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May 15, 2008

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Supervisor, Consumer Safety Office
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-255),
Center for Food Safety and Applied Nutrition
Food and Drug Administration
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Delivered via courier

SUBJECT: GRAS EXEMPTION CLAIM: REBAUDIOSIDE A (REBIANA)

Dear Dr. Martin:

Cargill, Incorporated is pleased to submit the attached GRAS Exemption claim for rebiana, high purity rebaudioside A, which is one of several naturally occurring steviol glycosides present in the leaves of the *Stevia rebaudiana* (Bertoni) plant. Rebiana has been determined by Cargill to be safe and Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures and has been evaluated by experts qualified by scientific training and experience to assess the safety of rebiana under the conditions of its intended use in food. Therefore, the use of rebiana in food as described in this GRAS Exemption Claim is exempt from the requirement of premarket approval.

The attached GRAS Notification provides a comprehensive evaluation of studies related to the safety of Cargill's food grade rebiana (not less than 97% rebaudioside A) product, intended for use as a general purpose sweetener. Included with this GRAS Notification submission you'll find three (3) hard copies of the GRAS Notification dossier which include the signed Expert Panel Consensus Statement, and three (3) CD ROMs with studies and critical reviews specifically concerning rebiana. The information in this cover letter is provided to aid your review of the Notification by identifying historical safety gaps related to stevia-based ingredients and by providing references to scientific reviews and data developed to address those gaps. The attached published paper authored by Carakostas *et*

al., 2008, *"Overview: the history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages,"* is also provided for historical and international regulatory perspectives that may prove useful to your review. A brief synopsis of the historical gaps and their resolution is provided below.

Historical Issue #1: Lack of a consistent food grade specification supported by a reproducible manufacturing process

Earlier attempts to gain a permitted regulatory status and JECFA permanent ADI have been unsuccessful due to the variability in steviol glycoside content of available products and poorly defined specifications for commercial products. Many early safety studies also suffered from poorly defined stevia-derived test material.

Rebiana, the subject of the attached GRAS Notification, is a fully-characterized, high-purity (> 97% rebaudioside A) ingredient that is manufactured using a consistent and well-defined process resulting in a final product that meets defined food grade specifications. Rebiana specifications fall within the current JECFA specifications for steviol glycoside content. Analyses across multiple batches have demonstrated the rigor of the manufacturing and control processes defined in our Notification dossier. In addition, contaminant levels, including both pesticides and heavy metals, range from very low to not detectable and are carefully monitored as part of the manufacturing quality control process.

Historical Issue #2: Reproductive effects reported from studies carried out with poorly-characterized stevia-derived test materials

A number of published studies have reported reproductive toxicity following administration of crude or ill-characterized stevia materials. The findings from these studies have been widely disseminated into the scientific and regulatory communities. However, other previously conducted safety studies on purified steviol glycosides reported no such adverse reproductive outcomes.

The determination of reproductive safety of rebiana is based on the lack of reproductive and developmental effects reported for studies carried out with *purified* steviol glycosides (Mori *et al.*, 1981, Yodyingyuad and Bunyawong, 1991, and Usami *et al.*, 1995) as well as chronic toxicity studies (Xili *et al.*, 1992 and Toyoda *et al.*, 1997). Conclusions from these earlier studies are supported by two recent studies, a subchronic oral toxicity study reported in the paper by Curry and Roberts, 2008 and a 2-generation reproductive safety study reported by Curry *et al.*, 2008. Both studies included specific investigations of male reproductive organs and no adverse effects were reported at the highest dietary concentrations tested in both studies. The No-Adverse-Effect-Levels for both studies were the highest dietary concentrations tested for both male and female rats and exceeded 2000 mg/kg body weight/day.

Historical Issue #3: Safety for special populations, e.g., consumers with diabetes or low blood pressure

Several researchers have attempted to demonstrate a therapeutic benefit of stevia in subjects with diabetes or hypertension and some have reported positive results. This has led to concerns about the unintended effects of wide-spread use of stevia as a food ingredient. However, the existing scientific literature also demonstrates that effects on glucose homeostasis or the cardiovascular system reported by some researchers have not been confirmed by others. In recent studies, Ferri and colleagues (2006) failed to demonstrate an effect of a crude steviol glycoside extract on blood pressure, and Jeppesen and colleagues (2006b) failed to demonstrate an effect on glucose homeostasis in subjects with type 2 diabetes. Barriocanal *et al.*, 2008 conducted a single clinical study in normal subjects and subjects with either type 1 or type 2 diabetes. They found no impact of steviol glycoside consumption on either blood pressure or glucose homeostasis in any of the groups.

Recent studies employing rebiana affirm that steviol glycosides do produce pharmacologic effects in healthy adults with normal and low-normal blood pressure and also in subjects with type 2 diabetes. In studies reported by Maki *et al.*, 2008, subjects consumed 1000 mg of rebiana per day which is more than three times the mean estimated intake for adults with diabetes who are heavy users.

Reports of effects of steviol glycosides on glucose or blood pressure from *in vitro* or animal studies were of limited applicability to oral consumption of rebiana by humans. Exposure concentrations or routes of administration were largely not relevant for human dietary exposure.

Historical Issue #4: Results from *in vitro* studies or studies using parenteral administration of stevia-derived test materials have led to questions about renal effects

Reports of adverse or physiological effects of stevioside or steviol on the kidney are present in the scientific literature and have led to concerns about renal toxicity. Some reports have extrapolated renal toxicity concerns from *in vitro* studies that used exposure levels far above those that could occur from dietary exposure. In addition, several of the *in vitro* studies reported no adverse effects on the kidney from stevioside exposure, but some effect from steviol exposure which would not occur with normal dietary intake and hepatic metabolism. Studies using animals in which stevioside or steviol were administered *via* an intravenous or subcutaneous route have also been used to raise concerns about toxicity from dietary exposure. These studies are not relevant to human dietary consumption of rebiana due to the high blood levels that result from parenteral administration and the lack of first-pass hepatic metabolism that converts steviol to a stable detoxification product.

Wheeler *et al.*, 2008 demonstrated that steviol is rapidly converted to its glucuronide in the human liver so that there is virtually no exposure to steviol beyond the portal system. Therefore, both *in vitro* studies and studies in which steviol is directly administered into the blood are not relevant to human dietary exposure. Additionally, recent subchronic oral

toxicity studies conducted at exposure levels exceeding 4,000 mg/kg/day resulted in no adverse effects in any organ system, including renal and urinary, as reported by Curry and Roberts, 2008.

Our safety research program was focused on critically examining and resolving the historical safety gaps and uncertainty areas concerning the safety of high purity steviol glycosides intended for use to sweeten food and beverages. The published literature reviewed in the Notification for high purity steviol glycosides and specifically on Cargill's rebiana product addresses all of the previous concerns raised by various regulatory bodies and supports the conclusion that consumption of food grade rebiana as a general purpose sweetener in foods is generally recognized as safe for its intended use.

Cargill looks forward to the Agency's review of the accompanying dossier and is prepared to respond to any questions that arise in the review process. We are confident in our safety determination of rebiana for its intended use as a general purpose sweetener and trust that the Agency will concur with this conclusion.

Sincerely,

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GRAS EXEMPTION CLAIM FOR REBIANA (REBAUDIOSIDE A)

Summary of Data Concerning the Safety and GRAS Determination of Rebiana (Rebaudioside A) for Use as a General Purpose Sweetener

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
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May 14, 2008

GRAS EXEMPTION CLAIM FOR REBIANA (REBAUDIOSIDE A)

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GRAS EXEMPTION CLAIM FOR REBIANA (REBAUDIOSIDE A)

EXECUTIVE SUMMARY

Cargill, Incorporated (Cargill) intends to market food-grade rebiana, comprised of not less than 97% rebaudioside A, as a general purpose use sweetener. Rebaudioside A is one of several naturally-occurring steviol glycoside constituents of the *Stevia rebaudiana* (Bertoni) plant. Rebaudioside A has high sweetness intensity (approximately 200x sweetness equivalence when compared to sucrose), is stable across extremes of food processing and delivers favorable sweetness in food systems. Cargill's rebiana is Generally Recognized as Safe (GRAS) for use as a general purpose use sweetener because:

- It is a high-purity, well-characterized ingredient that is consistently produced to a food grade specification and is stable in food and beverage systems;
- Its safety for its intended use in foods and beverages is supported by an extensive database of published studies conducted with purified steviol glycosides, including comprehensive studies of metabolism, pharmacokinetics, and animal toxicology, as well as human studies conducted to assess potential effects on glucose homeostasis in subjects with type 2 diabetes and on hemodynamics in subjects with normal to low-normal blood pressure. Supplemental animal and human studies carried out specifically on Cargill's high-purity rebaudioside A (rebiana) further corroborate its safety;
- An independent Expert Panel comprised of scientists with expertise and training in the evaluation of the safety of food ingredients has critically examined the safety database concerning *Stevia*, purified steviol glycosides, and rebaudioside A and concluded based on scientific procedures that rebiana is GRAS and safe for its intended use. The opinion of the Panel has also been corroborated in a number of deliberations of the Joint FAO/WHO Committee on Food Additives (JECFA); and
- The data and critical reviews supporting the safety of rebiana are generally available in the published literature.

Cargill's rebiana product is produced in accordance with current good manufacturing practice (cGMP). The manufacturing process for rebiana consists of extraction, isolation, and step-wise purification to consistently produce a product that meets food-grade specifications. Non-consecutive batch analyses confirm the consistency of the product and reproducibility of the manufacturing process.

JECFA has previously evaluated the safety database on steviol glycosides and established a temporary Acceptable Daily Intake (ADI) of 2 mg/kg body weight (expressed as steviol equivalents), equivalent to 6 mg/kg body weight/day for rebaudioside A[1]. Intake estimates for average and high consumers of rebiana are below the temporary ADI. Using published dietary exposure data for other approved high intensity sweeteners (HIS) (*e.g.*, aspartame) and adjusting for relative sweetness intensity, mean intake of rebiana (assuming 100% rebaudioside A content) was predicted to range from 1.3 mg/kg body weight/day (0.43 mg/kg body weight/day as steviol equivalents) for non-diabetic adults to 3.4 mg/kg body weight/day (1.12 mg/kg body weight/day as steviol equivalents) for children with diabetes. Predicted intakes for heavy intake consumers ranged from 3.4 mg/kg body weight/day (1.12 mg/kg body weight/day as steviol equivalents) for non-diabetic adults to 5.0 mg/kg body weight/day (1.64 mg/kg body weight/day as steviol equivalents) for non-diabetic children.

The metabolism and pharmacokinetic profile for steviol glycosides has been established and is described in the publicly-available literature. Rat and human metabolism studies carried out by Cargill using high-purity rebaudioside A and stevioside affirm their metabolic equivalence. Because of this equivalence, safety studies conducted with stevioside may be used to support the safety assessment of rebiana.

Steviol glycosides are not readily absorbed from the upper small intestine of the rat or human following oral administration. Human digestive enzymes are not capable of hydrolyzing β-glycosidic bonds and thus steviol glycosides are expected to escape digestion in the upper gastrointestinal tract. Microbes of the *Bacteroidaceae* family (predominantly Bacteroides) transform rebaudioside A and stevioside to steviol in the large intestine of the rat and human. Following absorption, steviol enters the hepatic circulation and undergoes conjugation with glucuronic acid to form steviol glucuronide. In rats, steviol, administered as steviol or available following cleavage of glycosides in the gut, has been shown to be primarily excreted in the feces *via* the bile (generally within 48 hours), with smaller amounts in the urine (less than 3%) (Wingard *et al.*, 1980; Nakayama *et al.*, 1986; Sung, 2002; Roberts and Renwick, 2008). In humans, the major excretory route is urinary (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Geuns *et al.*, 2006, 2007; Wheeler *et al.*, 2008) due to the lower molecular weight threshold for biliary excretion in rats (325) as compared to humans (500 to 600) (Renwick, 2008a).

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[1] *Temporary ADI*
Used by JECFA when data are sufficient to conclude that use of the substance is safe over the relatively short period of time required to generate and evaluate further safety data, but are insufficient to conclude that use of the substance is safe over a lifetime. A higher-than-normal safety factor is used when establishing a temporary ADI and an expiration date is established by which time appropriate data to resolve the safety issue should be submitted to JECFA (Annex 1, reference 76, page 114 <http://www.inchem.org/documents/ehc/ehc/ehc70.htm>). Studies reported in this safety summary include human clinical data that, pending favorable review by JECFA, will obviate the need for the additional 2-fold safety factor currently applied to the ADI for steviol glycosides. A future ADI of 12 mg/kg body weight/day for rebaudioside A is anticipated on the basis of the favorable safety database on purified steviol glycosides and the supplemental studies reported in this safety review.

Acute, subchronic, and chronic feeding studies indicate the lack of toxicity of purified steviol glycosides following dietary administration. Steviol glycosides are not acutely toxic by the oral route of exposure to mice, rats, or hamsters (Medon *et al.*, 1982; Toskulkao *et al.*, 1997).

Subchronic feeding studies of 4 to 13 weeks in duration in rats with high-purity rebaudioside A and stevioside have reported no evidence of systemic toxicity (Aze *et al.*, 1991; Curry and Roberts, 2008; Nikiforov and Eapen, 2008). Another 90-day multiple-dose toxicity study conducted in rats on stevioside of lower purity without any observed toxicity has been reported (Xili *et al.*, 1992). Likewise, no adverse effects were observed in rats administered *Stevia* extracts in studies with dosing periods ranging from 56 to 90 days (Akashi and Yokoyama, 1975; Lee *et al.*, 1979;).

In a study designed to meet FDA Redbook Guidelines and conducted according to GLP-compliance standards, rebiana was reported not to present any evidence of systemic toxicity when provided to both sexes of Han Wistar rats at dietary concentrations of up to 100,000 ppm (9,938 and 11,728 mg/kg body weight/day for males and females, respectively) for 4 weeks or 50,000 ppm (4,161 and 4,645 mg/kg body weight/day for males and females, respectively) for 13 weeks (Curry and Roberts, 2008). The results of the 13-week toxicity study of rebiana (Curry and Roberts, 2008) closely echo those of a similar study in Sprague-Dawley rats using test material of similar purity (Nikiforov and Eapen, 2008). Nikiforov and Eapen (2008) reported that feeding of rebaudioside A in the diet *ad libitum* to produce target doses 500, 1,000, or 2,000 mg/kg body weight/day was without adverse effect on body weight gain, terminal body weights, clinical and functional observational battery observations, or on the results of the hematology, serum chemistry, or urinalysis evaluations. Treatment was reportedly not associated with any organ weight or macroscopic or microscopic tissue changes.

Single- and multi-generation reproductive and developmental studies conducted with high-purity rebaudioside A and stevioside (Akashi and Yokoyama, 1975; Mori *et al.*, 1981; Usami *et al.*, 1995; Curry *et al.*, 2008) and slightly lower purity stevioside (Yodyingyuad and Bunyawong; 1991) have shown a lack of reproductive or developmental toxicity in rats and hamsters.

