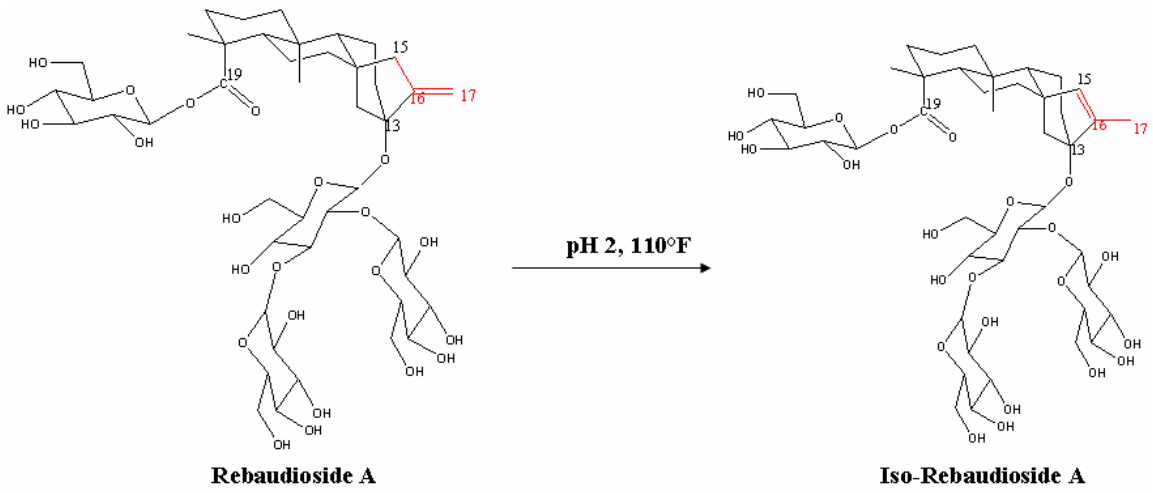
Identification of Compound X

As follow up to the stability studies, Compound X was prepared, isolated, purified and crystallized for characterization and identification at ChemPharma Int'l., LLC (Eisenberg, 2007 [Unpublished]). Experiments were conducted to prepare quantities of the Compound X from Reb A using high temperature and low pH conditions as used in the stability studies. After approximately 70% conversion of Reb A to Compound X, the material was desalted, and Compound X was isolated using preparative column chromatography and crystallized. A crystal structure of Compound X was obtained and the chemical structure elucidated. The ChemPharma report (Eisenberg, 2007 [Unpublished]) concludes:

> "Rebaudioside A has been shown to undergo an alkene isomerism in acidic conditions at elevated temperatures from an external alkene in Rebaudioside A to an internal double bond in iso-Rebaudioside A. The isomer has been characterized by HPLC/MS, 1H-NMR, and single crystal x-ray crystallography. Small amounts were isolated and purified by semi-preparative reversed-phase HPLC and further crystallized to produce a single crystal suitable for x-ray analysis. The x-ray structure clearly established the three-dimensional structure of iso-Rebaudioside A as having an internal double bond in the five-membered ring, with an external methyl group. Rebaudioside A is reported as having an exocyclic double bond. The x-ray crystal structure obtained for Rebaudioside A confirmed the structure, and the position of the exocyclic double bond."

Reb A and the elucidated structure of Compound X (iso-Reb A) are shown in the following figure.

Figure III.-7: Degradation of Reb A to iso-Reb A at low pH with heat



Summary

Reb A, rebaudioside B, stevioside, steviolbioside, and steviol concentrations in three purified Reb A products (Chrysanta® 99-P, Rebaudioside A-92% and Rebaudioside A-98%) were identified and quantitated using validated assays. The results support the reported test article purity values for Chrysanta® 99-P (Certificate of Analysis (C of A) purity = 95.7%), Rebaudioside A-98% (C of A purity = 99.5%) and Rebaudioside A-92% (C of A purity = 96.2%).

During incubation in aqueous citric acid solutions (50mM) at various pH values (2.0, 4.0 and 7.0 ± 0.5) for 15 weeks, no significant Reb A degradation was detected in 1) the Chrysanta® 99-P and Rebaudioside A-98% pH 7 solutions at 40, 70, 90 or 110ºF, 2) the Chrysanta® 99-P and Rebaudioside A-98% pH 4 solutions at 40, 70 or 90ºF and 3) the Chrysanta® 99-P and Rebaudioside A-98% pH 2 solutions at 40ºF. However, solutions prepared at pH 4 and incubated at 110ºF or at pH 2 and incubated at 70, 90 and 110ºF, resulted in significant Reb A degradation. Reb A degradation rate increased with increasing incubation temperature and with decreasing solution pH. At intermediate pH

values (2.5, 3.0 and 3.5 ± 0.5), aqueous citric acid solutions (50mM) incubated at 40, 70, 90 and 110ºF for 25 weeks demonstrated results fitting logically between those for pH 2 and 4. There were no significant differences in the stability (or instability) of Reb A observed with solutions of Chrysanta® 99-P or Rebaudioside A-98%.

Reb A degradation under severe pH and temperature storage conditions resulted in two major degradant peaks (i.e. Compounds X and Y) in the HPLC/UV chromatograms. The data indicate that the initial step in Reb A degradation at low pH and high temperature is the formation of Compound X, and that Compound Y is the direct product of Compound X degradation. The data and other observations associated with the degradation of Reb A at low pH and high temperature conditions indicate that Compound X (iso-Reb A) and Compound Y (iso-rebaudioside B) are structural isomers of Reb A and rebaudioside B, respectively.

During incubation for 15 weeks, rebaudioside B, stevioside, steviolbioside and steviol, which initially were present in both Chrysanta® 99-P and Rebaudioside A-98% in trace amounts, failed to accumulate to significant levels even under severe incubation conditions of test article solutions at 110ºF and pH 2.

Under severe pH and temperature storage conditions, four minor (in apparent concentration) degradant peaks appeared in the LC/MS/MS chromatograms. Three of the four unknown peaks chromatographically eluted immediately following the chromatographic peaks for stevioside, steviolbioside and steviol, and were designated iso-stevioside, iso-steviolbioside and isosteviol II. The fourth unknown peak was detected at the mass ion of steviol and isosteviol II, and eluted about 2 minutes after steviol. This unknown peak is concluded to be isosteviol, the isomer identified in the published literature as the product of the acid-catalyzed Wagner-Meerwein rearrangement of steviol.

Appendix IV: Estimated Human Intake

Appendix IV.-1: NHANES 2003-2004 Food Codes and Reb A Use Level Included in EDI Analysis

Appendix IV.-1: NHANES 2003-2004 Food Codes and Reb A Use Level Included in EDI Analysis