The existing data pertaining to the genotoxicity of rebaudioside A and stevioside demonstrate a lack of genotoxic activity for both compounds *in vitro* and *in vivo*. One comet assay conducted with stevioside (88.6% purity) indicated a positive result, but this study was subsequently shown to be uninterpretable (Geuns, 2007; Williams, 2007). Another comet assay (Sekihashi *et al.*, 2002) demonstrated no evidence of genotoxicity of *Stevia* extract comprising 52% stevioside and 22% rebaudioside A when orally administered to mice at up to 2,000 mg/kg body weight/day. The lack of genotoxic potential is confirmed by the results of 2 carcinogenicity studies on stevioside which show no oncogenic or toxicological effects (Xili *et al.*, 1992; Toyoda *et al.*, 1997).

Collectively, the results of the human studies (Alvarez *et al.*, 1981; Chan *et al.*, 2000; Gregersen *et al.*, 2001, 2004; Hsieh *et al.*, 2003; Temme *et al.*, 2004; Ferri *et al.*, 2006; Cavalcante da Silva *et al.*, 2006; Jeppesen *et al.*, 2006a; Geuns *et al.*, 2007; Maki *et al.*, 2007, unpublished; Wheeler *et al.*, 2008; Maki *et al.*, 2008a,b) demonstrate that steviol glycosides are safe and well-tolerated in groups of normotensive individuals and subjects with type 2 diabetes following long-term consumption at doses of up to 1.5 g/day, or about 25 mg/kg body weight/day. Human studies specifically on rebiana showed no effects on glucose homeostasis or blood pressure at doses of up to 1,000 mg/day (about 16 mg/kg body weight/day), a dose more than 3-fold greater than the predicted intake of rebaudioside A in adults and children with diabetes (4.5 mg/kg body weight/day).

There are no concerns for rebiana with respect to allergenicity or toxicity associated with degradation products or other related steviol glycosides in the final product. Based on the totality of the available data, it is concluded that there is no scientific evidence supporting the fact that purified steviol glycosides, including rebaudioside A, have any allergic or toxic potential.

The weight of evidence clearly supports the safety of rebiana, when produced in accordance with cGMP to a food-grade specification for its intended use as a general purpose use sweetener.

GRAS EXEMPTION CLAIM FOR REBIANA (REBAUDIOSIDE A)

I GRAS EXEMPTION CLAIM

I.A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)]

Rebiana, a highly purified form of rebaudioside A, which is one of several naturally occurring steviol glycosides present in the leaves of the *Stevia rebaudiana* (Bertoni) plant, has been determined by Cargill, Incorporated (hereafter Cargill) to be Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use in food, among experts qualified by scientific training and expertise. Therefore, the use of rebiana in food as described below is exempt from the requirement of premarket approval.

Signed,

Leslie Lake Curry
Director, Regulatory & Scientific Affairs
Cargill, Incorporated
15407 McGinty Road West
Wayzata, MN 55345

Date

I.B Name and Address of Notifier

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I.C Common Name of the Notified Substance

The common name of the notified substance that has been determined to be GRAS and thus is the subject of this GRAS notification is rebiana, a highly purified form of rebaudioside A with small amounts of other related steviol glycosides.

I.D Conditions of Intended Use in Food and Consumer Exposure

I.D.1 History of Use and Current Regulatory Status

Rebaudioside A and stevioside (a closely related structural analog) are typically identified as the principal sweetening constituents found in *S. rebaudiana* and are accompanied by smaller amounts of other steviol glycosides. *S. rebaudiana* and stevioside have been consumed for hundreds of years by humans, in various countries, as sweeteners in foods and beverages (Geuns, 2003). There have been no reports of adverse effects following the use of these natural sweeteners (Lee, 1979; Ferlow, 2005).

Currently, rebaudioside A is not permitted for addition to food and beverages in either the United States or the European Union but is permitted in a number of countries. Stevioside and *S. rebaudiana* leaf extracts are accepted for general use as sweeteners in a variety of foods and beverages including pickling gum, pickles, dried seafood, meat, fish, soy sauce, bean pastes, sugarless chewing gums, juices, cola, table-top sweeteners, and ice cream in Japan (Marie, 1991; Das *et al.*, 1992; Ferlow, 2005). In Korea, stevioside is widely used in cookies, sugar products, beverages, seasonings, soy sauce, honey, and *so-ju* (traditional liquor made of starch). Stevioside, *S. rebaudiana* leaves, and highly refined extracts also are permitted for use as low-calorie sweeteners in Argentina, Paraguay, and Brazil. Recently, the Food Standards Australia New Zealand (FSANZ) recommended that steviol glycosides be approved for use as sweeteners in Australia and New Zealand (FSANZ, 2007). Furthermore, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2006) allocated a temporary Acceptable Daily Intake (ADI) of 0-2 mg/kg body weight/day, expressed as steviol, for steviol glycosides based on a no-observed-adverse-effect level (NOAEL) of 970 mg/kg body weight from a 2-year study in rats (Toyoda *et al.*, 1997). However, the Committee noted at that time that available human data indicated evidence of pharmacological effects in patients with hypertension or type 2 diabetes and requested further information regarding the potential pharmacological effects of steviol glycosides. Steviol glycosides are scheduled for re-evaluation by JECFA in June 2008.

Stevia became a popular herbal tea ingredient in the United States in the 1980s (Blumenthal, 1995; Ferlow, 2005). In 1995, the use of stevioside in Asia was reported to be approximately 160,000 metric tons as sucrose equivalence (SE), while in 1999, such use reportedly increased to approximately 200,000 metric tons SE (Anonymous, 2001). Stevioside (containing other

steviol glycosides) has been used as a sweetener in Japan for more than 30 years, and its use has been reported to be safe, without the occurrence of adverse effects (Ferlow, 2005).

I.D.2 Functionality

Rebiana is intended for use as a general purpose sweetening agent, in accordance with cGMP.

According to the literature, rebaudioside A is 200 to 300 times more potent than sucrose. However, sweetness depends heavily on concentration for all high intensity sweeteners (HIS). At a SE of 6% (realistic use-levels of HIS are generally in the range of 4 to 8% SE), the potency of rebaudioside A is 200 times that of sucrose (DuBois *et al.*, 1991). This is similar to the sweetness of aspartame, an artificial HIS that is widely marketed and approved for addition to numerous foods and beverages in several countries. In comparison to sucrose, aspartame is 180 times as sweet at 6% SE (DuBois *et al.*, 1991).

I.D.3 Intended Use of Rebiana and Levels of Use in Foods

Considering that rebiana is characterized by a sweetness potency that is largely comparable to that of aspartame, the uses and use-levels for rebiana are likely to reflect use levels for aspartame in the United States, with a few minor exceptions. The permitted uses of aspartame prior to its acceptance as a general purpose sweetener and corresponding levels of rebiana are summarized in Table I.D.3-1. The anticipated use-levels for rebiana are to be lower than those of aspartame for baked goods, baking mixes, ready-to-eat cereals, and granola bars due to the known stability issues with aspartame and the technical requirement to add an overage to provide appropriate sweetening capability which are not necessary for rebiana. This is because rebiana exhibits greater stability than aspartame during thermal processing, thus eliminating the technical requirement to add an overage to account for losses. Increased use-levels of rebiana are anticipated for chewing gum, breath freshening mints, soft candy, nougats, and marzipans. Only one new category of use, soybean-based beverages, has been proposed. These small differences will have no impact of the estimated consumption of rebiana.

Table I.D.3-1 Summary of the Individual Food-Uses and Use-Levels for Aspartame in the United States Prior to Regulatory Approval as a General Purpose Sweetener and Proposed Food-Uses and Use-Levels for Rebiana

Food Category	Proposed Food Uses	Use-Level (%)	
		Aspartame	Rebiana
Alcoholic Beverages	Aromatized Alcoholic Beverages (Excluding Beer)	0.06	0.06
Baked Goods and Baking Mixes	Cakes	0.17	0.1
	Cookies	0.17	0.1
	French Toast, Pancakes, and Waffles	0.17	0.1
	Muffins, Scones and Doughnuts	0.17	0.1
	Pastries and Pie Crust	0.17	0.1
	Sweet Breads and Rolls	0.17	0.1
Beverages and Beverage Bases	Carbonated Beverages	0.06	0.06
	Coffee and Tea Drinks	0.06	0.06
	Fruit Flavored Drinks	0.06	0.06
	Energy, Sport, and Electrolyte Drinks	0.06	0.06
	Meal Replacements (non-milk based), Not for Weight Reduction	0.06	0.06
	Meal Replacements (non-milk based) for Weight Reduction	0.08	0.08
Breakfast Cereals	Ready to Eat Breakfast Cereals	0.1	0.1
	Instant and Regular Hot Breakfast Cereals	0.1	0.1
Chewing Gum	Chewing Gum	0.55	1.5
Condiments and Relishes	Mustard	0.035	0.035
	Ketchup	0.035	0.035
Confections and Frostings	Cocoa Mixes	0.1	0.1
	Cocoa-Based Spreads and Fillings	0.1	0.1
	Frostings, Icings, and Coatings	0.1	0.1
Dairy Product Analogs	Soybean-Based Beverages	N/A	0.06
Fats and Oils	Emulsified Sauces	0.035	0.035
	Fat-Based Desserts	0.1	0.1
Frozen Dairy Desserts and Mixes	Ice Cream, Novelties, and Frozen Milk Desserts	0.1	0.1
	Frozen Yogurt	0.1	0.1
Fruit and Water Ices	Edible Ices, Sherbet, and Sorbet	0.08	0.08
Gelatins, Puddings, and Fillings	Puddings and Other Milk-Based Desserts	0.1	0.1
	Flans, Custards, and Other Egg-Based Desserts	0.1	0.1
Grain Products and Pastas	Cereal and Granola Bars	0.17	0.10
	Energy, Meal Replacement, and Fortified Bars	0.2	0.2
Gravies and Sauces	Water and Milk-Based Sauces, Gravies, and Dressings, Including Mixes	0.035	0.035
	Clear Sauces	0.035	0.035

Table I.D.3-1 Summary of the Individual Food-Uses and Use-Levels for Aspartame in the United States Prior to Regulatory Approval as a General Purpose Sweetener and Proposed Food-Uses and Use-Levels for Rebiana

Food Category	Proposed Food Uses	Use-Level (%)	
		Aspartame	Rebiana
Hard Candy	Breath-Freshening Micro Mints with No Added Sugar	0.6	1.0
	Hard Candy	0.1	0.1
	Freshening Throat Pastilles with No Added Sugar	0.2	0.2
Jams and Jellies	Jams, Jellies, Preserves, and Marmalades	0.1	0.1
Milk Products	Fermented Milks, Plain	0.1	0.1
	Flavored Milk, Milk Drinks, and Mixes (not cocoa)	0.06	0.06
	Milk-Based Meal Replacements, Not for Weight Reduction	0.06	0.06
	Milk-Based Meal Replacements, For Weight Reduction	0.08	0.08
	Yogurt	0.1	0.1
	Yogurt Drinks	0.06	0.06
Nut and Nut Products	Nut Spreads	0.1	0.1
	Processed Whole Nuts, Coated Nuts, and Mixtures	0.05	0.05
Processed Fruits and Fruit Juices	Canned or Bottled Fruit	0.1	0.1
	Coconut Milk and Coconut Cream	0.1	0.1
	Fruit Fillings For Pastries	0.1	0.1
	Fruit Puree	0.1	0.1
	Fruit-Based Desserts	0.1	0.1
Processed Vegetables and Vegetable Juices	Vegetable Purees	0.1	0.1
Soft Candy	Cocoa and Chocolate Products	0.2	0.2
	Soft Candy, Nougats, and Marzipans	0.1	0.15
Sugar Substitutes	Table Top Sugar Substitutes	GMP	GMP
Sweet Sauces, Toppings, and Syrups	Cocoa Syrups	0.1	0.1
	Fruit Sauces, Syrups, and Toppings	0.1	0.1
	Sweet Sauces and Toppings (not fruit, not syrups)	0.1	0.1

N/A = not applicable

I.D.4 Estimated Dietary Consumption of Rebiana Based upon Intended Food Uses

Based on production data, the *per capita* consumption of caloric sweeteners in the United States is 216.5 g/day (USDA, 2007). Assuming that rebiana would replace all sugar consumption, and assuming that rebiana is 200 times as sweet as sugar, this corresponds to a rebiana intake of approximately 18 mg/kg body weight/day (average body weight 60 kg assumed) or 5.9 mg steviol equivalents/kg body weight/day (average body weight of 60 kg assumed). However, these estimated intakes are highly conservative since it is unlikely that rebiana would entirely replace sugar consumption.

Since there have been numerous studies in the United States, Canada, Australia/New Zealand, and countries in the European Union that identify the intakes of aspartame and other HIS through post-market surveillance data, a more realistic, but conservative approach is to estimate rebiana intake based on the intake figures reported in these published studies. The intake of rebaudioside A was estimated by Renwick (2008b) using published data on dietary exposures to approved intense sweeteners, such as aspartame from post-market surveillance studies, with adjustment for their relative sweetness intensities, assuming a relative sweetness for rebiana of 200 times that of sucrose (Renwick, 2008b). For the purposes of this assessment, it was assumed that the composition of rebiana is 100% rebaudioside A.

The calculated intakes of intense sweeteners (as sucrose equivalents) based on published data, and the corresponding predicted intake of rebiana, assuming complete replacement of other intense sweeteners and a relative rebiana sweetness of 200 times that of sucrose, were determined for non-diabetic and adults and children with diabetes. Using this approach, estimated intake for average users was highest for children with diabetes at 3.4 mg rebiana/kg body weight/day (672 and 1.12 mg/kg body weight/day, expressed as sucrose equivalents and steviol equivalents, respectively), and lowest for non-diabetic adults at 1.3 mg rebiana/kg body weight/day (255 and 0.43 mg/kg body weight/day, expressed as sucrose equivalents and steviol equivalents, respectively). On a heavy intake consumer basis, non-diabetic children were determined to have the highest calculated intake of intense sweeteners (as sucrose equivalents), rebiana, and rebiana expressed as steviol equivalents (990, 5.0, and 1.64 mg/kg body weight/day, respectively), while non-diabetic adults were determined to have the lowest calculated intakes on a heavy user basis (675, 3.4, and 1.12 mg/kg body weight/day, respectively). The predicted intakes of rebiana are all below the current temporary ADI defined by the JECFA for steviol glycosides (JECFA, 2005) of 0-2 mg/kg body weight/day as steviol.

I.E Basis for the GRAS Determination

Pursuant to 21 CFR §170.35, rebiana has been determined by Cargill to be GRAS under the intended use as a general purpose sweetener limited by cGMP on the basis of scientific procedures (U.S. FDA, 2007). This determination is based on the views of experts who are qualified by scientific training and experience to evaluate the safety of rebiana as a component

of food [see Appendix I, **EXPERT PANEL REPORT CONCERNING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF REBAUDIOSIDE A (REBIANA) FOR USE AS A GENERAL PURPOSE SWEETENING AGENT**].

At the request of Cargill, Incorporated (Cargill), an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened on March 27 and 28, 2008 to conduct a critical and comprehensive evaluation of the available pertinent data and information on rebaudioside A (rebiana), and determine whether rebiana under the conditions of intended use as a general purpose sweetening agent in conventional foods (when not otherwise precluded by a Standard of Identity), is safe and GRAS, based on scientific procedures.

The Panel consisted of the following qualified scientific experts: Henry Black, M.D. (New York University School of Medicine), Samuel Cohen, Ph.D., M.D. (University of Nebraska Medical Center), Morey Haymond, M.D. (Baylor College of Medicine), Glenn Sipes, Ph.D. (University of Arizona), and William Waddell, M.D. (University of Louisville).

The Expert Panel convened on behalf of Cargill, independently and collectively, critically evaluated the data and information summarized herein and concluded that the proposed use of rebiana as a general purpose sweetener in foods and beverages (when not otherwise precluded by a Standard of Identity), produced consistently with cGMP and meeting appropriate food-grade specifications described herein, is safe. They further concluded that the proposed use of rebiana as a general purpose sweetening agent is GRAS under the Food, Drug, and Cosmetic Act (FDCA) based on scientific procedures.

It is also Cargill's opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion.

Because rebiana is GRAS based on scientific procedures for its intended use as a general purpose sweetening agent, it is excluded from the definition of a food additive and thus may be marketed and sold for its intended purpose in the United States without the promulgation of a food additive regulation under 21 CFR.

I.F Availability of Information

The detailed data and information that serve as a basis for this GRAS determination will be provided to the FDA upon request, or are available for the Food and Drug Administration's review and copying during reasonable business hours at the offices of:

Leslie Lake Curry
Director, Regulatory & Scientific Affairs

Cargill, Incorporated
15407 McGinty Rd W
Wayzata, MN 55345

Telephone: 952-742-5371
Facsimile: 952-742-7573
Email: Leslie_Curry@cargill.com

Should the Unites States Food and Drug Administration (FDA) have any questions or additional information requests regarding this notification, Cargill will supply these data and information.

II. DETAILED INFORMATION ABOUT THE IDENTITY OF THE SUBSTANCE

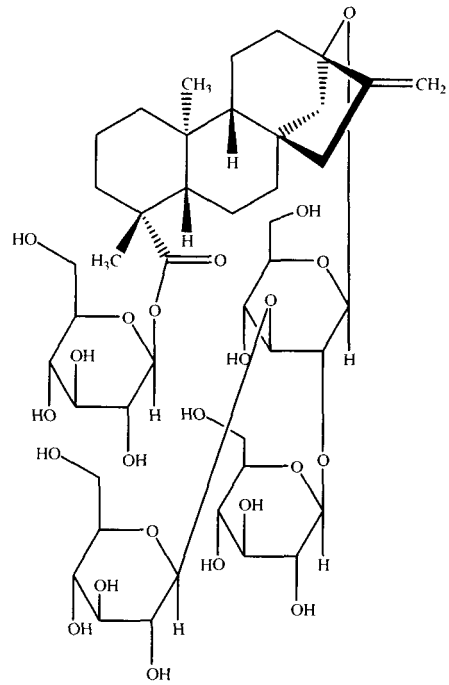
II.A Identity

II.A.1 Chemical Description

Cargill intends to market rebiana as a general purpose sweetener. Rebiana is high-purity rebaudioside A, which is one of several steviol glycosides (steviol conjugated with glucose, xylose, and/or rhamnose) known to occur naturally as constituents of the *Stevia rebaudiana* (Bertoni) plant. *S. rebaudiana*, also known as honey leaf and sweet herb, is a perennial shrub of the *Compositae* family, which has been native to Northeastern Paraguay, Brazil, and other South American regions for over 1,500 years (Geuns, 2003; Ferlow, 2005). At least 10 different steviol glycosides have been identified in the leaves of *S. rebaudiana* (stevioside, rebaudioside A, B, C, D, E, and F, dulcoside A, rubusoside, and steviolbioside). Rebaudioside A accounts for approximately 2 to 4% of the plant leaf composition (SCF, 1999). The glycosides can be obtained by extracting *Stevia* leaves with hot water, followed by solvent purification of the water-soluble extract. The water extracts, obtained from the crushed *Stevia* leaves, have a long history of use primarily for their sweetening properties. Rebaudioside A and stevioside (a closely related structural analog) are typically identified as the principal sweetening constituents and are accompanied by smaller amounts of other steviol glycosides. Depending on the production process, individual steviol glycosides can be isolated and high-purity products can be obtained. Cargill's rebaudioside A product, rebiana is comprised of not less than 97% rebaudioside A.

Rebiana is produced as a white to off-white powder, with a characteristic sweet taste. It is freely soluble in water, but not soluble in ethanol.

Common or Usual Name: Rebiana; Rebaudioside A
Chemical Name: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid β-D-glucopyranosyl ester
Chemical Abstracts Service (CAS) Number: 58543-16-1
Empirical Formula and Formula Weight: $C_{44}H_{70}O_{23}$
Molecular Weight: 967.014 g/mol
Structural Formula:

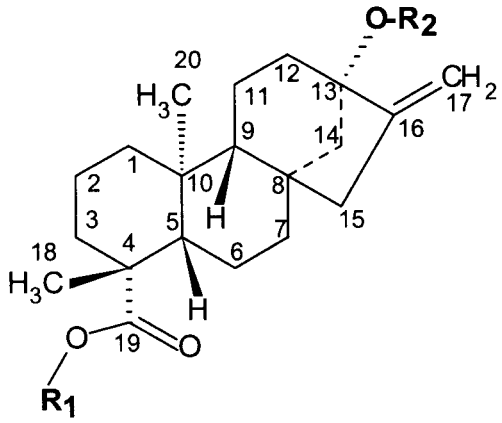


II.A.2 Related Steviol Glycosides

Several other related steviol glycosides of rebaudioside A have been identified in small amounts by high performance liquid chromatography (HPLC) in the rebiana bulk material, as well as at the start of the storage periods in representative food and mock beverage samples to which rebiana was added for assessment of stability (see Section II.C.3). The other related steviol glycosides included rebaudioside B (CC-00202), rebaudioside F (CC-00216), IMP-2 (CC-00218), IMP-4 (CC-00220), and DAQ3 (CC-00208). More sensitive liquid chromatography tandem mass spectroscopy (LC-MS/MS) analyses of 5 lots of rebiana (Lot Nos. 1001 to 1005) further revealed the presence of trace amounts of DAQ1 (CC-00207), DAQ4 (CC-00210), DAQ5 (CC-00212), and DAQ7 (CC-00222). As listed in Section II.C.1, proposed specifications for rebiana limit the level of the related steviol glycosides to not more than 3.0% of the final rebiana

product. Levels of some of these related steviol glycosides increase over time, thus also classifying them as degradation products (see Section II.C.3).

Many of these related steviol glycoside products share the same general steviol aglycone backbone structure as rebaudioside A (see Figure II.A.2-1), while a few others are characterized by slight structural differences to the aglycone.



Steviol: R_1 and R_2 = H

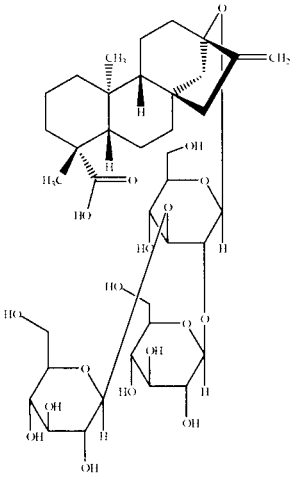
Figure II.A.2-1 Aglycone Structure of Rebaudioside A and Related Steviol Glycosides

The structural characteristics (aglycone backbone structure and number of monosaccharide units) for each identified related steviol glycoside are summarized in Table II.A.2-1 (for discussion of the degradation products listed in the Table, see Section II.C.3). The structures for each identified related steviol glycoside are presented in Figure II.A.2-2.

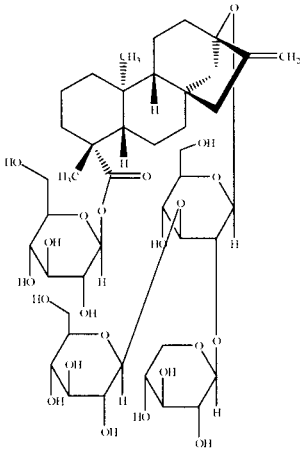
Table II.A.2-1 Summary of Other Related Steviol Glycosides and Degradation Products of Rebiana and their Structural Properties

Steviol Glycoside	'CC' Number	Classification	Aglycone (Backbone Structure)	No. of Mono-Saccharide Units
Rebaudioside A	CC-00201	--	Steviol	4 Glu
Rebaudioside B	CC-00202	Related Steviol Glycoside/Degradation Product	Steviol	3 Glu
Rebaudioside F	CC-00216	Related Steviol Glycoside	Steviol	3 Glu, 1 Xyl
IMP-2	CC-00218	Related Steviol Glycoside /Degradation Product	Endocyclic double bond and a hydroxyl group at C-17	4 Glu
IMP-4	CC-00220	Related Steviol Glycoside	Steviol	5 Glu
DAQ1	CC-00207	Related Steviol Glycoside [1]/ Degradation Product	Hydroxyl group at C-16	4 Glu
DAQ3	CC-00208	Related Steviol Glycoside /Degradation Product	Endocyclic double bond	4 Glu
DAQ4	CC-00210	Related Steviol Glycoside [1]/ Degradation Product	Endocyclic double bond	3 Glu
DAQ5	CC-00212	Related Steviol Glycoside [1]/ Degradation Product	Isosteviol	1 Glu
DAQ7	CC-00222	Related Steviol Glycoside [1]/ Degradation Product	Steviol	1 Glu
DAQ2	CC-00209	Degradation Product	Hydroxyl group at C-16	3 Glu
DS-1	CC-00219	Degradation Product	Steviol	5 Glu
DAQ6	CC-00221	Degradation Product	Steviol	2 Glu

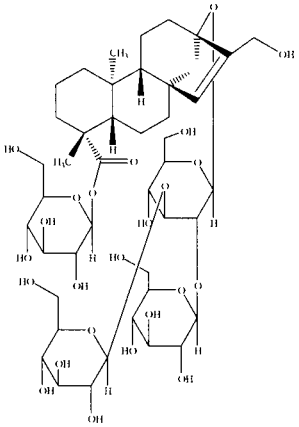
Glu = Glucose; Xyl = Xylose.
[1] As demonstrated by LC-MS/MS analysis.



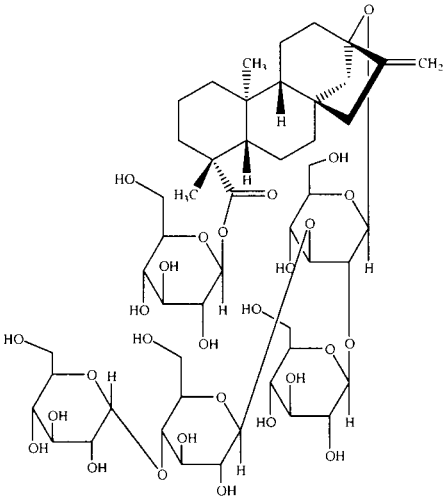
Rebaudioside B (CC-00202)



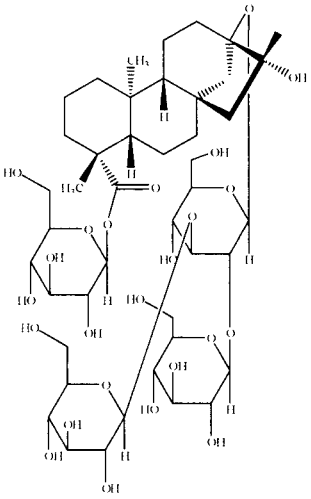
Rebaudioside F (CC-00216)



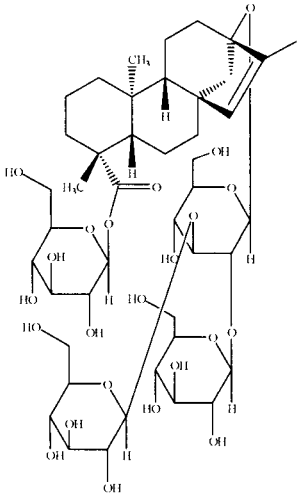
IMP-2 (CC-00218)



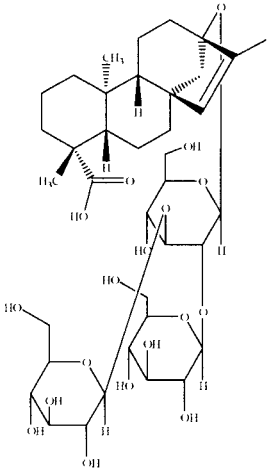
IMP-4 (CC-00220)



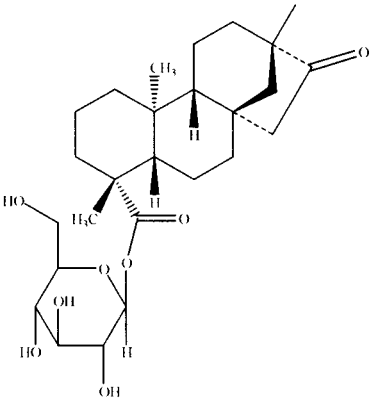
DAQ1 (CC-00207)



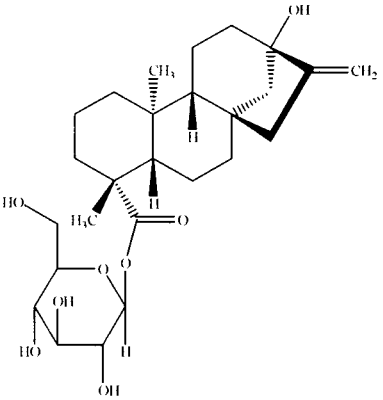
DAQ3 (CC-00208)



DAQ4 (CC-00210)



DAQ5 (CC-00212)