Food Code	Food Description	Food Category	Reb A Use level (ppm)
53540000	Breakfast bar, NFS	Cereal bar	150
53540200	Breakfast bar, cereal crust with fruit filling, lowfat	Cereal bar	150
53540250	Breakfast bar, cereal crust with fruit filling, fat free	Cereal bar	150
53540500	Breakfast bar, date, with yogurt coating	Cereal bar	150
53540600	Milk 'n Cereal bar	Cereal bar	150
53541200	Meal replacement bar	Cereal bar	150
53542100	Granola bar, oats, sugar, raisins, coconut	Cereal bar	150
53542200	Granola bar, oats, fruit and nuts, lowfat	Cereal bar	150
53542210	Granola bar, nonfat	Cereal bar	150
53543100	Granola bar, peanuts, oats, sugar, wheat germ	Cereal bar	150
53544200	Granola bar, chocolate-coated	Cereal bar	150
53544210	Granola bar, with coconut, chocolate-coated	Cereal bar	150
53544220	Granola bar with nuts, chocolate-coated	Cereal bar	150
53544250	Granola bar, coated with non-chocolate coating	Cereal bar	150
53544300	Granola bar, high fiber, coated with non-chocolate yogurt coating	Cereal bar	150
53544400	Granola bar, with rice cereal	Cereal bar	150
57000000	Cereal, NFS	Cold breakfast cereal	150
57000100	Oat cereal, NFS	Cold breakfast cereal	150
57100100	Cereal, ready-to-eat, NFS	Cold breakfast cereal	150
57117000	Cap'n Crunch	Cold breakfast cereal	150
57117500	Cap'n Crunch's Christmas Crunch	Cold breakfast cereal	150
57119000	Cap'n Crunch's Crunch Berries	Cold breakfast cereal	150
57120000	Cap'n Crunch's Peanut Butter Crunch	Cold breakfast cereal	150
57126500	Cocoa Blasts, Quaker	Cold breakfast cereal	150
57304100	Life (plain and cinnamon)	Cold breakfast cereal	150
57316710	Oh's, Honey Graham	Cold breakfast cereal	150
57316750	Oh's, Fruitangy, Quaker	Cold breakfast	150

Food Code	Food Description	Food Category	Reb A Use level (ppm)
		cereal	
57319000	100% Natural Cereal, plain, Quaker	Cold breakfast cereal	150
57320500	100 % Natural Cereal, with oats, honey and raisins, Quaker	Cold breakfast cereal	150
57321500	100 % Natural Wholegrain Cereal with raisins, lowfat, Quaker	Cold breakfast cereal	150
57323000	Sweet Crunch, Quaker (formerly called Popeye)	Cold breakfast cereal	150
57323050	Sweet Puffs, Quaker	Cold breakfast cereal	150
57327450	Quaker Oat Bran Cereal	Cold breakfast cereal	150
57327500	Quaker Oatmeal Squares (formerly Quaker Oat Squares)	Cold breakfast cereal	150
57328000	Quisp	Cold breakfast cereal	150
57413000	Wheat germ, with sugar and honey	Cold breakfast cereal	150
92400000	Soft drink, NFS	Diet soft drink	150
92400100	Soft drink, NFS, sugar-free	Diet soft drink	500
92410110	Carbonated water, sweetened	Diet soft drink	150
92410250	Carbonated water, sugar-free	Diet soft drink	250
92410315	Soft drink, cola type, reduced sugar	Diet soft drink	150
92410320	Soft drink, cola-type, sugar-free	Diet soft drink	500
92410350	Soft drink, cola-type, decaffeinated, sugar-free	Diet soft drink	500
92410370	Soft drink, pepper-type, sugar-free	Diet soft drink	500
92410400	Soft drink, pepper-type, decaffeinated, sugar-free	Diet soft drink	500
92410420	Cream soda, sugar-free	Diet soft drink	500
92410520	Soft drink, fruit-flavored, sugar free, caffeine free	Diet soft drink	500
92410560	Soft drink, fruit flavored, caffeine containing, sugar-free	Diet soft drink	500
92410620	Ginger ale, sugar-free	Diet soft drink	500
92410720	Root beer, sugar-free	Diet soft drink	500
92410820	Chocolate-flavored soda, sugar-free	Diet soft drink	500
92411610	Cola with fruit or vanilla flavor, sugar-free	Diet soft drink	500
92411620	Cola with chocolate flavor, sugar-free	Diet soft drink	500
92650000	Red Bull Energy Drink	Energy Drinks	150
92651000	Energy drink	Energy Drinks	150
94210100	Propel Fitness Water	Flavored Water	150
92431000	Carbonated juice drink, NS as to type of juice	Fruit Juice drinks	150
92432000	Carbonated citrus juice drink	Fruit Juice drinks	150

Food Code	Food Description	Food Category	Reb A Use level (ppm)
92433000	Carbonated noncitrus juice drink	Fruit Juice drinks	150
92510120	Apple-cherry drink	Fruit Juice drinks	150
92510150	Apple juice drink	Fruit Juice drinks	150
92510170	Apple-cranberry-grape juice drink	Fruit Juice drinks	150
92510200	Apple-orange-pineapple juice drink	Fruit Juice drinks	150
92510220	Apricot-pineapple juice drink	Fruit Juice drinks	150
92510310	Banana-orange drink	Fruit Juice drinks	150
92510410	Black cherry drink	Fruit Juice drinks	150
92510610	Fruit drink	Fruit Juice drinks	150
92510630	Fruit juice drink, NFS	Fruit Juice drinks	150
92510720	Fruit punch, made with fruit juice and soda	Fruit Juice drinks	150
92510810	Grapeade and grape drink	Fruit Juice drinks	150
92510820	Grape juice drink	Fruit Juice drinks	150
92510910	Grapefruit juice drink	Fruit Juice drinks	150
92510950	Guava juice drink	Fruit Juice drinks	150
92511000	Lemonade, frozen concentrate, not reconstituted	Fruit Juice drinks	150
92511010	Lemonade	Fruit Juice drinks	150
92511020	Lemon-limeade	Fruit Juice drinks	150
92511110	Limeade	Fruit Juice drinks	150
92511200	Orange-mango juice drink	Fruit Juice drinks	150
92511220	Orange drink	Fruit Juice drinks	150
92511230	Orange-apricot juice drink	Fruit Juice drinks	150
92511240	Orange-lemon drink	Fruit Juice drinks	150
92511250	Citrus fruit juice drink	Fruit Juice drinks	150
92511260	Orange-cranberry juice drink	Fruit Juice drinks	150
92511270	Orange-peach juice drink	Fruit Juice drinks	150
92511280	Orange-grape-banana juice drink	Fruit Juice drinks	150
92511290	Papaya juice drink	Fruit Juice drinks	150
92511310	Pineapple-grapefruit juice drink	Fruit Juice drinks	150
92511340	Pineapple-orange juice drink	Fruit Juice drinks	150
92511350	Orange-raspberry juice drink	Fruit Juice drinks	150
92511400	Raspberry-flavored drink	Fruit Juice drinks	150
92511510	Strawberry-flavored drink	Fruit Juice drinks	150
92520410	Fruit drink, low calorie	Fruit Juice drinks	500
92520810	Grape drink, low calorie	Fruit Juice drinks	500
92520910	Lemonade, low calorie	Fruit Juice drinks	500
92530210	Black cherry drink with vitamin C added	Fruit Juice drinks	150
92530310	Cherry drink with vitamin C added	Fruit Juice drinks	150
92530410	Citrus drink with vitamin C added	Fruit Juice drinks	150
92530510	Cranberry juice drink with vitamin C added	Fruit Juice drinks	150