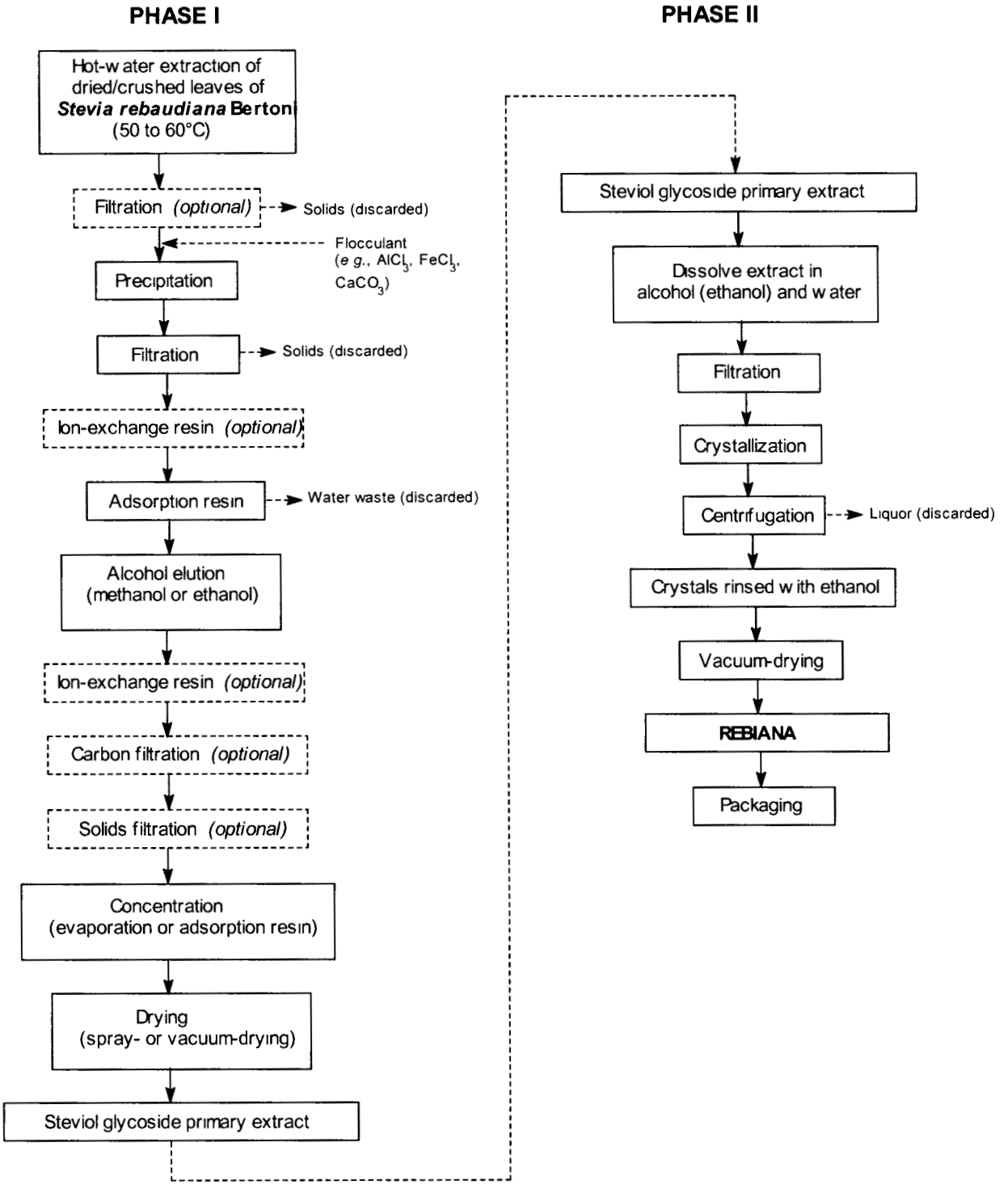


DAQ7 (CC-00222)

Figure II.A.2-2 Structures of Related Steviol Glycosides in Rebiana

II.B Method of Manufacture

Rebiana is obtained by hot water-extraction of leaves from the *S. rebaudiana* plant. The process can be divided into 2 phases, with the first phase involving the extraction of the leaves and preliminary purification to yield the steviol glycoside primary extract, followed by a second phase which involves re-crystallization of the steviol glycoside primary extract from a water/alcohol mixture to obtain a final product with a high rebaudioside A content (*i.e.*, rebiana). The production process of rebiana is illustrated in Figure II.B-1.



$AlCl_3$ = Aluminum chloride; $CaCO_3$ = Calcium carbonate; $FeCl_3$ = Ferric chloride.

Figure II.B-1 Schematic Overview of the Production Process for Rebiana

Cargill obtains the steviol glycoside primary extract, meeting appropriate food-grade specifications outlined by Cargill based on the Chinese National Standards for steviol glycosides (see Table II.B-1), from other manufacturers. Ethanol (≥99.5%) used in the 2nd phase of the production process of rebiana to dissolve the steviol glycoside primary extract prior to recrystallization is approved for food use.

Table II.B-1 Cargill's Specifications for the Steviol Glycoside Primary Extract

Specification Parameter	Specification	Analytical Method
Appearance	White, loose powder or crystals	Visual inspection
Total steviol glycosides content	≥90%	GB 8270 -1999 (5.2)[1]
Rebaudioside A content	≥40%	GB 8270 -1999 (5.2)
Loss on drying	≤4%	GB/T 5009.3
Residue on ignition	≤0.1%	GB/T 5099.4
Absorbance	≤0.05%	GB 8270-1999 (5.2)
Heavy metals		
Lead (Pb)	≤1 ppm	GB/T 5099.12
Arsenic (As)	≤1 ppm	GB/T 5099.11
Microbiological parameters		
Mesophilic bacteria	≤100 CFU/gram	GB 4789.2
Mold and yeast	≤10 CFU/gram	GB 4789.5
Escherichia coli	Negative (MPN) per 100 gram	GB 4789.3
Pathogens (*Salmonella*)	Negative (CFU) per 25 gram	GB 4789.4

CFU = Colony Forming Unit; MPN = Most Probable Number.
[1] Chinese National Standard Method Number.

II.C Specifications for Food Grade Material

II.C.1 Chemical and Microbiological Specifications

The chemical and microbiological specifications for rebiana are presented in Table II.C.1-1. Analyses of representative, non-consecutive lots demonstrated compliance with final product chemical and microbiological specifications.

Table II.C.1-1 Chemical and Microbiological Specifications for Rebiana		
Specification Parameter	**Specification**	**Method**
Chemical Parameters		
Identity	Conforms to IR standard	FCC V[1]
Solubility	Freely soluble in water	FCC V
pH	Between 4.5 and 7.0 (1% solution; wt/v)	FCC V
Assay (Rebaudioside A content)	Not less than 97.0% and not more than 102.0% (wt/wt) (on an anhydrous basis)	HPLC method (Cargill method No. STV-001-01)
Other related steviol glycosides	Not more than 3.0% (wt/wt)	HPLC method (Cargill method No. STV-001-01)
Loss on drying	Not more than 6.0% (105 °C)	FCC V
Residue on ignition (ash)	Not more than 1.0% (wt/wt)	FCC V, Method I
Specific rotation (water 0.5 wt%) $[\alpha]_D^{25}$	Between -29 and -31° (on anhydrous basis)	FCC V
Lead (Pb)	Not more than 1 ppm	AOAC, 2000[2] (ICP; method 993.14)
Solvent residues		
Ethanol	Not more than 0.5% (wt/wt)	FCC V
Methanol	Not more than 0.02% (wt/wt)	FCC V
Microbiological Parameters		
Standard plate count	Not more than 1,000 CFU	BAM[1], Chapter 3
Total coliforms	Not more than 3 CFU	BAM[1], Chapter 4
Fecal coliforms	Not more than 3 CFU	BAM[1], Chapter 4
Escherichia coli	Not more than 10 CFU	BAM[1], Chapter 4
Listeria	Negative (in 11 grams)	BAM[1], Chapter 10
Salmonella	Negative (in 25 grams)	BAM[1], Chapter 5
Staphylococcus	Not more than 10 CFU	BAM[1], Chapter 12
Yeast	Not more than 100 CFU	BAM[1], Chapter 18
Mold	Not more than 100 CFU	BAM[1], Chapter 18

CFU = Colony Forming Unit; HPLC = High Performance Liquid Chromatography; ICP-MS = Inductively Coupled Plasma Mass Spectrometry; IR = Infrared.
[1] FCC. 2003. Food Chemicals Codex (5th Ed.). National Academy Press (NAP); Washington, DC.
[2] AOAC. 1998. Official Methods of Analysis of the Association of Official Analytical Chemists (16th Ed.). Association of Official Analytical Chemists (AOAC), Inc.; Arlington, VA.
[3] U.S. FDA. *Bacteriological Analytical Manual Online* (BAM). College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), 2001-2005. Available from: http://www.cfsan.fda.gov/~ebam/bam-toc.html [Jan. 2001, Last Updated: Oct. 3, 2005].

II.C.2 Additional Analyses

Rebiana is routinely screened for commonly used pesticides in cultivation practices, including organochlorine, organophosphorus, *N*-containing, benzimidazole-type, and phenoxycarboxylic acid and other acidic pesticides, as well as for the herbicide, glyphosate. Multi-residue pesticide screens revealed no pesticide residues in the steviol glycoside primary extract or rebiana

(detection limits varied among different groups and among individual compounds within a group between 0.005 and 1.0 mg/kg). In addition to lead, rebiana also is routinely screened for arsenic, cadmium, and mercury, all of which have been demonstrated not to occur at levels of 1 ppm or greater.

II.C.3 Stability

A series of studies assessed the bulk stability of rebiana (dry) under various storage conditions, as well as under a range of pH and temperatures. Studies were also conducted in representative food matrices (real food/beverages at both room and elevated temperatures) to determine whether rebiana remains stable in foods and beverages at a range of pH values and following processing. The photostability of rebiana was also examined under dry and aqueous conditions. The collective results from stability studies with rebiana demonstrate its stability in foods representing a broad spectrum of pH and temperature conditions.

II.C.3.1 Bulk Stability of Rebiana (dry powder)

In a 5-year study designed to evaluate the bulk stability of rebiana (dry powder; Lot Nos. 1002, 1003, and 1004), slight reductions in rebaudioside A content were observed at interim 52- and 104-week analyses when rebiana was stored in glass bottles (in the range of less than 1% to not more than 1.5%) or in polyethylene bags (ranging from 1.5 to 2.7%) at 25°C and 60% relative humidity. The levels of rebaudioside A were shown to decrease by not more than 6.4% over the course of the 65-week storage period under accelerated storage conditions (40°C and 75% relative humidity) in sealed glass bottles. The slight reductions in the levels of rebaudioside A were accompanied by increasing levels of rebaudioside B (CC-00202) and DS-1 (CC-00219), with the latter not detected in the rebiana sample material at the beginning of the storage period. DS-1 (CC-00219) is likely formed as a result of free glucose released from the degradation of other related steviol glycosides reacting with rebaudioside A (see Figure II.C.3.4-1 for DS-1 structure).

Rebiana (dry powder) was also demonstrated to be photostable after exposure to fluorescent (1.2 million lux hours; equivalent to approximately 2 weeks) and near-UV (not less than 200 watt hours/m^2) light in a light chamber set at 25°C and 60% relative humidity.

II.C.3.2 Stability of Rebiana in Phosphate Buffer

The general stability of rebiana (500 mg/L; Lot No. 1001) to different pH and temperature conditions was assessed in 0.1 M phosphate buffer at temperatures ranging from 5 to 80°C and at pH values from 2 to 8. The extent and rate of rebiana degradation were shown to be dependent on pH, temperature, and time. In general, rebiana was more stable at pHs between 4 and 6 and at temperatures between 5 and 25°C than under other pH and temperature conditions.

II.C.3.3 Stability of Rebiana in Foods and Beverages

A 3-dimensional model previously validated for aspartame (Pariza *et al.*, 1998), was developed to evaluate the stability of rebiana in representative examples of the intended food and beverage uses. The model considers a range of moisture levels, temperatures, and pH values and which are typically considered as the primary factors affecting the stability of an ingredient in food. The extremes of the 3-dimensional model were represented by the following food types: table-top product (low moisture, low heat, medium pH), mock beverages (high moisture, low heat, low pH), yogurt (high moisture, medium heat, low pH), thermally processed beverages (high moisture, medium-to-high heat, low pH), and white cake (moderate moisture, high heat, medium pH).

Rebiana (Lot No. 1001) stability was demonstrated for a period of 26 weeks under normal (25°C and 50% relative humidity) and accelerated storage conditions (40°C and 75% relative humidity) when evaluated in a table-top product at a rebaudioside A target concentration of 1% (10,000 mg/kg) (table-top product also contained 98.7% erythritol and 0.3% silica). At the end of the 26-week storage period, concentrations of 105 and 99.5% of rebaudioside A were quantified relative to baseline values under normal and accelerated storage conditions, respectively.

The stability of rebiana in mock beverages (500 mg/mL), assessed under various pH conditions reflective of a range of potential end food-uses [pH 2.8 (cola drinks), pH 3.2 (cola drinks), pH 3.8 (lemon-lime soft drinks), and pH 4.2 (root beer soft drinks)], and at different storage temperatures (5, 20, 30, and 40°C) for periods of up to 26 weeks, was demonstrated to be pH-, temperature-, and time-dependent. Solutions with a pH of 3.2 were stored at 20°C for 8 weeks, conditions considered by the FDA as representative for the evaluation of non-nutritive sweetener stability in carbonated soft drinks (U.S. FDA, 1998). At 8 weeks and either 5 or 20°C, rebaudioside A loss was <3.5% at all 4 pH values. At 20°C, rebaudioside A loss was slightly increased from 2.2% at week 8 to 4.1% at week 26 in the pH 3.2-beverage. At the higher temperatures (30 or 40°C) and the lower pH values (2.8 and 3.2) degradation ranged from >4.5% to <27% at week 8 and from >11% to <62% at week 26. DAQ1 (CC-00207) and DAQ3 (CC-00208) were identified as the major degradation products, with the former increasing from levels below detection at study beginning. Levels of rebaudioside B (CC-00202) were detected in the mock beverage samples at the start of the storage period and increased over time, particularly in all samples stored at 30 and 40°C. Other degradation products detected in mock beverages following storage of up to 26 weeks included DAQ2 (CC-00209), DAQ4 (CC-00210), DAQ5 (CC-00212), DAQ6 (CC-00221), and DAQ7 (CC-00222), which were detected only at higher temperatures (30 or 40°C) at all pH values [DAQ2 (CC-00209) and DAQ6 (CC-00221) detected only in the pH 2.8 and 3.2 beverages].

Rebiana in mock beverages (pH 3.8) exposed to fluorescent (minimum of 1.2 million lux hours; equivalent to approximately 2 weeks) and near-UV (minimum 200 watt hours/m^2) light was also

demonstrated to be photostable. Rebaudioside A content decreased slightly from 505 mg/L in unexposed samples to 498 mg/L in light-treated beverages. Apart from rebaudioside B (CC-00202), DAQ3 (CC-00208), rebaudioside F (CC-00216), and IMP-4 (CC-00220), which were detected in both covered (control) and uncovered (test) samples, DAQ1 (CC-00207) (0.654 mg/L) and IMP-2 (CC-00218) (1.22 mg/L) also were identified in the light-exposed samples. The low levels of DAQ1 (CC-00207) and IMP-2 (CC-00218) detected in the uncovered, but not covered samples could be reflective of analytical error, rather than degradation given that the limit of detection was 0.500 mg/L.

Short-term thermal processing of a rebiana-containing (Lot No. 1005) mock beverage at low (3.2) and high (6.5) pH conditions typically used in pasteurization resulted in less than 1% loss of rebaudioside A. The minimal decrease in rebaudioside A content in the mock beverage at pH 3.2 was accompanied by a small increase in the levels of DAQ3 (CC-00208). In the mock beverage at pH 6.5, a small increase in the levels of rebaudioside B (CC-00202) was noted.

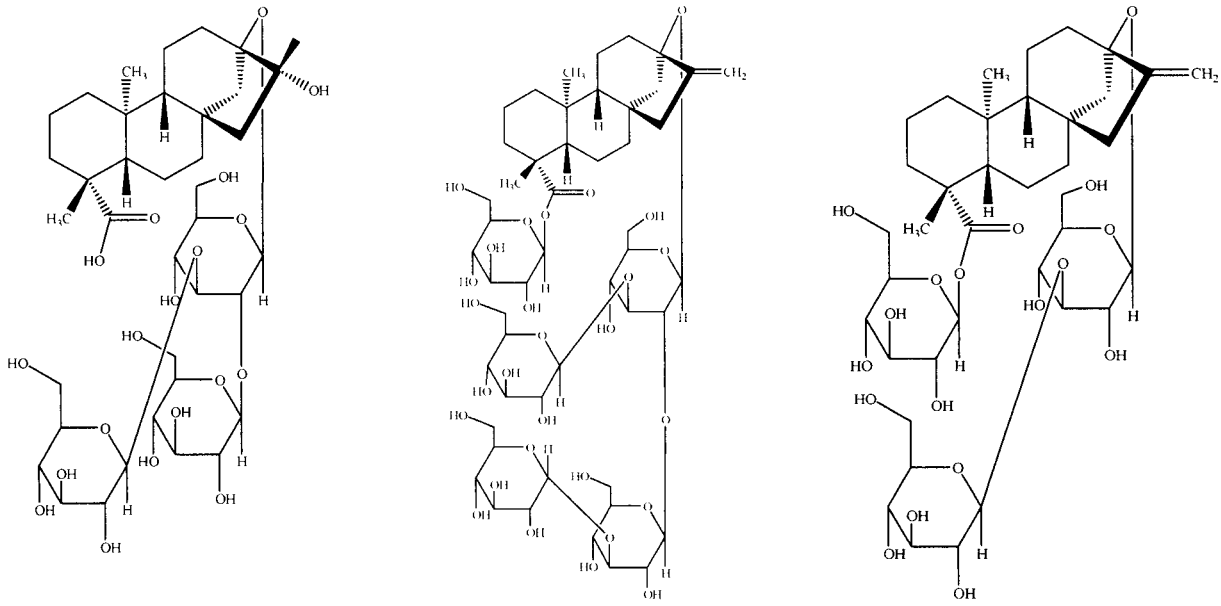
Rebiana (Lot No. 1002) was demonstrated to be stable when added to plain yogurt at a rebaudioside A target concentration of 0.034% (340 mg/kg), pasteurized (190°F for 5 minutes), fermented, and stored for a period of 6 weeks at 5±3°C. Relative to baseline values, 106% rebaudioside A was identified in yogurt at the end of the 6-week storage period; however, the levels of rebaudioside A in the yogurt were reported to be nearly 20% lower than levels in the yogurt sample after pasteurization. The decrease in levels of rebaudioside A after blending was determined to be the result of the experimental conditions rather than loss at processing and not due to degradation.

Rebiana (Lot No. 07Y28ISB201) added to cake batter at a rebaudioside A target concentration of 0.0673% (0.2491 g rebaudioside A/370 g batter or 673 mg/kg), which was subsequently baked for 20 to 25 minutes in a conventional oven at 360°F or in a convection oven at 335°F and stored for 5 days at 25°C and 60% relative humidity, was demonstrated to remain stable during the baking process and after the 5-day storage period. Following the 5-day storage period, 99.9% of rebaudioside A was identified in the cake sample. Overall, the results of these studies confirm the stability of rebiana in various food matrices following several days or weeks of storage.

II.C.3.4 Degradation Products of Rebiana Identified in the Stability Studies

Based on the results of the stability studies, the following compounds were identified as degradation products of rebiana: rebaudioside B (CC-00202), IMP-2 (CC-00218), DAQ1 (CC-00207), DAQ3 (CC-00208), DAQ4 (CC-00210), DAQ5 (CC-00212), DAQ7 (CC-00222), DAQ2 (CC-00209), DS-1 (CC-00219), and DAQ6 (CC-00221). The first 7 compounds are other related steviol glycoside identified in rebiana that also increase over time under certain conditions (see Section II.A.2; Table II.A.2-1 and Figure II.A.2-2). Conversely, DAQ2 (CC-00209), DS-1 (CC-00219), and DAQ6 (CC-00221) have not been identified in the bulk

rebiana material by either HPLC or LC-MS/MS. The chemical structures of these 3 degradation products are presented in Figure II.C.3.4-1. As with the related steviol glycosides discussed in Section II.A.2, degradation products CC-00219 (DS-1) and CC-00221 (DAQ6) share the same steviol aglycone backbone structure as rebaudioside A and differ only with respect to the number of glucose units. DS-1 (CC-00219) has an additional glucose moiety relative to rebaudioside A, whereas DAQ6 (CC-00221) is formed by the loss of a glucose unit. DAQ2 (CC-00209) possess a hydroxyl group at C-16 and one less glucose unit than rebaudioside A.



DAQ2 (CC-00209) **DS-1 (CC-00219)** **DAQ6 (CC-00221)**

Figure II.C.3.4-1 Structures of Degradation Products[2] Identified in Rebiana Stability Studies

III. SELF-LIMITING LEVELS OF USE

The use of rebiana in food is largely limited by the desired sweetness intended for a particular food or beverage product. Thus, the use of rebiana in foods at upper use-levels is largely self-limiting based on its organoleptic properties.

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[2] Structures of related steviol glycosides present in rebiana that also increase over time/under certain conditions of processing (rebaudioside B, DAQ1, DAQ3, DAQ4, DAQ5, and DAQ7) are presented in Figure II.A.2-2.

IV. BASIS FOR GRAS DETERMINATION

IV.A Introduction

The determination that rebiana is GRAS is on the basis of scientific procedures. The safety of rebiana is based on an extensive toxicology database that includes published metabolism and pharmacokinetic studies, acute toxicity studies, short- and long-term toxicity and carcinogenicity studies, reproductive and developmental toxicology studies, genotoxicity studies, and studies to determine safety in humans performed with purified steviol glycosides. The safety of rebiana is further supported by studies conducted with Cargill's high-purity rebaudioside A product, including 4- and 13-week rat feeding studies, developmental and reproductive toxicity studies, as well as acute and longer-term human studies.

Animal and human metabolism studies indicate that steviol glycosides, including rebaudioside A and stevioside, a structural analog to rebaudioside A (rebaudioside A has one additional glucose moiety), are not degraded by digestive enzymes but are metabolized by lower gut microflora to the aglycone steviol by successive removal of the attached glucose moieties. Steviol subsequently is absorbed from the colon, glucuronidated in the liver, and excreted primarily in the feces (*via* bile) of rats or urine of humans (as steviol glucuronide). Rat and human metabolism studies carried out by Cargill using high-purity steviol glycosides affirm the metabolic equivalence of rebaudioside A and stevioside. Because of this equivalence, safety studies conducted with stevioside may be used to support the safety assessment of rebiana.

There are numerous studies available on rebaudioside A (rebiana), stevioside, steviol, other steviol glycosides, as well as on *Stevia* extracts, that may be pertinent to the safety assessment of rebiana. These studies used various protocols, routes of exposure, and varied widely in the degree of reporting, purity of the material tested, use of OECD or FDA Redbook Guidelines, and adherence to GLP standards. Studies conducted with steviol glycoside test materials of high purity (≥95% purity as per JECFA's specifications for steviol glycosides) and applying oral routes of exposure were considered to be of greatest relevance to the determination of a GRAS status.

Studies using materials of lower or unknown purity (either rebaudioside A or stevioside) or mixtures of various steviol glycosides such as *Stevia* extract or *Stevia* leaves were considered to be less relevant to the safety assessment of pure rebaudioside A (*i.e.*, rebiana). Similarly, studies that used non-oral routes of exposure to steviol glycosides are of no relevance since steviol glycosides, following oral exposure, are hydrolyzed and absorbed from the colon in the form of steviol. As a result, studies on impure materials, *Stevia* extract, and those that used non-oral routes of exposure are noted for the sake of completeness, but are not discussed in greater detail in the main sections of the report, except where this information is needed to address specific safety-related concerns. The results of all studies considered for the safety assessment of rebiana are presented in summarized, tabular form, in Appendix II. Those

studies on pure rebaudioside A or stevioside, and which utilized appropriate routes of exposure (*i.e.*, oral in the case of preclinical studies) and which were conducted in accordance with OECD or FDA Redbook guidelines and/or GLP-conditions, as well as well-designed clinical studies are presented in greater detail in the following sections and form the basis of the safety assessment for rebiana.

The weight of the available scientific evidence supports the safety of the proposed uses of Cargill's rebiana [see Appendix I, **EXPERT PANEL REPORT CONCERNING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF REBAUDIOSIDE A (REBIANA) FOR USE AS A GENERAL PURPOSE SWEETENING AGENT**].

IV.B Absorption, Distribution, Metabolism, and Elimination

IV.B.1 Digestion, Absorption, and Distribution

Following oral administration, steviol glycosides are not readily absorbed from the upper small intestine. Human digestive enzymes are not capable of hydrolyzing β-glycosidic bonds and thus steviol glycosides are expected to escape digestion in the upper gastrointestinal tract. *In vitro* assays have confirmed the inability of digestive enzymes such as salivary or pancreatic α-amylase or pepsin, pancreatin solutions, or intestinal brush border membrane enzymes from humans, mice, rats, hamsters, or pigs to metabolize stevioside (Hutapea *et al.*, 1997). Steviol glycosides, therefore, pass undigested through the upper portion of the gastrointestinal tract and enter the colon intact where they are subject to microbial degradation. This has been further confirmed by the results of various *in vitro* studies using gut microflora (Wingard *et al.*, 1980; Hutapea *et al.*, 1997; Gardana *et al.*, 2003; Geuns *et al.*, 2003a; Koyama *et al.*, 2003a). Essentially, the glycosides are sequentially hydrolyzed, removing one glucose molecule at a time. This means that rebaudioside A is first converted to stevioside which is then further degraded to steviol which provides further support for the use of stevioside safety studies in rebaudioside A safety assessment.

Microbes of the *Bacteroidaceae* family (predominantly Bacteroides) have previously been identified as being responsible for the biotransformation of rebaudioside A and stevioside. Since absorption of steviol is dependent on its release from the glycoside by bacterial activity, inter- and intra-species variability in the amounts of steviol available for uptake may result from differences in the composition of the microflora among different species and individuals. However, with the exception of a lower degradation of stevioside to steviol in chickens (Geuns *et al.*, 2003a) than that observed in rodents and humans, no other apparent species differences have been identified in the microflora degradation of steviol glycosides. Hydrolysis of rebaudioside A and stevioside occurs as result of the action of intestinal microflora and this process appears to be similar in rats and humans, which is consistent with the observation that the composition of the human and rat microflora is similar (Luckey, 1972; Brown *et al.*, 1978). The hydrolysis of rebaudioside A to steviol appears to be slower than that of stevioside

(Wingard *et al.*, 1980; Koyama *et al.*, 2003a), which is partly due to the presence of 1 additional glucose moiety and to differences in structural complexities. As suggested by Koyama *et al.* (2003a), rebaudioside A is first converted to either stevioside (major pathway) or rebaudioside B (minor pathway) prior to being ultimately degraded to steviol. Stevioside is further degraded to steviolbioside, steviolmonosides, and finally steviol, with glucose released with each sequential hydrolysis (Nakayama *et al.*, 1986; Koyama *et al.*, 2003a). Since glucose is released in the lower segment of the gut (colon in humans/cecum in rodents), it is not expected to be a significant source of energy.

Studies conducted to assess the absorption of intact steviol glycosides demonstrate that the degree of glycoside transport across the epithelium is very low (Geuns *et al.*, 2003b; Koyama *et al.*, 2003b). However, quantifiable amounts of stevioside were identified in the plasma of rats following oral administration of a *Stevia* product containing 70% of stevioside (Sung, 2002). This report, however, is in contrast to other data, including a pharmacokinetic study conducted with rebiana in the rat (Roberts and Renwick, 2008).

Whereas steviol glycosides are not expected to be absorbed intact in significant amounts, *in vitro* and *ex vivo* studies confirmed that steviol is rapidly absorbed from the gastrointestinal tract (Geuns *et al.*, 2003b; Koyama *et al.*, 2003b). Following absorption, steviol is taken up systemically by the portal vein (Koyama *et al.*, 2003b) and transported to the liver for further metabolism, with low distribution to other organs (Nakayama *et al.*, 1986). In studies in which Wistar or Sprague-Dawley rats were orally dosed with radiolabeled steviol glycosides, radioactivity in plasma appeared slowly, with maximum levels attained at approximately 8 hours (Nakayama *et al.*, 1986; Koyama *et al.*, 2003b; Roberts and Renwick, 2008). In another study, plasma steviol concentrations were noted to increase over the course of 24 hours after oral stevioside administration (Wang *et al.*, 2004). In comparison, steviol was observed in plasma within 15 to 30 minutes of oral administration of the aglycone (Koyama *et al.*, 2003b; Roberts and Renwick, 2008). Generally, the delay between the occurrence of radioactivity in the plasma and the time of administration of steviol glycosides is due to the fact that glycosides are first cleaved to steviol before absorption, as confirmed by high levels of radioactivity in the lower gastrointestinal tract for up to several hours after oral administration.

A comparable pharmacokinetic profile was observed following gavage treatment of male and female Sprague-Dawley rats with radiolabeled rebaudioside A (rebiana) or stevioside (Roberts and Renwick, 2008). With either steviol glycoside, the time of maximum plasma concentration (T_{max}) for radioactivity occurred within 2 to 8 hours of dosing for both rebaudioside A (rebiana) and stevioside. Peak plasma radioactivity levels attained with stevioside were slightly higher than those observed in rats treated with rebaudioside A which was expected given the structure of the rebaudioside A molecule. Stevioside is expected to be more easily degraded to steviol than rebaudioside A as a result of one less glucose unit. This results in greater amounts of

steviol being released more quickly from stevioside and thus systemic absorption of steviol occurs more rapidly as compared to rebaudioside A metabolism.

IV.B.2 Metabolism *in vitro* and in animals

Several *in vitro* studies have been conducted to assess potential metabolic pathways for stevioside and steviol in the liver (Compadre *et al.*, 1988; Ishii-Iwamoto and Bracht, 1995; Koyama *et al.*, 2003b). In the liver, steviol has been shown to primarily undergo conjugation with glucuronic acid to form steviol glucuronide. *In vitro* studies with liver microsomal preparations indicated that steviol also is metabolized to a number of hydroxy and dihydroxy derivatives (steviol-16,17α-epoxide, 15α-hydroxysteviol) *via* CYP-dependent pathways (Compadre *et al.*, 1988; Koyama *et al.*, 2003b). *In vivo*, these steviol metabolites have been identified in hamsters (Hutapea *et al.*, 1999), but not in rats (Roberts and Renwick, 2008) or humans (Geuns and Pietta, 2004; Simonetti *et al.*, 2004; Geuns *et al.*, 2007).