Food Code	Food Description	Food Category	Reb A Use level (ppm)
92530520	Cranberry-apple juice drink with vitamin C added	Fruit Juice drinks	150
92530610	Fruit punch, fruit drink, or fruitade, with vitamin C added	Fruit Juice drinks	150
92530710	Grape drink with vitamin C added	Fruit Juice drinks	150
92530810	Grapefruit juice drink with vitamin C added	Fruit Juice drinks	150
92530840	Guava juice drink with vitamin C added	Fruit Juice drinks	150
92530910	Lemonade with vitamin C added	Fruit Juice drinks	150
92530950	Vegetable and fruit juice drink, with vitamin C added	Fruit Juice drinks	150
92531010	Orange drink and orangeade with vitamin C added	Fruit Juice drinks	150
92531020	Orange breakfast drink, made from frozen concentrate	Fruit Juice drinks	150
92531030	Orange breakfast drink	Fruit Juice drinks	150
92531110	Pineapple-grapefruit juice drink with vitamin C added	Fruit Juice drinks	150
92531120	Pineapple-orange juice drink with vitamin C added	Fruit Juice drinks	150
92531150	Pineapple-orange-grapefruit juice drink with vitamin C added	Fruit Juice drinks	150
92531210	Strawberry-flavored drink with vitamin C added	Fruit Juice drinks	150
92550050	Apple-white grape juice drink, low calorie, with vitamin C added	Fruit Juice drinks	500
92550110	Cranberry juice drink, low calorie, with vitamin C added	Fruit Juice drinks	500
92550210	Cranberry-apple juice drink, low calorie, with vitamin C added	Fruit Juice drinks	500
92550300	Grapefruit juice drink, low calorie, with vitamin C added	Fruit Juice drinks	500
92550610	Fruit-flavored drinks, punches, ades, low calorie, with vitamin C added	Fruit Juice drinks	500
92551700	Juice drink, low calorie	Fruit Juice drinks	500
92552100	Orange-cranberry juice drink, low calorie, with vitamin C added	Fruit Juice drinks	500
92553000	Fruit-flavored thirst quencher beverage, low calorie	Fruit Juice drinks	500
92560000	Fruit-flavored thirst quencher beverage	Fruit Juice drinks	150
92570100	Fluid replacement, electrolyte solution	Fruit Juice drinks	150
92570500	Fluid replacement, 5% glucose in water	Fruit Juice drinks	150
92582000	Fruit-flavored drink, low calorie, calcium fortified	Fruit Juice drinks	500
92582100	Citrus juice drink, calcium fortified	Fruit Juice drinks	150
92582110	Orange breakfast drink, calcium fortified	Fruit Juice drinks	150
56202960	Oatmeal, cooked, NS as to regular, quick or instant; NS as to fat added in cooking	Oatmeal	150
56202970	Oatmeal, cooked, quick (1 or 3 minutes), NS as to fat added in cooking	Oatmeal	150
56202980	Oatmeal, cooked, regular, NS as to fat added in cooking	Oatmeal	150
56203000	Oatmeal, cooked, NS as to regular, quick or instant, fat not added in cooking	Oatmeal	150
56203010	Oatmeal, cooked, regular, fat not added in cooking	Oatmeal	150

Food Code	Food Description	Food Category	Reb A Use level (ppm)
56203020	Oatmeal, cooked, quick (1 or 3 minutes), fat not added in cooking	Oatmeal	150
56203030	Oatmeal, cooked, instant, fat not added in cooking	Oatmeal	150
56203040	Oatmeal, cooked, NS as to regular, quick, or instant, fat added in cooking	Oatmeal	150
56203050	Oatmeal, cooked, regular, fat added in cooking	Oatmeal	150
56203060	Oatmeal, cooked, quick (1 or 3 minutes), fat added in cooking	Oatmeal	150
56203070	Oatmeal, cooked, instant, fat added in cooking	Oatmeal	150
56203080	Oatmeal, cooked, instant, NS as to fat added in cooking	Oatmeal	150
56203110	Oatmeal with maple flavor, cooked	Oatmeal	150
56203200	Oatmeal with fruit, cooked	Oatmeal	150
56203210	Oatmeal, NS as to regular, quick, or instant, made with milk, fat not added in cooking	Oatmeal	150
56203220	Oatmeal, NS as to regular, quick, or instant, made with milk, fat added in cooking	Oatmeal	150
56203230	Oatmeal, NS as to regular, quick, or instant, made with milk, NS as to fat added in cooking	Oatmeal	150
56203540	Oatmeal, made with evaporated milk and sugar, Puerto Rican style	Oatmeal	150
56203600	Oatmeal, multigrain, cooked, NS as to fat added in cooking	Oatmeal	150
56203610	Oatmeal, multigrain, cooked, fat not added in cooking	Oatmeal	150
56203620	Oatmeal, multigrain, cooked, fat added in cooking	Oatmeal	150
92301060	Tea, NS as to type, presweetened with sugar	Sweetened tea	90
92301080	Tea, NS as to type, presweetened with low calorie sweetener	Sweetened tea	450
92301130	Tea, NS as to type, presweetened, NS as to sweetener	Sweetened tea	450
92302200	Tea, leaf, presweetened with sugar	Sweetened tea	90
92302300	Tea, leaf, presweetened with low calorie sweetener	Sweetened tea	450
92302400	Tea, leaf, presweetened, NS as to sweetener	Sweetened tea	450
92306000	Tea, herbal	Sweetened tea	90
92306020	Tea, herbal, presweetened with sugar	Sweetened tea	90
92306030	Tea, herbal, presweetened with low calorie sweetener	Sweetened tea	450
92306040	Tea, herbal, presweetened, NS as to sweetener	Sweetened tea	450
91105010	Fructose sweetener, sugar substitute, dry powder	Table Top Sweetener	30000
91106000	Sugar substitute, sugar-aspartame blend, dry powder	Table Top Sweetener	30000
91107000	Sucralose-based sweetener, sugar substitute	Table Top Sweetener	30000
91108000	Sugar substitute, herbal extract sweetener, powder	Table Top	30000

Food Code	Food Description	Food Category	Reb A Use level (ppm)
		Sweetener	
91200000	Sugar substitute, low-calorie, powdered, NFS	Table Top Sweetener	30000
91200020	Sugar replacement, saccharin-based, dry powder	Table Top Sweetener	30000
91200030	Brown sugar replacement, saccharin-based, dry powder	Table Top Sweetener	30000
91200040	Sugar substitute, saccharin-based, dry powder and tablets	Table Top Sweetener	30000
91201010	Sugar substitute, aspartame-based, dry powder	Table Top Sweetener	30000

Appendix V: Safety Evaluation

Appendix V.-1: Rat 90-Day Oral (Dietary) Toxicity Study of Reb A

Appendix V.-2: Reb A Dog 6-Month Oral (Dietary) Toxicity Study

Appendix V.-3: Data Tables from Aze *et al.* (1991)

Appendix V.-4: Two-Generation Dietary Reproduction Toxicity Study in Rats

Appendix V.-5: Oral (Gavage) Embryo/Fetal Developmental Toxicity Study in Rats

Appendix V.-1: Rat 90-Day Oral (Dietary) Toxicity Study of Reb A

A 90-day oral (dietary) toxicity study was conducted in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practice Regulations (21 CFR Part 58) and the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice [C (97) 186/Final]. The study protocol was written to comply with the FDA Redbook 2000 test guideline IV.C.4a. (U.S. FDA, 2000b). The purity of the test article was 99.5% (Eapen, 2007 [Unpublished]; Nikiforov and Eapen, 2008).

The objectives of this study were to evaluate the potential toxic effects of Reb A when administered via the diet to rats for 90 days. This study included evaluation of potential neurotoxicity by functional observational battery and motor activity assessment.

Reb A was administered for a minimum of 90 consecutive days on a continuous basis in the diet to 3 groups (Groups 2-4) of Crl:CD (SD) rats. Target dosage levels were 500, 1000 and 2000 mg/kg/day. Dietary concentrations were adjusted weekly based on expected average weight and current food consumption. Concentration of Reb A in the formulated test diet reached 35.93 and 30.42 g/kg (35,930 and 30,420 ppm) for high-dose group males and females, respectively, by study week 12. A concurrent control group (Group 1) received the basal diet, PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 (meal), on a comparable regimen. Each group consisted of 20 animals/sex. Following at least 90 days of dietary exposure, all animals were euthanized.

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded weekly. Functional observational battery and locomotor activity data were recorded for 10 animals/sex/group during study week 12. Ophthalmic examinations were performed during study weeks -1 and 12. Blood samples were collected for hematology and serum

chemistry evaluations from 10 animals/sex/group during study weeks 2 and 5 and clinical pathology evaluations (hematology, serum chemistry and urinalysis) were performed on the same 10 animals/sex/group at the scheduled necropsy (study week 13). Complete necropsies were conducted on all animals, and selected organs were weighed at the scheduled necropsy. Selected tissues were examined microscopically from all animals in the control and 2000 mg/kg/day groups.

There were no test article-related effects on clinical observations, food consumption or functional observational battery or locomotor activity parameters. There were no test article-related macroscopic, organ weights or microscopic findings. Average daily test article consumption for the target dosage levels of 500, 1000 and 2000 mg/kg/day were 517, 1035 and 2055 mg/kg/day for males and 511, 1019 and 2050 mg/kg/day for females, respectively.

Lower body weight gains were noted in the 2000 mg/kg/day group males throughout the study (statistically significant at weeks 12 and 13). These lower mean body weight gains resulted in statistically significant lower cumulative body weight gains and a statistically significant mean body weight difference that was 9.1% lower compared to the control group at the end of the dosing period (study week 13). The lower body weights were not considered to be adverse due to the small magnitude of difference from the control group value. The study authors concluded that because of the proportion of basal diet that was replaced with the test article containing little caloric value, the lower mean body weight gains were likely the result of the males not consuming an equivalent number of calories as the concentration of the test article in the diet increased over the course of the study. A similar trend in mean body weights did not occur in the female test article-treated groups; however, this may be explained by the fact that the overall inclusion rates of test article in the diet were lower for the females as compared to the males throughout the study. The food efficiency data for males demonstrate that body weight gained as a percent of feed consumed is statistically significantly decreased at 2000 mg/kg/day as compared to the control group for the following intervals: week 0 to 1, week 3 to 4, and week 7 to 8. In contrast, the food efficiency data for females demonstrate that body

weight gained as a percent of feed consumed is generally similar for test article-treatment groups as compared to the control group.

Dietary administration of Reb A to Crl:CD (SD) rats for 13 consecutive weeks at average dosage levels of 517, 1035 and 2055 mg/kg/day to males and 511, 1019 and 2050 mg/kg/day to females resulted in mildly lower mean body weights in the high-dose group males. This finding was not considered to be adverse due to the magnitude of change and may have been the result of the amount of basal diet that was replaced with the test article containing little caloric value rather than a direct action of the test article itself. Therefore, based on the results of this study, the NOAEL was considered to be at least 2055 and 2050 mg/kg/day for males and females, respectively, the highest average dosage levels examined.

Based on the results of this study, the NOAEL was considered to be at least 2055 and 2050 mg/kg/day for males and females, respectively, the highest average dosage levels examined.

As discussed throughout the Reb A Safety Evaluation, decreases in food efficiency data, body weight and/or body weight gain compared to controls are observed at the highest dietary dose levels of Reb and stevioside in rat subchronic toxicity (Eapen, 2007 [Unpublished]; Nikiforov and Eapen, 2008; Aze *et al*., 1991), rat chronic toxicity/carcinogenicity (Xili *et al*., 1992; Toyoda *et al*., 1997) and rat or hamster reproduction toxicity (Sloter, 2008a [Unpublished]; Yodyingyuad and Bungawong, 1991; Mori *et al*., 1981) studies. It may be concluded that decreases in body weight and/or body weight gain in these studies are due to the extremely high levels of Reb A or stevioside administered via the dietary route which provide no caloric value in the diet, resulting in a decrease in total calorie consumption. Effects on body weight, body weight gain, and/or feed efficiency have been reported for other high intensity sweeteners when they are administered in the diet to test animals at similarly high dietary concentrations. For example, the reductions in body weight gain that have been reported for neotame, sucralose, and saccharin ranged from 3.7 to >20% in comparison to controls. It was

concluded that body weight gain decreases are not an appropriate basis for a no-observed-adverse-effect level (NOAEL) (Flamm *et al.,* 2003).

In the absence of toxicity, JECFA has concluded that changes in body weight gain are not appropriate for establishing NOAELs when they are associated with lower food consumption or food efficiency. It is the normal practice of JECFA to recognize when body weight is affected by reduced palatability of food containing high concentrations of test material. For example, JECFA noted that lower body weight gain for sucralose at the high dose in the long-term rat study (1500 mg/kg bw/day) was due to poor palatability of the diet and did not consider this finding adverse when setting the NOAEL and an ADI of 0-15 mg/k bw/day. Similarly, the highest dose of acesulfame-potassium, 1500 mg/kg bw/day was likewise associated with lower body weight gain. JECFA did not consider this an adverse finding when establishing an ADI for acesulfame-potassium of 0-15 mg/kg bw/day (WHO, 1983 as summarized in Flamm, *et al*., 2003).

Appendix V.-2: Reb A Dog 6-Month Oral (Dietary) Toxicity Study

A 6-month oral (dietary) toxicity study in dogs was conducted in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practice Regulations (21 CFR Part 58) and the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice [C (97) 186/Final]. The study protocol was written to comply with the FDA Redbook 2000 test guideline IV.C.4b. (U.S. FDA, 2000c). The purity of the test article was 95.7% (Eapen, 2008 [Unpublished]).

The objective of this study was to evaluate the potential toxic effects of Reb A (supplied as Chrysanta® 99-P) when administered via the diet to Beagle dogs for 182 days. Reb A was administered as a dietary admix during the normal daily feeding period for 3 groups (Groups 2-4) of beagle dogs. Target dosage levels were 500, 1000 and 2000 mg/kg/day. Dietary concentrations were adjusted weekly based on expected average weight and food consumption. Concentration of Reb A in the formulated test diet reached 61.70 and 68.15 g/kg (61,700 and 68,150 ppm) for high-dose group males and females, respectively, by study week 24. A concurrent control group (Group 1) received the basal diet on a comparable regimen. All groups each consisted of 4 males and 4 females. Following a minimum 182-day feeding period, all animals were euthanized.

The animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly. Individual body weights were recorded weekly. Food consumption was recorded daily and reported weekly. Functional observational battery testing was conducted on all dogs during pretest and near the end of the dosing period. Clinical pathology evaluations (hematology, serum chemistry and urinalysis) were performed prior to the initiation of dose administration (study week -1) and during study weeks 4, 8, 12, 16 and 24. Blood, urine and fecal samples for toxicokinetic evaluation were collected during study weeks 1, 12 and 24. Ophthalmic examinations were performed during study weeks -1 and 25. Complete necropsies were performed on all animals, and selected

organs were weighed at the scheduled necropsy. Selected tissues were examined microscopically from all animals.

There were no unscheduled deaths during the course of the study. No test article-related clinical observations were reported. In-cage, open field, and functional observations and/or measurements were unaffected by test article administration. No test article-related hematology findings, serum chemistry findings, or urinalysis findings were noted. No test article-related gross necropsy observations, alterations in final body weight, alterations in organ weights, or histologic changes were noted at the scheduled necropsy.

Based on the results of this study, no systemic toxicity of Reb A administered as a dietary admix to beagle dogs for a minimum of 182 days was observed at dosage levels up to 2000 mg/kg/day. Therefore, the no-observed-adverse-effect level (NOAEL) for oral (dietary) administration of Reb A to beagle dogs for 182 consecutive days was at least 2000 mg/kg/day.