Following oral administration, free steviol (82 to 86% of chromatographed radioactivity 8 hours post-dosing), steviol glucuronide (10 to 12% of chromatographed radioactivity 8 hours post-dosing), and 2 unidentified metabolites (5 to 6% of chromatographed radioactivity 8 hours post-dosing) were identified in the plasma of rats following treatment with either rebaudioside A or stevioside (Roberts and Renwick, 2008). When steviol was administered, steviol glucuronide appeared faster in plasma compared to when rats were administered the glycosides. The 2 unidentified metabolites also were identified in the plasma of steviol-treated rats.

In rats, steviol, administered as steviol or available following cleavage of glycosides in the gut, has been shown to be primarily excreted in the feces *via* the bile (generally within 48 hours), with smaller amounts in the urine (less than 3%) (Wingard *et al.*, 1980; Nakayama *et al.*, 1986; Sung, 2002; Roberts and Renwick, 2008).

Nakayama *et al.* (1986) identified 2 conjugates of steviol as metabolites of stevioside in the bile of rats, 1 which was hydrolyzed by a weak acid and another which was hydrolyzed by a weak acid and β-glucuronidase. Following elimination in the bile, steviol is available to be released again from its conjugated form by the action of the microflora and may enter enterohepatic circulation. Consequently, free and conjugated steviol are excreted in the feces, along with any unhydrolyzed fraction of the administered glycosides. Significant amounts of unchanged rebaudioside A (29 and 19% of the dose in males and females, respectively) and stevioside 3 and 4% of the dose in males and females, respectively) were excreted in the feces of rats treated with rebaudioside A (Roberts and Renwick, 2008). Fecal samples of rebaudioside A-treated rats also contained stevioside (rebaudioside A is first hydrolyzed to stevioside). Similarly, unchanged stevioside is excreted in the feces following its oral administration to rats. Other unidentified metabolites also were present in fecal samples of rats treated with either glycoside.

Stevioside also was shown to be completely degraded to steviol in pigs (Geuns *et al.*, 2003b) while in roosters and chickens, stevioside has been shown to be eliminated largely untransformed (Pomaret and Lavieille, 1931; Geuns *et al.*, 2003a). Distribution and metabolism studies have been conducted with stevioside (Cardoso *et al.*, 1996) and steviol (Wingard *et al.*, 1980) using the parenteral route of exposure, however these are of no relevance to the safety assessment of rebiana following oral exposure.

Overall, the data demonstrate that rebaudioside A and stevioside show similar pharmacokinetics in the rat; they are both metabolized in the gut to steviol prior to absorption followed by glucuronidation in the liver and excretion in the feces *via* the bile. Therefore, the results of toxicology studies on stevioside can be used to support the safety assessment of rebaudioside A.

IV.B.3 Metabolism in Humans

In humans, steviol glucuronide and, in some cases, low concentrations of the unchanged glycoside were detected in the plasma following ingestion of stevioside or rebaudioside A (Geuns and Pietta, 2004; Simonetti *et al.*, 2004; Geuns *et al.*, 2007). In one study, very low levels of steviol were detected in the plasma of one of eight subjects consuming either stevioside or rebiana (97% rebaudioside A) (Wheeler *et al.*, 2008). Significant inter-individual variability in maximum plasma steviol glucuronide levels, and in the time required to reach peak plasma levels, was noted in study participants following stevioside ingestion (Geuns *et al.*, 2007). Such variations can likely be attributed to differences in the time required to release steviol from the glycoside in the gut as a result of inter-individual variability in the microflora composition or gastric emptying. None of the dihydroxy or monohydroxy metabolites of steviol that were identified in plasma, urine, and feces of hamsters following gavage administration of stevioside and *in vitro* following incubation of steviol with liver enzymes (steviol-16,17α-epoxide, 15α-hydroxysteviol, or 15-oxo-steviol), were detected in the plasma or urine of humans consuming steviol glycosides orally (Geuns and Pietta, 2004; Simonetti *et al.*, 2004), confirming that in humans, steviol epoxide formation was not likely to occur. As in the rat, the T_{max} of steviol glucuronide in humans was shorter following the ingestion of stevioside as compared to rebaudioside A due to the simpler structure and faster bacterial degradation of stevioside (Wheeler *et al.,* 2008). In addition, the maximum plasma concentration (C_{max}) for steviol glucuronide was lower following a single dose of rebaudioside A as compared to a single oral dose of stevioside.

Human data indicate that in contrast to rats, steviol glycosides are excreted primarily as steviol glucuronide in the urine (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Geuns *et al.*, 2006, 2007; Wheeler *et al.*, 2008). Additionally, very small amounts of the unchanged glycoside (Geuns and Pietta, 2004; Simonetti *et al.*, 2004) or steviol (Wheeler *et al.*, 2008) also were recovered in urine. In the feces, small amounts of the unchanged steviol glycoside and glucuronide conjugate (secreted back into the gut *via* the bile) were detected (Kraemer and

Maurer, 1994). Relative to amounts recovered in urine, larger amounts of steviol (unabsorbed steviol released from the steviol glycosides in the colon or from small amounts of steviol glucuronide secreted back into the gut *via* the bile) also were eliminated in the feces (Geuns and Pietta, 2004; Simonetti *et al.*, 2004; Geuns *et al.*, 2007; Wheeler *et al.*, 2008).

The inter-species difference in the route of elimination of systemically absorbed steviol as steviol glucuronide (*via* the bile in rats and in the urine in humans) occurs as a result of the lower molecular weight threshold for biliary excretion in rats (325 Da) as compared to humans (500 to 600 Da; molecular weight of steviol glucuronide is 495 Da) (Renwick, 2008a). Notably, in bile-duct ligated rats, excretion of steviol glucuronide occurred primarily in the urine (Wingard *et al.*, 1980). While the primary routes of elimination of steviol glucuronide differs between rats and humans, the metabolism of steviol glycosides and pharmacokinetics are quite similar, which confirms the rat as an acceptable model for risk assessment in humans. The difference in the route of elimination is considered to be of no toxicological significance due to the fact that the water soluble phase II metabolites are rapidly cleared in both species.

IV.C Toxicological Studies

IV.C.1 Acute Studies

Acute toxicity studies were conducted in several species including mice, rats, hamsters, and dogs to determine oral LD_{50} values for rebaudioside A, related steviol glycosides (*e.g.*, stevioside, rebaudioside B, and steviolbioside), *Stevia* extracts, steviol, and isosteviol (structural isomer of steviol). A summary of oral LD_{50} values for rebaudioside A, stevioside, rebaudioside B, *Stevia* extracts, steviol, and isosteviol in mice, rats, hamsters, and dogs, is presented in Appendix II, Table A.II-1. Rebaudioside A (purity not specified), administered as a single gavage dose of 2 g/kg body weight to male Swiss-Webster mice, was reported to produce no toxic effects (Medon *et al.,* 1982). Similarly, stevioside (96% purity) was not associated with any adverse effects following gavage administration at dose levels of up to 15 g/kg body weight in mice, rats, and hamsters (Medon *et al.*, 1982; Toskulkao *et al.*, 1997). Doses of 2 g/kg body weight of rebaudioside B and steviolbioside (unspecified purities) produced no signs or symptoms of toxicity in mice (Medon *et al.*, 1982). In earlier studies conducted with *Stevia* extracts of lower purity, LD_{50} values of 3.4 and 17 to >42 g/kg body weight (depending on stevioside content) were reported in rats and mice, respectively (Akashi and Yokoyama, 1975; Lee *et al.,* 1979). Steviol administered by gavage at a dose of 15 g/kg body weight to rats and mice was not associated with any adverse effects, while dosing of hamsters resulted in LD_{50} values of 5.2 and 6.1 g/kg body weight in males and females, respectively (Toskulkao *et al.*, 1997). Bazotte *et al.* (1986) also reported the absence of any signs or symptoms of toxicity in mice, rats, or dogs treated with isosteviol (a structural isomer of steviol) at a dose level of 500 mg/kg body weight. The results of these studies demonstrate that rebaudioside A, other related steviol glycosides, steviol, and isosteviol, and by analogy rebiana, are not acutely toxic to doses in the range of 15 g/kg body weight.

Other acute studies conducted with lower purity stevioside (90% purity) administered orally to hamsters (Panichkul *et al.*, 1988) or non-orally to rats or hamsters (Toskulkao *et al.*, 1994a) and with high doses of steviol (5 g/kg body weight/day) administered orally to hamsters (Toskulkao *et al.*, 1997) have reported nephrotoxic effects (for study results see Appendix II, Table A.II-2). However, stevioside of higher than 96% purity administered orally to hamsters, the most sensitive species to steviol glycosides, showed no evidence of nephrotoxicity following oral doses of 15 g/kg body weight (Toskulkao *et al.*, 1997). This indicates that the nephrotoxic effects observed in the earlier studies may have been the result of other impurities in the stevioside test materials. The increased sensitivity to stevioside and steviol in hamsters may be due to a species-specific difference in the metabolism of steviol. Specifically, while steviol glucuronide was consistently the only metabolite of steviol observed in rats and humans following oral intake of steviol glycosides, in hamsters, metabolism of steviol *in vivo* was shown to involve formation of dihydroxy and monohydroxy metabolites (Hutapea *et al.*, 1999).

No microscopic changes of the renal tissues or liver enzyme variations were noted in dogs following single dose intravenous administration of stevioside (purity not specified) (26.1 mg/kg body weight) (Krejci and Koechel, 1992).

In an OECD-compliant study, but conducted with stevioside of unspecified purity, Balb/c mice of either sex did not display any changes in general behavior or mortality following oral administration (presumably gavage) of single doses of the test material (up to 25 mg/kg body weight) (Sehar *et al.*, 2008). Full study details are summarized in Appendix II, Table A.II-2.

IV.C.2 Subchronic and Chronic Studies

Several animal toxicity studies were identified that assessed the potential short- and long-term toxicity of rebiana (rebaudioside A) (Curry and Roberts, 2008), rebaudioside A of high purity (Nikiforov and Eapen, 2008), stevioside of high purity (Aze *et al.*, 1991; Geuns *et al.*, 2003a), and other related steviol glycosides of low or undefined purity and/or used non-oral routes of exposure (Pomaret and Lavieille, 1931; Akashi and Yokoyama, 1975; Lee *et al.*, 1979; Xili *et al.*, 1992; Wood *et al.*, 1996). All subchronic and chronic studies are discussed below and are summarized in greater detail in Appendix II, Table A.II-3.

Overall, the subchronic toxicity study data on rebiana corroborate the findings of other studies conducted with related steviol glycosides. No specific toxicity concerns were noted with rebiana at concentrations in the diet of up to 100,000 ppm for 4 weeks, providing 9,938 mg rebiana/kg body weight/day for males and 11,728 mg rebiana/kg body weight/day for females, and at concentrations in the diet of up to 50,000 ppm for 13 weeks, providing 4,161 mg rebiana/kg body weight/day for males and 4,645 mg rebiana/kg body weight/day for females.

IV.C.2.1 Oral Subchronic and Chronic Studies Conducted with High Purity (>95% pure) Steviol Glycosides

In a dose-range finding study, rebiana [97% rebaudioside A; Lot No. 1001 (weeks 1 to 3) and Lot No. 1002 (week 4)] was administered *via* the diet to HsdBR1 Han:Wist (Han Wistar) rats (10/sex/group) at levels of 0 (control), 25,000, 50,000, 75,000, or 100,000 ppm for a period of 4 weeks (Curry and Roberts, 2008). Based on data reported by the authors, these levels were equivalent to mean intakes of 0 (control), 2,367, 4,842, 7,143, and 9,938 mg rebiana/kg body weight/day, respectively, for males and 0 (control), 2,616, 5,422, 8,190, and 11,728 mg rebiana/kg body weight/day, respectively, for females. In a subsequent 13-week toxicity study, HsdRcc Han:Wist rats (20 rats/sex/group) were administered rebiana (97% rebaudioside A; Lot No. 1002) in the diet at concentrations of 0 (control), 12,500, 25,000, or 50,000 ppm (Curry and Roberts, 2008). The dietary concentrations were established on the basis of the 4-week study, described above. At each of the 3 dietary concentrations, males consumed daily doses of 1,506, 3,040, and 5,828 mg rebiana/kg body weight/day, respectively, during the first week of the study, and 698, 1,473, and 3,147 mg/kg body weight/day, respectively, during the last week. In females, doses of 1,410, 2,841, and 5,512 mg rebiana/kg body weight/day were achieved at each of the 3 dietary concentrations, respectively, during the first week and 980, 1,914, and 3,704 mg/kg body weight/day during week 13.

In these studies, no evidence of systemic toxicity was reported following administration of rebiana to both sexes of Han Wistar rats at dietary concentrations of up to 100,000 ppm (9,938 and 11,728 mg/kg body weight/day for males and females, respectively) for 4 weeks or 50,000 ppm (4,161 and 4,645 mg/kg body weight/day for males and females, respectively) for 13 weeks (Curry and Roberts, 2008). In the 4-week study, body weight gains were decreased in both sexes at the 50,000 and 100,000 ppm dose levels, but terminal body weights were significantly lower only in the high-dose females. In the 13-week study, there were statistically significant decreases in body weight gains despite no clear differences in food consumption and only minimal effects on food conversion efficiency.

The authors concluded that the decreases in body weight gains during the initial study period were due to the acclimation of the intensely sweet diets, as well as the lower caloric density of the rebiana-containing diets and not related to any adverse effects of rebiana. In the 13-week study of Curry and Roberts (2008), food consumption, adjusted for caloric density, was reduced in nearly all weeks of the study in the high-dose group. Food consumption was decreased by about 5% in both high-dose males and females. Early in the study (days 1 to 14) when the rats adjusted to the diet during a period of rapid growth, food conversion efficiency was significantly decreased in treated males. Lesser decreases also occurred in females during this period, but did not attain statistical significance. However, overall, the effects of treatment on food conversion efficiency were minimal, with food consumption efficiency values significantly decreased only in high-dose males over the course of the study. The initial decrease in food consumption noted in the 13-week study also is consistent with the results of the 2-generation

reproductive toxicity study on rebiana (Curry *et al.*, 2008) where body weight gain decrements in the high-dose groups in comparison to controls occurred during the first 2 weeks of the weaning period.

The interpretation of the effects of rebiana on body weight gain can be guided by previous evaluations as described by Flamm *et al.* (2003). Flamm and associates have presented a procedure for assessing palatability/body weight gain issues and have described a number of criteria to establish that decreases in body weight gain and/or food consumption are not adverse. These include:

a) Treatment does not affect food conversion efficiency during the phase of rapid growth (*i.e.*, first 13 weeks);

b) The test substance affects palatability at concentrations that cause reductions in body weight and/or food consumption;

c) There is consistency between effects of palatability and patterns of reduced food consumption;

d) Changes in body weight gain occur without a dose-response over a wide-range of doses with no other signs of toxicity.

As stated, the overall effects of rebiana treatment on food conversion efficiency are minimal, with the most notable effects occurring in the first 2 weeks of the study. Furthermore, rebiana has effects on food consumption and body weight that are associated in a fairly consistent manner with palatability in the first days of the study. Food consumption (adjusted for caloric density of the diet) of the treated-males and in the mid- and high-dose females was consistently lower than controls and can substantively explain the decrements in body weight gain noted in the study. Based on the procedure outlined by Flamm *et al.* (2003), the calculated 5% decrease in food consumption related to the decreased caloric density in high-dose males could explain up to a 15% decrease in weight gain in a 13-week study. Finally, despite the observations of reduced food consumption and body weight gain, no toxicity was observed over the dose-range in the study.

Based on World Health Organization's (WHO) guidance (WHO, 1987) in regard to the interpretation of lower body weight gain in the absence of other toxicity due to consumption of a test material with known nutritive and palatability effects, the body weight effects observed in both studies were not considered an adverse effect of rebiana. Similar effects have been reported in safety studies with other HIS dosed at high levels which were likewise determined to be of no toxicological significance.

In the 13-week toxicity study on rebiana (Curry and Roberts, 2008), several changes in clinical chemistry and hematological parameters were recorded. In the clinical chemistry, mean plasma

urea and creatinine concentrations were slightly higher in several treated groups in both studies. Given the previous reports of nephrotoxicity in some studies on *Stevia* extracts (Toskulkao *et al.*, 1994a, 1997), these findings were evaluated closely. The increases in mean plasma urea and creatinine were relatively small and within the normal historical control range. In this regard, they are typical of dehydration or osmotic loading of the blood rather than frank renal toxicity (Car *et al.*, 2006). This effect would not be expected to occur at anticipated rates of human exposure. Further evidence for the lack of renal toxicity is provided by the macroscopic and microscopic evaluation of the kidneys; no significant alterations were noted in either evaluation. Furthermore, increases in plasma urea and creatinine that are related to renal impairment *per se* are almost always associated with histological evidence of kidney toxicity (Hall and Everds, 2007).

Significantly decreased bile acids also were observed in the 4-week and 13-week rebiana studies, which were within the normal range for historical controls except for males administered the highest dose of rebiana for 13 weeks. The authors considered the reductions in bile acids the result of metabolism and/or excretion of large amounts of the test article, and as evidenced by biliary elimination being the predominant excretory pathway in rats. It is interesting to note, however, that in the 13-week subchronic study, decreases in plasma triglycerides were observed in all treated males on day 89 as well as decreases in cholesterol among females administered 25,000 and 50,000 ppm rebiana on day 89 were observed. The triglycerides and cholesterol values were within the historical control range. The authors attributed the decreases in bile acids and cholesterol to the excretion of large amounts of administered rebiana and its metabolites which may have decreased the enterohepatic recirculation of the bile acids with their subsequent excretion in the feces. In addition, no biologically significant differences in serum liver enzymes were reported and histopathology also revealed no significant differences. Overall, the decrease in bile acids was determined by the authors to be of no biological or toxicological consequence.

All statistically significant hematological findings were of no biological significance as they were not dose-dependent, did not occur in both sexes or at all time points, were small in magnitude, and were within the normal historical control range. Given the preceding data and the guidance with respect to interpretation of toxicological data, including clinical chemistry and hematological endpoints (WHO, 1987; Wilson *et al.*, 2001; Lewis *et al.*, 2002), rebiana was considered to have had no adverse effect on the clinical evaluations.

The lack of toxicity related to short-term treatment of animals with rebiana corroborate the results of a 13-week study conducted with rebaudioside A (99.5% purity) in Sprague-Dawley rats (Nikiforov and Eapen, 2008) or stevioside (95.6% purity) in F344 rats (Aze *et al.*, 1991). Nikiforov and Eapen (2008) administered rebaudioside A in the diet to produce target doses of 500, 1,000, or 2,000 mg/kg body weight/day. Animals were allowed *ad libitum* access to the diets. No adverse effects on body weight gain, terminal body weights, clinical and functional

observational battery observations, or on the results of the hematology, serum chemistry, or urinalysis evaluations were reported. Treatment was reportedly not associated with any organ weight variations or macroscopic or microscopic tissue changes. Similar to Curry and Roberts (2008), a slight decrease in food conversion efficiency was noted in the high-dose males, an effect associated with decreased terminal body weight and body weight gain. According to Nikiforov and Eapen (2008), the decrement in food conversion efficiency and in terminal body weights was suggested to possibly be the result of the inclusion of a high concentration of a non-nutritive substance in the diet. Other observations reported in Nikiforov and Eapen (2008) also tend to mirror those of Curry and Roberts (2008), namely, an indication of decreased serum bile acids, a tendency to decreased urine volumes in treated animals, and similar slight changes in serum electrolytes. No clear effects of palatability were reported in the Nikiforov and Eapen (2008) study likely due to the fact that lower dietary concentrations were used overall. Furthermore, since dietary concentrations were adjusted to achieve target doses in terms of mg/kg body weight/day, the dietary concentrations in the Nikiforov and Eapen (2008) study were lowest in the early part of the experiment, a time when adjustment to the palatability of the diet would have the greatest impact, as the rats were consuming the highest amount of diet in relation to their body weight. Nikiforov and Eapen (2008) considered minor decreases in body weight and food conversion efficiency as non-adverse and ascribed a NOAEL of 2,000 mg/kg body weight/day, the highest dose tested.

Stevioside (95.6% purity) was administered to F344 rats (10/sex/group) *via* the diet at concentrations of 0 (control), 0.31, 0.62, 1.25, 2.5, or 5% [approximately, 0, 155, 310, 625, 1,250, and 2,500 mg/kg body weight/day, respectively (U.S. FDA, 1993)] for a period of 13 weeks (Aze *et al.,* 1991). No deaths occurred during the treatment period and there were no differences between groups in body weight gains or food intakes; however terminal body weights were significantly decreased in the female 2.5% dose group and in the male and female 5% dose group in comparison to the controls. Statistically significant increases or decreases in blood urea nitrogen (BUN), choline, creatinine, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), total protein, plasma potassium, phosphorus, chloride, aspartate aminotransferase (AST), alanine aminostransferase (ALT), hematocrit percentage, and platelet counts were reported by the authors in males and/or females; however, the increases were sporadic and considered by the study authors to be toxicologically insignificant. No significant differences compared to the control group were reported among male rats; however, among females, significantly increased absolute and relative liver weights were reported in the 3 highest dose groups. Other significant increases in organ weights included relative brain and spleen weights in high-dose females and absolute right and left kidney weights in the 2 highest dose groups. Upon histopathological examination of the liver, males in all treatment groups had increased incidences of single cell necrosis of the liver (significance not reported). The authors considered the above results not to be attributable to the stevioside treatment due to the lack of clear dose-response patterns, relatively low severity, and the limitations to only one sex. Based on the results of this study, the authors reported that a concentration of 5% stevioside in the diet

(approximately 2,500 mg/kg body weight/day or 956 mg steviol equivalents/kg body weight/day) was an appropriate maximum tolerated dose of stevioside to be used in a 2-year carcinogenicity study in rats.

Stevioside (96% purity) fed to 8 Cobb broiler chickens and 4 Hisex brown laying hens in their diet at levels of 667 mg stevioside/kg diet (approximately 137 mg stevioside/kg body weight/day and 78 mg stevioside/hen/day; body weight of Hisex brown laying hens not reported) for 2 weeks and 10 days, respectively, had no effects on feed consumption, body weight gains, or weights of the eggs produced compared to hens receiving an unsupplemented diet (Geuns *et al.*, 2003a). Stevioside or steviol was not detected in the blood samples of the hens.

IV.C.2.2 Oral Subchronic and Chronic Studies Conducted with Lower Purity (<95% pure) Steviol Glycosides and Uncharacterized Stevia Materials

Weanling Wistar rats (10/sex/group) were administered diets containing 0 (control), 3, or 5% stevioside (85% purity) [approximately 0, 1,500, and 2,500 mg/kg body weight/day, respectively (U.S. FDA, 1993)] for a period of 90 days in a dose range-finding study for a carcinogenicity study (discussed in Section IV.C.5) (Xili *et al.,* 1992). The authors reported that stevioside had no significant effects on body weight gain, food conversion efficiency, and no signs or symptoms of toxicity or abnormal behavior were observed; however, the authors selected lower doses for the 2-year study due to the cost of the ingredient. Based on the results of this study, a NOAEL of 5% stevioside (2,500 mg/kg body weight/day) was established.

In a 13-week oral subchronic toxicity study, male and female SLC-Wistar rats (15/sex/group) were administered 0 (control), 0.28, 1.4, or 7% of a refined *Stevia* extract (STEVIX) containing 53.1% stevioside in the diet (Akashi and Yokoyama, 1975). The reported stevioside intake was 0, 112, 590, and 2,988 mg/kg body weight/day for males and 0, 115, 629, and 3,026 mg/kg body weight/day for females at each of the 3 dietary concentrations of the extract, respectively. No biologically relevant changes in hematology, serum biochemistry, or histopathological observations were reported. Statistically significant increases or decreases in relative organ weights also were deemed to be of little toxicological significance as they were not accompanied by meaningful changes in hematology, serum biochemistry, or histopathological observations. A NOAEL for the *Stevia* extract for this study was determined to be 7%, approximately 2,988 and 3,026 mg stevioside/kg body weight/day for males and females, respectively.

Male and female rats (8 to 10/sex/group; strain not specified) were administered a *Stevia* extract (50% stevioside) in the diet at doses of 0 (control), 1,250, or 2,500 mg/kg body weight/day for up to 56 days to determine the safety of the *Stevia* extract (Lee *et al.*, 1979). The authors reported no biologically significant differences in body weight gain, number of red or white blood cells, concentrations of hemoglobin, hematocrit, total serum protein, albumin, α_1-globulin, α_2-globulin, γ-globulin, glucose, triglycerides, total cholesterol, creatinine, urea, calcium, ALP,

AST, and LDH levels, and albumin/globulin ratios. Microscopic examination of the liver was reported to reveal no significant changes in the hepatocytes or Kupffer cells, or in the areas of portal fibrosis in treated rats compared to controls. Based on these results, a NOAEL of 2,500 mg *Stevia* extract/kg body weight/day (approximately 1,250 g stevioside/kg body weight/day) was established for rats under the conditions of this study.

Pure stevioside, administered to chickens (4 g or 1.6 g steviol equivalents) over a period of 2 days also was not associated with any signs of toxicity (Pomaret and Lavieille, 1931). Chickens fed 3.8 µg/kg body weight/day of a *Stevia* extract (content not reported) from the age of 1-day old to 42-days old were reported to have significantly decreased body weights; however, at higher dietary levels (up to 38 µg/kg body weight/day) of the *Stevia* extract, no differences in body weights were reported (Wood *et al.*, 1996). Furthermore, no effects on feed intake or food conversion efficiency were observed.

IV.C.2.3 Non-Oral Subchronic and Chronic Studies Conducted with Steviol Glycosides

Pure stevioside, administered to rabbits (2.2 g or 0.88 g steviol equivalents) over a period of 15 days by intravenous or subcutaneous injection was not associated with any signs of toxicity (Pomaret and Lavieille, 1931). However, as steviol glycosides are not detected in the systemic circulation following oral administration to any appreciable degree (see Section IV.B), the results of studies employing non-oral routes of exposure are of limited relevance to the safety of oral steviol glycoside preparations.

IV.C.3 Developmental and Reproductive Toxicity Studies

Single- and multi-generation reproductive and developmental studies conducted with high-purity stevioside (Akashi and Yokoyama, 1975; Mori *et al.*, 1981; Usami *et al.*, 1995) or stevioside of slightly lower purity (Yodingyuad and Bunyawong, 1991) have shown a lack of reproductive and developmental toxicity in rats and/or hamsters. This viewpoint was supported in 2006 by JECFA who concluded that in light of the absence of any adverse reproductive or developmental effects in several different rodent species treated with purified stevioside, the adverse reproductive effects associated with the administration of aqueous extracts of *S. rebaudiana* were unlikely to be caused by steviol glycosides. This conclusion also is corroborated by the findings of the 2-generation reproductive toxicity study conducted with rebiana (Curry *et al.*, 2008), which demonstrated no adverse effects on reproductive function or reproductive organs at dietary concentrations of up to 25,000 ppm, or approximately 2,048 (males) and 2,273 (females) mg/kg body weight/day based on pre-mating intakes and body weights. Full study details of all developmental and reproductive toxicity studies are provided in Appendix II, Table A.II-4.

IV.C.3.1 Oral Developmental and Reproductive Toxicity Studies Conducted with High Purity (>95% pure) Steviol Glycosides

In a preliminary reproduction study, rebiana (>97% rebaudioside A; Lot No. 1002) was administered to adult F_0 female HsdBrl:Han Wistar rats and their litters (juvenile F_1 HsdBrl:Han Wistar rats) at dietary concentrations of 0 (control), 25,000, 37,500, or 50,000 ppm. Food and water were provided *ad libitum* and fresh diets were prepared weekly (Curry *et al.*, 2008). The F_0 females (6/dose level) were treated from the 14th to the 21st day of lactation, resulting in the offspring being treated with rebiana from day 14 post-partum. A total of 10 rats/sex were selected from each treatment level (a maximum of 2 rats/sex selected from each litter) for continuation of treatment from weaning (day 21) until the 35th day post-partum. At each of the dietary concentrations of rebiana (0, 25,000, 37,500, or 50,000 ppm), during the first 4 days of lactation, female F_0 rats were reported to achieve calculated doses of 0 (control), 4,711, 8,021, and 9,484 mg/kg body weight/day, respectively. The doses achieved in female F_0 rats from day 17 to 20 of lactation were reported to be 0 (control), 6,291, 10,045, and 11,386 mg/kg body weight/day, respectively, with the higher intakes reported likely due to consumption by the litter during this time. In order to minimize effects of maternal body weight change on reproductive outcomes, caused by diet palatability when high concentrations were used, and due to the very high exposure on a mg/kg body weight/day basis likely achieved during lactation and in the young rat pups during pre-weaning, the 25,000 ppm dietary concentration was considered suitable as the top dose for use in the main 2-generation reproductive toxicity study.

In the definitive 2-generation study, rebiana (97% rebaudioside A; Lot Nos. 1002 and 1003) was administered *via* the diet to male and female HsdRcc:Han Wistar rats (30/sex/group) at concentrations of 0 (control), 7,500, 12,500, or 25,000 ppm for 2 generations to determine its potential reproductive and developmental effects (Curry *et al.*, 2008). Male and female F_0 rats received the respective test diets for a period of 10 weeks prior to mating. Mating occurred within the same treatment group and lasted for a period of up to 3 weeks. Dams continued to receive the test diets for the duration of gestation and lactation. Offspring were weaned on day 21 and on day 25 a minimum of 1 male and 1 female was randomly selected from as many litters as possible within each group until the required number of F_{1P} animals for breeding of the second generation (F_2) offspring was attained (30/sex/group). Pups not selected for the F_{1P} generation were necropsied on post-natal day 30.

The average doses of rebiana achieved during different phases of the multi-generational reproductive study are summarized in Table IV.C.3.1-1.