Appendix V.-3: Data Tables from Aze *et al.* (1991)

Table 1. Serum biochemical and hematological data of male rats treated with stevioside

Item / Dose (%)	0	0.31	0.62	1.25	2.5	5
Effective No.	10	10	10	10	10	10
Serum biochemistry						
TP (mg/dl)	6.28±0.08	6.28±0.14	6.28±0.10	6.45±0.14*	6.42±0.15	6.37±0.12
Cho (g/dl)	52.1±2.0	52.9±3.8	54.3±4.3	57.0±4.5*	57.9±3.6*	58.3±4.7**
BUN (mg/dl)	14.1±1.4	15.6±0.8*	15.9±1.5**	16.1±1.1**	17.4±1.1**	17.2±0.9**
CRN (mg/dl)	0.57±0.07	0.57±0.05	0.62±0.04	0.64±0.05*	0.70±0.05**	0.72±0.06**
Ca (mg/dl)	10.3±0.3	10.3±0.3	10.3±0.3	10.5±0.3	10.5±0.3	10.4±0.4
P (mg/dl)	5.98±0.18	6.10±0.21	5.79±0.29	5.42±0.28**	5.5±0.27 **	5.42±0.26**
Na (mEQ/dl)	145±1.2	145±0.8	145±0.9	145±1.2	145±0.5	144±0.9
K (mEQ/dl)	4.24±0.20	4.37±0.16	4.44±0.14	4.48±0.22**	4.33±0.11	4.51±0.23**
Cl (mEQ/dl)	105±0.9	104±1.1	105±0.3	104±1.4	104±1.1	103±0.5
G-GT (IU/l)	0.1±0.3	0.3±0.5	0.1±0.3	0.6±0.7	0.4±0.5	0.4±0.7
Cho-E (IU/l)	3.9±1.4	4.2±2.1	4.1±2.1	4.7±2.0	4.5±2.2	5.2±2.1
GOT (IU/l)	78.7±8.3	85.6±7.0	78.8±6.1	88.4±5.7*	80.6±7.2	87.8±21.4
GPT (IU/l)	46.1±5.1	46.0±2.4	43±3.1	45.1±3.0	41.6±2.8	47.9±16.2
LDH (IU/l)	538±249	900±309*	654±219	1075±251**	875±310*	1035±234**
ALP (IU/l)	271±22	289±19	292±18	311±19**	344±19**	345±22**
Alb (g/dl)	4.64±0.12	4.73±0.12	4.74±0.13	4.78±0.08	4.79±0.15	4.78±0.09
A/G	2.83±0.18	3.07±0.38	3.08±0.24	2.87±0.22	2.96±0.21	3.02±0.22
Hematology						
RBC ($x10^4/mm^3$)	976±17	977±18	914±132	950±30	961±31	966±28
Ht. (%)	50.1±0.67	50.3±0.92	46.7±6.75*	48.4±1.27*	49.3±1.57	49.6±1.49
WBC ($x10^2/mm^3$)	45.4±6.0	44.8±4.4	39.2±8.1	40.4±3.5	45.7±4.0	44.6±5.1
PLT ($x10^4/mm^3$)	76.5±5.4	78.4±2.6	75.1±11.1	78.4±2.8	79.0±3.9	83.1±4.5*

Data represent mean values ± S.D.
* : Significantly different from respective control value at $p<0.05$.
** : Significantly different from respective control value at $p<0.01$.

Table 2. Serum biochemical and hematological data of female rats treated with stevioside

Item / Dose (%)	0	0.31	0.62	1.25	2.5	5
Effective No.	10	10	10	10	10	10
Serum biochemistry						
TP (mg/dl)	6.22±0.23	6.34±0.28	6.27±0.19	6.59±0.28**	6.63±0.26**	6.48±0.24
Cho (g/dl)	94.2±8.0	89.5±10.4	81.0±8.7**	88.3±9.7	88.3±8.7	84.0±9.3
BUN (mg/dl)	14.3±1.5	14.7±1.7	14.1±1.7	15.5±1.8	15.6±1.7	15.7±1.4
CRN (mg/dl)	0.57±0.09	0.62±0.10	0.58±0.08	0.64±0.07	0.57±0.07	0.59±0.06
Ca (mg/dl)	10.3±0.3	10.3±0.3	10.3±0.3	10.5±0.3	10.6±0.3	10.4±0.4
P (mg/dl)	5.71±0.92	5.72±0.85	5.44±1.00	5.17±0.64	5.95±0.39	5.89±0.77
Na (mEQ/dl)	146±1.8	147±1.7	145±0.9	146±1.1	145±1.6	146±1.8
K (mEQ/dl)	4.29±0.66	4.03±0.26	4.49±0.77	4.5±0.49	4.21±0.17	4.64±0.42
Cl (mEQ/dl)	108±1.4	106±1.7	107±1.1	105±2.3**	105±1.1**	105±1.3**
G-GT (IU/l)	0.5±0.7	0.5±0.7	0.4±0.7	0.4±0.5	0.3±0.5	1.2±1.4
Cho-E (IU/l)	8.3±1.8	7.0±1.2	6.9±2.1	7.4±2.9	7.2±1.8	7.2±1.5
GOT (IU/l)	70.7±4.5	74.8±5.1	83.8±10.9*	74.9±4.4	76.6±9.2	79.5±4.1*
GPT (IU/l)	32.4±5.1	32.9±2.0	36.3±3.5	37.2±2.5*	3.69±2.4*	35.4±4.1
LDH (IU/l)	503±195	684±211	901±349**	544±247	722±274	793±290
ALP (IU/l)	163±18	169±23	182±27	181±24	183±23.0	205±15**
Alb (g/dl)	4.73±0.13	4.82±0.23	4.75±0.16	5.02±0.24*	4.95±0.24	4.91±0.21
A/G	3.18±0.20	3.17±0.19	3.12±0.23	3.21±0.26	2.93±0.22	3.14±0.16
Hematology						
RBC ($x10^4/mm^3$)	912±25	901±19	914±31	890±41	909±24	901±45
Ht. (%)	49.4±6.2	39.7±8.7	29.9±5.7	36.0±6.3	49.2±0.8	49.2±2.4
WBC ($x10^2/mm^3$)	34.1±2.6	31.8±5.6	36.0±6.3	29.9±5.7	39.7±8.7	36.7±6.2
PLT ($x10^4/mm^3$)	83.2±2.7	84.9±4.8	86.2±3.2	80.7±18.7	90.2±3.4*	92.6±5.8*

Data represent mean values ± S.D.
* : Significantly different from representive control value at $p<0.05$.
** : Significantly different from representive control value at $p<0.01$

Table 3. Absolute and relative organ weights of male rats treated with stevioside

Item / Dose (%)	0	0.31	0.62	1.25	2.5	5
Effective No.	10	10	10	10	10	10
Body weight	329±14	317±11	316±15	325±15	322±9	309±22*
Brain	1.99±0.03 (0.61)	1.98±0.07 (0.63)	2.01±0.07 (0.64)	2.03±0.03 (0.63)	1.99±0.06 (0.62)	2.00±0.05 (0.65)
Salivary gl.	0.54±0.05 (0.17)	0.53±0.05 (0.17)	0.53±0.05 (0.17)	0.56±0.03 (0.17)	0.52±0.12 (0.16)	0.55±0.04 (0.18)
Thymus	0.24±0.04 (0.07)	0.21±0.04 (0.07)	0.20±0.03 (0.06)	0.22±0.03 (0.07)	0.26±0.11 (0.08)	0.22±0.03 (0.07)
Lung (R)	0.70±0.03 (0.21)	0.69±0.06 (0.22)	0.65±0.06 (0.21)	0.66±0.03 (0.20)	0.66±0.05 (0.20)	0.66±0.05 (0.21)
(L)	0.36±0.01 (0.11)	0.35±0.02 (0.11)	0.34±0.20 (0.11)	0.36±0.02 (0.11)	0.35±0.02 (0.11)	0.34±0.03 (0.11)
Heart	1.01±0.06 (0.31)	0.95±0.07 (0.30)	0.94±0.04 (0.30)	0.95±0.04 (0.29)	0.97±0.07 (0.30)	0.93±0.06 (0.30)
Spleen	0.64±0.04 (0.19)	0.63±0.03 (0.20)	0.62±0.03 (0.20)	0.65±0.03 (0.20)	0.66±0.03 (0.21)	0.65±0.06 (0.21)
Liver	7.68±0.45 (2.3)	7.22±0.34 (2.3)	7.44±0.52 (2.4)	7.94±0.50 (2.4)	8.01±0.46 (2.5)	8.06±0.45 (2.6)
Adrenal (R)	0.017±0.003 (0.005)	0.018±0.004 (0.006)	0.016±0.002 (0.005)	0.017±0.002 (0.005)	0.017±0.003 (0.005)	0.019±0.005 (0.006)
(L)	0.020±0.003 (0.006)	0.019±0.003 (0.006)	0.017±0.002 (0.005)	0.019±0.002 (0.006)	0.020±0.002 (0.006)	0.020±0.004 (0.006)
Kidney (R)	1.00±0.06 (0.30)	0.94±0.05 (0.30)	0.94±0.10 (0.30)	0.96±0.04 (0.30)	0.96±0.06 (0.30)	0.95±0.06 (0.31)
(L)	1.00±0.08 (0.30)	0.95±0.05 (0.30)	0.92±0.06 (0.29)	0.98±0.03 (0.30)	0.97±0.07 (0.30)	0.94±0.06 (0.31)
Testis (R)	1.52±0.05 (0.46)	1.51±0.06 (0.48)	1.46±0.07 (0.46)	1.51±0.07 (0.46)	1.47±0.03 (0.46)	1.47±0.04 (0.48)
(L)	1.54±0.03 (0.47)	1.51±0.03 (0.48)	1.49±0.04 (0.47)	1.52±0.05 (0.47)	1.52±0.08 (0.47)	1.52±0.04 (0.49)

Data represent mean values ± S.D.
Relative organ weights are shown in parentheses (%)
* : Significantly different from respective control value at $p<0.05$.
** : Significantly different from respective control value at $p<0.01$.

Table 4. Absolute and relative organ weights of female rats treated with stevioside

Item / Dose(%)	0	0.31	0.62	1.25	2.5	5
Effective No.	10	10	10	10	10	10
Body weight	177±11	176±8	177±10	173±11	165±10*	163±5**
Brain	1.85±0.08 (1.0)	1.83±0.04 (1.0)	1.83±0.05 (1.0)	1.84±0.05 (1.1)	1.84±0.05 (1.1)	1.84±0.06 (1.1*)
Salivary gl.	0.36±0.06 (0.20)	0.38±0.07 (0.22)	0.36±0.03 (0.20)	0.34±0.07 (0.20)	0.33±0.02 (0.21)	0.34±0.02 (0.21)
Thymus	0.18±0.02 (0.10)	0.19±0.02 (0.11)	0.19±0.01 (0.11)	0.18±0.02 (0.11)	0.18±0.02 (0.11)	0.18±0.02 (0.11)
Lung (R)	0.51±0.04 (0.29)	0.48±0.02 (0.27)	0.49±0.04 (0.28)	0.51±0.04 (0.29)	0.49±0.03 (0.30)	0.48±0.04 (0.30)
(L)	0.26±0.02 (0.15)	0.25±0.02 (0.14)	0.26±0.02 (0.15)	0.26±0.02 (0.15)	0.25±0.02 (0.15)	0.25±0.01 (0.16)
Heart	0.61±0.04 (0.35)	0.61±0.06 (0.35)	0.65±0.07 (0.37)	0.62±0.05 (0.36)	0.58±0.04 (0.36)	0.56±0.03 (0.35*)
Spleen	0.40±0.03 (0.23)	0.41±0.03 (0.23)	0.41±0.05 (0.23)	0.40±0.03 (0.23)	0.39±0.02 (0.24)	0.41±0.04 (0.25*)
Liver	3.91±0.29 (2.2)	3.96±0.27 (2.2)	4.00±0.24 (2.3)	4.13±0.35* (2.4*)	4.25±0.22* (2.6**)	4.38±0.25** (2.7**)
Adrenal (R)	0.021±0.003 (0.012)	0.020±0.003 (0.011)	0.022±0.004 (0.012)	0.021±0.003 (0.012)	0.022±0.003 (0.014)	0.020±0.003 (0.013)
(L)	0.023±0.004 (0.013)	0.23±0.003 (0.013)	0.023±0.004 (0.013)	0.024±0.002 (0.014)	0.024±0.003 (0.014)	0.023±0.002 (0.014)
Kidney (R)	0.57±0.04 (0.32)	0.56±0.02 (0.32)	0.57±0.04 (0.32)	0.55±0.03 (0.32)	0.52±0.01* (0.32)	0.52±0.03* (0.32)
(L)	0.58±0.06 (0.33)	0.57±0.03 (0.33)	0.58±0.05 (0.33)	0.56±0.03 (0.32)	0.53±0.02* *(0.32)	0.53±0.03* (0.33)

Data represent mean values ± S.D.
Relative organ weights are shown in parentheses (%)
* : Significantly different from respective control value at $p<0.05$.
** : Significantly different from respective control value at $p<0.01$.

Table 5. Histopathological findings of male rats treated with stevioside

Findings / Dose (%)	0				0.31				0.62				1.25				2.5				5			
	−	±	+	++	−	±	+	++	−	±	+	++	−	±	+	++	−	±	+	++	−	±	+	++
Heart																								
Myocardiopathy	2	3	5	0	6	2	2	0	4	0	4	2	6	2	2	0	3	4	3	0	5	3	1	1
Lung																								
Calcification of pulmonary ar.	4	3	3	0	3	3	4	0	5	2	3	0	7	2	1	0	5	5	0	0	8	2	0	0
Liver																								
Single cell nec.	2	8	0	0	2	7	1	0	1	7	2	0	2	5	3	0	0	1	9	0	0	5	5	0
Cell infiltration	9	1	0	0	10	0	0	0	9	1	0	0	9	1	0	0	10	0	0	0	10	0	0	0
Focal necrosis	10	0	0	0	9	0	1	0	10	0	0	0	10	0	0	0	9	0	1	0	10	0	0	0
Micro-granuloma	10	0	0	0	9	0	1	0	10	0	0	0	9	1	0	0	10	0	0	0	8	2	0	0
Spleen																								
Brown pigment	9	1	0	0	10	0	0	0	9	1	0	0	9	1	0	0	10	0	0	0	10	0	0	0

− ; Normal ± ; Minimal + ; Slight ++ ; Moderate

Table 6. Histopathological findings of female rats treated with stevioside

Findings / Dose (%)	0				0.31				0.62				1.25				2.5				5			
	−	±	+	++	−	±	+	++	−	±	+	++	−	±	+	++	−	±	+	++	−	±	+	++
Heart																								
Myocardiopathy	10	0	0	0	10	0	0	0	10	0	0	0	10	0	0	0	9	0	1	0	10	0	0	0
Lung																								
Calcification of pulmonary ar.	6	3	1	0	8	2	0	0	5	3	2	0	7	3	0	0	9	1	0	0	6	4	0	0
Liver																								
Single cell nec.	3	7	0	0	8	2	0	0	6	4	0	0	4	6	0	0	5	5	0	0	7	3	0	0
Cell infiltration	9	1	0	0	10	0	0	0	10	0	0	0	10	0	0	0	10	0	0	0	10	0	0	0
Focal necrosis	9	0	1	0	10	0	0	0	10	0	0	0	10	0	0	0	9	0	1	0	10	0	0	0
Micro-granuloma	8	1	1	0	10	0	0	0	10	0	0	0	9	1	0	0	9	0	1	0	8	1	1	0
Spleen																								
Brown pigment	1	1	7	1	2	0	4	4	2	5	3	0	3	3	3	1	2	2	4	2	0	4	6	0
Kidney																								
Calcification	9	1	0	0	8	2	0	0	7	3	0	0	9	1	0	0	8	1	1	0	7	3	0	0
Harderian gland																								
Cell infiltration	10	0	0	0	10	0	0	0	10	0	0	0	10	0	0	0	10	0	0	0	9	0	0	1

− ; Normal ± ; Minimal + ; Slight ++ ; Moderate

Appendix V.-4: Two-Generation Dietary Reproduction Toxicity Study in Rats

This study was conducted in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practice Regulations (21 CFR Part 58) and the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice [C(97) 186/Final]. The study protocol was written to comply with the FDA Redbook 2000 testing guideline IV.C.9.a. (U.S. FDA, 2000a). In addition, the study was conducted in accordance with the OECD Guideline 416 (OECD, 2001) with the exception that histopathology of the protocol-specified tissues from the F_0 and F_1 parental animals was performed on 10 rats/sex/group in the control and high-dose groups instead of 30 rats/sex/group; however, tissues from the remaining rats were preserved for possible future examination. The purity of the test article was 95.7% (Sloter, 2008a [Unpublished]).

This study was conducted to evaluate the potential adverse effects of Reb A (supplied as Chrysanta® 99-P) on reproduction in a two-generation study. This included determining the effects of the test article on male and female reproductive processes, including gonadal function, estrus cyclicity, mating behavior, conception, gestation, parturition, lactation and weaning and on growth and development of the offspring. One litter per dam was produced in each generation.

Four groups of male and female Crl:CD(SD) rats (30/sex/group) were offered either basal diet or the test article, Reb A, continuously in the diet for at least 70 consecutive days prior to mating. Target test article concentrations were 0, 500, 1000 and 2000 mg/kg/day for the F_0 and F_1 generations. F_0 animals were approximately 7 weeks of age at the initiation of test diet exposure. The test diet was offered to the offspring selected to become the F_1 generation following weaning (beginning on postnatal day [PND] 21). The F_0 and F_1 males continued to receive the test article throughout mating and continuing through the day of euthanasia. The F_0 and F_1 females continued to receive the test article throughout mating, gestation and lactation, and through the day of euthanasia.

For both generations (F_1 and F_2), 8 pups per litter (4 per sex, when possible) were selected on PND 4 to reduce the variability among the litters. Offspring (30/sex/group) from the pairing of the F_0 animals were selected on PND 21 to constitute the F_1 generation; an additional 1 pup/sex/litter was selected from 25 litters/test article-treated group and 4 litters in the control group on PND 21 for evaluation of toxicokinetic parameters (plasma, fecal and urine sample analysis). F_0 and F_1 males and females constituting the respective generations were each offered the test diet for 127 to 130 consecutive days; the F_1 animals in the toxicokinetic phase were offered the test diet until euthanasia following the final blood collection at 6 or 12 weeks of age.

All animals were observed twice daily for appearance and behavior. Clinical observations, body weights and food consumption were recorded at appropriate intervals for males throughout the study and for females prior to mating and during gestation and lactation. Vaginal lavages were performed daily for determination of estrus cycles beginning 21 days prior to pairing. All F_0 and F_1 females were allowed to deliver and rear their pups until weaning on lactation day 21. Clinical observations, body weights and sexes for F_1 and F_2 pups were recorded at appropriate intervals. Developmental landmarks (balanopreputial separation and vaginal patency) were evaluated for the selected F_1 rats. Nonselected F_1 and F_2 pups were necropsied on PND 21; selected organs from 1 pup/sex/litter were weighed. Each surviving F_0 and F_1 parental animal received a complete detailed gross necropsy following the completion of weaning of the F_1 and F_2 pups, respectively; selected organs were weighed. Spermatogenic endpoints (sperm motility, morphology and numbers) were recorded for all F_0 and F_1 males, and ovarian primordial follicle counts and corpora lutea counts were recorded for 10 F_0 and F_1 females in the control and high-exposure groups. Designated tissues from all F_0 and F_1 parental animals were examined microscopically.

For the toxicokinetic phase, urine and fecal samples were collected from F_1 males and females (3 animals/sex/group, including the control group) at approximately 6 and 12 weeks of age. Blood samples were collected from these same animals, if possible, 2-3 days following collection of urine and fecal samples at approximately 0600, 0900,

1200, 1500, 1800, 2100, and 2400 hours, as well as at approximately 0300 hours the following day. Blood samples from control group animals were collected at 0600 hours only. All surviving toxicokinetic phase animals received a complete necropsy following the final blood collection for each animal.

The average quantities of test article consumed during the F_0 and F_1 generations are presented below.

Mean Calculated Test Article Consumption (mg/kg/day[1])

	Males		***Females***		
Theoretical Dietary Level	**Prior to Mating**	**After Mating**	**Prior to Mating**	**Gestation**	**Lactation**
F_0 Generation					
500 mg/kg/day	517	494	500	573	1364
1000 mg/kg/day	1042	1001	1012	1056	2525
2000 mg/kg/day	2061	2009	2051	2085	5110
F_1 Generation					
500 mg/kg/day	527	492	513	453	1114
1000 mg/kg/day	1062	984	1027	945	2289
2000 mg/kg/day	2114	1988	2052	1881	4602

$$^{1} = \frac{\text{Summation of mean test article consumption for the specified interval}}{\text{Number of days or intervals assessed}}$$

F_0 and F_1 parental survival was unaffected by test diet administration at all exposure levels. No remarkable clinical findings were noted for F_0 or F_1 males and females in the 500, 1000 and 2000 mg/kg/day groups.

In the 2000 mg/kg/day group, mean body weight gains were generally lower for F_0 males during the pre-mating period (study weeks 0-10) and immediately following breeding (study week 12-13), and for F_1 males when the entire generation (study weeks 18-36) was evaluated. As a result, mean body weights in the F_0 and F_1 males were slightly lower than the control group during study weeks 7-18 and 28-36, respectively. The slight reduction in male body weight at 2000 mg/kg/day may be due to the proportion of the basal diet containing Reb A; which is non-nutritive. Therefore, the lower male body

weights were considered test article-related, but not adverse due to the small magnitude of difference from the control group value. Mean body weights, body weight gains and cumulative body weight gains in F_0 and F_1 males in the 500 and 1000 mg/kg/day groups and in F_0 and F_1 females in the 500, 1000 and 2000 mg/kg/day groups were unaffected by test diet exposure during the entire generation (males) and during the pre-mating, gestation and lactation periods (females).

Due to the non-nutritive nature of Reb A, mean food efficiency in the F_0 males was generally lower than the control group during the pre-mating period in the 1000 and 2000 mg/kg/day groups, and resulted in higher mean food consumption (primarily on g/kg/day basis). Mean food consumption and food efficiency in the F_0 males in the 500 mg/kg/day group were unaffected by test article administration prior to mating. Following the mating period, mean food consumption and food efficiency in F_0 males were similar to the control group in the 500, 1000 and 2000 mg/kg/day groups. In the F_1 males, mean food consumption and food efficiency in the 500, 1000 and 2000 mg/kg/day groups were generally similar to the control group. Therefore, the transient increase in food consumption affecting only the F_0 males was not considered to be toxicologically significant.

Mean food consumption and food efficiency in the F_0 and F_1 females were generally comparable to the control group during the pre-mating period. However, as these females entered gestation and lactation, food consumption in the 500, 1000 and/or 2000 mg/kg/day groups was higher than the control group values. These increases in mean food consumption were considered test article-related to Reb A, but not adverse due to the non-nutritive nature of reb A and increased demand for calories during pregnancy and nursing. Food efficiency in F_0 females in all test article-exposed groups and F_1 females in the 1000 and 2000 mg/kg/day groups was generally lower than the control group during gestation; the lower food efficiency was attributed to the lower caloric intake in these groups. Food efficiency during lactation was unaffected by test diet exposure in both generations.

No test article-related effects were observed on F_0 and F_1 reproductive performance (estrus cycles, mating, fertility, copulation or conception indices, the mean number of days between pairing and coitus and the mean length of gestation), parturition or the mean numbers of implantation sites and unaccounted-for sites. Spermatogenic endpoints (mean testicular and epididymal sperm numbers, sperm production rate, motility and the percentage of morphologically normal sperm) were also unaffected by test diet exposure in both generations.

There were no test article-related macroscopic findings or changes in mean organ weights in the F_0 or F_1 males and females in the 500, 1000 and 2000 mg/kg/day groups. Microscopic evaluation of the F_0 and F_1 males and females revealed no test article-related histopathological lesions. No test article-related effects on primordial follicle or corpora lutea counts were observed in the F_0 and F_1 females in the 2000 mg/kg/day group.

No test article-related effects were observed on the mean numbers of F_1 or F_2 pups born, the pup sex ratio, pup survival, or the general physical condition of the pups during the pre-weaning period. Mean body weight gains in F_1 male and female pups in the 1000 and 2000 mg/kg/day groups and F_2 male and female pups in the 2000 mg/kg/day group were lower than the control group at the end of the postnatal period (PND 14-21). These reductions in mean body weight gains were likely the result of the pups replacing their consumption of milk from nursing with Reb A-containing diet just prior to weaning.

No test article-related macroscopic findings were observed in F_1 or F_2 pups that were found dead, euthanized in extremis or at the scheduled necropsy. There were no test article-related effects on F1 or F2 pup organ weights on PND 21. Decreases in spleen weights noted in the F_1 and F_2 pups at 2000 mg/kg/day were not considered adverse as decreases were not observed in similarly exposed F_0 and F_1 adults, there were no histopathological correlates observed and pup spleen weights were comparable to control values in the laboratory's historical control database. The mean day of acquisition of balanopreputial separation or vaginal patency, and mean body weights on the day of acquisition in the F_1 test article-exposed pups were not affected by test article exposure.

There were no effects on reproduction (estrus cycles, mating, fertility, conception or copulation indices, number of days between pairing and coitus, gestation length, and spermatogenic endpoints). The only indications of parental toxicity, slight effects on mean body weights and body weight gains in F_0 and F_1 males at 2000 mg/kg/day and on mean food consumption and food efficiency in F_0 males at all exposure levels, were not considered to be adverse due to the magnitude of change and may have been the result of the amount of basal diet that was replaced with the test article containing little caloric value rather than a direct action of the test article itself. As the offspring of the F_0 and F_1 generations began to consume less milk from nursing and more of the diet containing Reb A, reductions in mean body weight gains (PND 14-21) were noted in male and female F_1 pups at 1000 and 2000 mg/kg/day and in male and female F_2 pups at 2000 mg/kg/day. As noted for the parental animals, and as noted for the 90-day study of Reb A with rats (Nikiforov and Eapen, 2008) these changes were not considered to be adverse or appropriate for setting a NOAEL (see Section V.A.2.b).

Therefore, an exposure level of at lest 2000 mg/kg/day was considered to be the no-observed-adverse-effect level (NOAEL) for parental systemic and reproductive toxicity, and an exposure level of at least 2000 mg/kg/day was considered to be the NOAEL for neonatal toxicity of Reb A when administered continuously in the diet to Crl:CD(SD) rats.

Appendix V.-5: Oral (Gavage) Embryo/Fetal Developmental Toxicity Study in Rats

This study was conducted in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practice Regulations (21 CFR Part 58) and the Organization for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice [C(97) 186/Final]. The protocol was written to comply with the FDA Redbook 2000 testing guideline IV.C.9.b. (U.S. FDA 2000g). The purity of the test article was 95.7% (Sloter, 2008b [Unpublished]).

The objective of this study was to determine the potential of the test article, Reb A (supplied as Chrysanta® 99-P), to induce developmental toxicity after maternal exposure during organogenesis, to characterize maternal toxicity at the exposure levels tested and to determine a NOAEL (no-observed-adverse-effect level) for developmental toxicity.

Reb A was administered orally by gavage to 3 groups of 25 bred female Crl:CD(SD) rats from gestation days 0 through 20. The animals were dosed twice daily (approximately 4 hours apart). Total daily dosage levels were 500, 1000 and 2000 mg/kg/day. Each of the 2 daily doses was administered at a dosage volume of 10 mL/kg. A concurrent control group composed of 25 females received the vehicle (reverse osmosis-purified water) on a comparable regimen. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded daily. On gestation day 21, a laparohysterectomy was performed on each female. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

All females in the control, 500, 1000 and 2000 mg/kg/day groups survived to the scheduled necropsy on gestation day 21. White discoloration of the feces was noted on at

least 1 occasion for 2 females in the 1000 mg/kg/day group and the majority of the females in the 2000 mg/kg/day group during gestation days 7-21; this finding was attributed to the test article but was not considered adverse. No test article-related clinical findings were noted in the 500 mg/kg/day group. No test article-related macroscopic findings were noted at any dosage level. The only internal finding, dark red uterine contents, was noted for 1 female in the 2000 mg/kg/day group and was not attributed to the test article.

Mean food consumption, evaluated as g/kg/day, was slightly higher than the control group during gestation days 6-9, 9-12 and 12-13 in the 1000 and 2000 mg/kg/day groups and gestation days 3-6 and 0-21 in the 2000 mg/kg/day group with most of these differences achieving statistical significance at $p<0.05$ or $p<0.01$. No corresponding effects on body weight were observed. These increases in food consumption were small in magnitude and did not occur in a dose-related pattern; therefore, the differences in food consumption were not considered test article-related. Mean body weights, body weight gains, net body weights, net body weight gain and mean gravid uterine weight were unaffected by test article administration at all dosage levels.

Intrauterine growth and survival were unaffected by test article administration in the 500, 1000 and 2000 mg/kg/day groups. There were no test article-related fetal malformations or developmental variations at any dosage level.

In the absence of maternal or developmental toxicity in this study, a total daily dosage level of at least 2000 mg/kg/day, the highest dosage level evaluated, was considered to be the no-observed-adverse-effect level (NOAEL) for maternal and embryo/fetal developmental toxicity when Reb A was administered orally, twice daily by gavage, to pregnant Crl:CD(SD) rats.

SUBMISSION END