Table IV.C.3.1-1	Dose Levels Achieved at Different Dietary Concentrations in Rats during Different Phases of the Reproductive/Developmental Study (Curry *et al.*, 2008)					
Group	F_0			F_{1P}		
	Concentrations (ppm)			Concentrations (ppm)		
	7,500	12,500	25,000	7,500	12,500	25,000
Males	586[1]	975	2,048	734	1,254	2,567
Females						
Pre-mating	669	1,115	2,273	798	1.364	2,768
Gestation	648-713	1,119-1,169	2,263-2,381	562-625	911-1,058	2,036-2,212
Lactation	715-1,379	1,204-2,388	2,602-5,019	976-1,406	1,752-2,394	3,289-4,893

[1] All values expressed as mg/kg body weight/day.

In Curry *et al.* (2008), no reproductive effects were found following treatment of parental rats and offspring at dietary concentrations of up to 25,000 ppm. In the dose-finding reproduction study, female rats administered rebiana in the diet during lactation displayed no toxic effects related to rebiana consumption. The offspring were reported to have decreased body weight gains at concentrations in the diet of higher than 37,500 ppm; however, the decreases in body weight gains in the offspring were accompanied by a concomitant decrease in food consumption (Curry *et al.*, 2008). The authors attributed the decreased food consumption to the palatability of the diet. Likewise, in the main multi-generational reproductive and developmental study, decreased body weight and body weight gains were reported in all of the generations at concentrations of 12,500 and 25,000 ppm in the diet (Curry *et al.*, 2008). While food consumption and food utilization efficiency were not significantly decreased compared to the control group, the authors also noted that the decreases in body weights and weight gains occurred within the first 2 weeks of administration of the diet in the immediate post-weaning period when the weanlings were first exposed to the intensely sweet diet. It is likely, therefore, that the decreases in body weight gains were due to acclimation of the diet, as decreases in body weight gains were not reported in rats following the first few weeks post-weaning.

Stevioside (95.6% purity) dissolved in distilled water was administered by gavage to pregnant female Wistar rats (21 to 24/group) at doses of 0 (control), 250, 500, or 1,000 mg/kg body weight/day from day 6 to 15 of gestation to investigate stevioside's potential teratogenic effects (Usami *et al.*, 1995). Rats were killed on day 20 of gestation and their fetuses were examined for abnormalities. No significant changes were reported in the body weight and food consumption in stevioside-treated pregnant females compared to the control group. The authors reported no increased incidences of fetal malformation, and no signs of toxicity in the pregnant rats and fetuses. Skeletal and visceral examinations in fetuses reportedly revealed no stevioside-related teratogenic effects. Based on these observations, the authors estimated the developmental NOAEL to be above 1,000 mg/kg body weight/day for both pregnant rats and the fetuses.

Male and female JCL:Wistar rats (22/sex/group) were administered diets containing 0 (control), 0.15, 0.75, or 3% stevioside (95.98% purity) (equivalent to 0, 100, 480, and 2,100 mg/kg body weight/day, respectively, for males and 0, 120, 530, and 2,100 , respectively, for females) for a fertility study (Mori *et al.*, 1981). Males received stevioside before and during mating for a total period of 60 days and females received stevioside for a period of 14 days before mating and for 7 days during gestation. On day 20 of gestation, pregnant females were killed and their fetuses were examined for abnormalities. No significant differences were reported in food and water consumption in both sexes of all treated groups compared with controls; however, a delayed increase in body weight in the early period of administration was reported in both sexes of the high-dose group (*i.e.*, 3%) compared with the control group. The authors reported stevioside treatment had no significant effects on mating performance and fertility, and external, internal, and skeletal examinations of the fetuses revealed no significant changes. The authors concluded that stevioside administration before and during the early gestation period had no adverse effect on fertility and the development of fetuses. Therefore, a NOAEL for stevioside was considered to be 3%, the highest dose tested in this study (approximately 2,100 mg/kg body weight/day).

IV.C.3.2 Oral Reproductive and Developmental Toxicity Studies Conducted with Lower Purity (<95% pure) Steviol Glycosides and Uncharacterized Stevia Material

The effects of 3 different stevioside preparations on the inhibition of pregnancy were examined in Sprague-Dawley rats (Akashi and Yokoyama, 1975). Males (5/group) and females (5/group) were administered 0.69% crude *Stevia* extract (20% stevioside), 0.35% refined *Stevia* extract (40 to 55% stevioside), or 0.15% crystallized stevioside (STEVIOSIN; 93 to 95% purity) in their diets for 21 days prior to mating. Based on feed consumption and body weights, these concentrations provided 83.4, 80.0, or 84.9 mg/kg body weight/day of stevioside for male rats, respectively, and 96.2, 101.7, or 101.2 mg/kg body weight/day of stevioside for female rats, respectively. Two (2) females were mated with 1 male. Pregnant females were allowed to deliver and the females and pups were observed weekly for 3 weeks. No significant differences in behavior, body weight, feed intake, pregnancy rate, or fetal condition were reported in comparison to control rats.

Stevioside (90% purity) was orally administered to male and female golden hamsters (*Mesocricetus auratus;* 1-month-old; 10/sex/group) in syrup by gavage at levels of 0 (control), 500, 1,000, or 2,500 mg/kg body weight/day over 3 generations to determine its effects on growth and reproduction (Yodyingyuad and Bunyawong, 1991). Each female was allowed to bear 3 litters during the experimental period and during late gestation and lactation, females then received stevioside *via* drinking water. After giving birth, females were allowed to feed their young for 1 month before being separated for a 2-week rest period followed by their next mating. The same number of young F_1 and F_2 hamsters (10/sex/group; 1-month-old) continued to receive the same dose of stevioside by gavage as the F_0 hamsters. The authors reported that stevioside treatment had no significant effects on growth and fertility in either sex, or on the

duration of pregnancy, number of fetuses, and number of young delivered. Females reportedly showed normal 4-day estrus cycles and histological examination of the reproductive tissues from both sexes of all experimental groups revealed no significant changes compared to control hamsters. The authors concluded that stevioside at a dose up to 2,500 mg/kg body weight/day had no effects on growth or reproduction in hamsters.

Two (2) other studies that evaluated the reproduction and developmental effects of *Stevia* extracts demonstrated no adverse effects (Oliveira-Filho *et al.*, 1989; Saenphet *et al.*, 2006). In a study to determine effects on reproductive organs and hormone levels, 30 prepubertal male Wistar rats (25 to 30 days old) were administered 2 mL of an aqueous *S. rebaudiana* extract (equivalent to 66.7 g of dried leaves/100 mL) twice daily by gavage for 60 days (Oliveira-Filho *et al.,* 1989). This provided the equivalent of approximately 14.58 g of dried leaves/kg body weight/day. The authors reported no differences in body weight, testis, prostate, submandibular gland, or adrenal weights as compared to the control group; however, the absolute and relative seminal vesicle weights were reported to be significantly decreased by approximately 52 and 62%, respectively, in the treated rats compared to the control rats. Serum levels of triiodothyronin (T_3) and thyroxine (T_4) and the T_3 retention index were reported not to be significantly different between the 2 groups. Compared to the control group, zinc levels in the prostate, testis, submandibular gland, and pancreas, and water content of the testis and prostate, were not significantly different among the treated rats. The authors reported no significant difference in the number of prostate binding sites in the control and treated rats.

The reproductive safety of an aqueous extract of *S. rebaudiana* (levels of steviol glycosides not specified) at concentrations of 0, 0.2, 1, or 10% in combination with an aqueous extract of *Aegle marmelos* (6%) (a Thai medicinal plant commonly consumed as a herbal tea) was examined in female Wistar rats (8/group) administered orally 1 mL of the test solutions once per day (approximately 0, 8.5, 41, and 433 mg *S. rebaudiana* extract/kg body weight/day, respectively) (route of administration not specified, but presumed to be gavage) for a period of 60 days prior to mating (Saenphet *et al.,* 2006). No significant differences were reported in the number of corpus lutea, implanted and dead fetuses, and sizes of the fetuses in any of the test groups compared with the control group. None of the pregnant rats exhibited any abnormalities. The authors concluded that the aqueous extract of *S. rebaudiana* mixed with *A. marmelos* at all doses used in this study (up to 433 mg/kg body weight/day of *S. rebaudiana* extract) had no adverse effects on female rat reproduction or fetal development.

Several studies conducted with aqueous *S. rebaudiana* extracts have reported contraceptive effects in female mice and rats following doses of up to 2,000 mg/kg body weight provided in the drinking water or by gavage (Mazzei-Planas and Kuc, 1968; Nunes and Pereira, 1988) and in male rats given a crude extract of *S. rebaudiana* at a dose corresponding to 1.34 g of dried leaves (Melis, 1999a). The procedures of these studies, however, have been criticized by several authors as male mice and rats were not administered the extract (Mazzei-Planas and

Kuc, 1968; Nunes and Pereira, 1988) and male rats were administered dried leaves without purification (Melis, 1999a). In a follow-up study to Mazzei-Planas and Kuc (1968), Shiotsu (1996) administered a *Stevia* extract in the drinking water of male and female Wistar rats before mating and to female Wistar rats during mating at a dose of 5,000 mg/kg body weight/day. No adverse effects related to reproduction were reported in either sex.

To study the developmental toxicity of steviol (approximately 90% purity), pregnant golden Syrian hamsters (*Mesocricetus auratus*; 20/group, except in the highest-dose and positive control groups, which had 12 and 6/group, respectively) were administered steviol (dissolved in corn oil) at doses of 0 (vehicle control), 250, 500, 750, or 1,000 mg/kg body weight/day by gavage on days 6 to 10 of gestation (Wasuntarawat *et al.*, 1998). High maternal mortality rates and a significant decrease in maternal body weight gain were reported in the 3 highest dose groups (*i.e.*, 500, 750, 1,000 mg/kg body weight/day) compared to the vehicle control. Histopathological examination of the maternal kidneys revealed dose-dependent pathological effects (*i.e.*, dilation and hyaline formation) on the convoluted tubules in the 3 highest dose groups. Significant decreases in the number of live fetuses per litter and mean fetal weight were reported in the 750 and 1,000 mg/kg body weight/day groups, and in the 500, 750, and 1,000 mg/kg body weight/day groups, respectively. One curved tail and 1 craniomeningocele were reported in fetuses in the vehicle control and steviol-treated (750 mg/kg body weight/day) groups, respectively. The skeletal and visceral development of all offspring in all dose groups were reportedly not affected by steviol-treatment and reported abnormalities in the skeletal and visceral development were judged as variations since effects also were seen in the vehicle control group and were not dose-dependent. No steviol treatment-related teratogenesis was observed in any of the offspring. The authors reported that a dose equal to or greater than 500 mg steviol/kg body weight/day was toxic to both dams and fetuses and a dose of 250 mg steviol/kg body weight produced no observable effects in maternal toxic condition and embryotoxicity.

Studies conducted on steviol at high doses, however, provide little relevance to the safety assessment of steviol glycosides, as steviol is absorbed immediately in the gastrointestinal tract following oral administration (see Section IV.B). In comparison, steviol glycosides are not readily absorbed in the gastrointestinal tract and are slowly hydrolyzed to the aglycone steviol. The plasma levels of steviol after administration of a high dose of steviol, therefore, would be expected to be much greater than the plasma levels of steviol following administration of a steviol glycoside. Moreover, hamsters have been noted to be more sensitive to steviol and steviol glycosides, which also limits the interpretability of this study to the safety of steviol glycosides.

IV.C.3.3 Non-Oral Reproductive and Developmental Toxicity Studies Conducted with Steviol Glycosides

Stevioside (98% purity) or steviol injected into developing broiler chick embryos did not display any developmental toxicities to the developing embryos (Geuns *et al.*, 2003c). However, as steviol glycosides are not detected in the systemic circulations following oral administration to any appreciable degree (see Section IV.B), the results of studies employing non-oral routes of exposure are of limited relevance to the safety of oral steviol glycoside preparations.

IV.C.4 Genotoxicity

Steviol glycosides, including rebaudioside A, stevioside and steviol, as well as *Stevia* extracts, have been subject to extensive genotoxicity testing, both *in vitro* and *in vivo*. The results of all the genotoxicity studies are summarized in tabular form in Appendix II (Tables A.II-5 to A.II-8). The most relevant studies, including those that used purified rebaudioside A, stevioside, other related steviol glycosides, and steviol, are presented in greater detail below.

Overall, the existing data pertaining to the genotoxicity of rebaudioside A and stevioside demonstrate a lack of genotoxic activity for both compounds *in vitro* and *in vivo*.

Rebaudioside A, stevioside, rebaudioside B, rebaudioside C, dulcoside A, and/or steviolbioside were demonstrated not to be mutagenic in reverse mutation assays in *Salmonella typhimurium* (*S. typhimurium*) strains TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, and TA1538 (Okumura *et al.,* 1978; Suttajit *et al.*, 1993; Matsui *et al.*, 1996a; Klongpanichpak *et al.*, 1997), and in forward mutation assays in *S. typhimurium* strain TM677 (Medon *et al.*, 1982; Pezzuto *et al.*, 1983, 1985; Matsui *et al.*, 1996a). Stevioside also tested negative in gene mutation assays in *S. typhimurium* TA1535/pk1002 and *Bacillus substilis* (*B. substilis*) (Okumura *et al.,* 1978; Matsui *et al.*, 1996a) and mouse lymphoma L5178Y cells (Oh *et al.,* 1999a,b) with or without metabolic activation. Tested in *Escherichia coli* (*E. coli*) WP2*uvr*A/pkM101 in the absence of metabolic activation, stevioside also was negative for reverse mutations (Matsui *et al.*, 1996a).

In vitro studies indicate that rebaudioside A or stevioside does not induce chromosomal aberrations in Chinese hamster lung fibroblasts at concentrations up to 12 mg/mL (Ishidate *et al.*, 1984; Matsui *et al.*, 1996a; Nakajima, 2000a). In contrast, human lymphocyte cultures treated with stevioside (purity not reported) at concentrations of 0.01 to 10 µM (approximately 0.008 to 8 µg/mL) exhibited significantly increased micronucleus formation rates compared to untreated control cells (with or without metabolic activation not reported) (Höhn and Zankl, 1990); however, no significant changes in the number of chromosome breaks or sister chromatid exchanges were reported (Flores *et al.*, 1987; Höhn and Zankl, 1990; Suttajit *et al.*, 1993)..

One comet assay conducted with stevioside (88.6% purity) demonstrated increased comet or "tail" scores in the blood cells and cells isolated from brain, liver, and spleen of Wistar rats

Appendix 1

APPENDIX I

EXPERT PANEL REPORT CONCERNING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF REBAUDIOSIDE A (REBIANA) FOR USE AS A GENERAL PURPOSE SWEETENING AGENT

EXPERT PANEL REPORT CONCERNING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF REBAUDIOSIDE A (REBIANA) FOR USE AS A GENERAL PURPOSE SWEETENING AGENT

March 27, 2008

INTRODUCTION

At the request of Cargill, Incorporated (hereafter Cargill), an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened on March 27 and 28, 2008 to conduct a critical and comprehensive evaluation of the available pertinent data and information on rebaudioside A (rebiana), and determine whether rebiana under the conditions of intended use as a general purpose sweetening agent in conventional foods (when not otherwise precluded by Standards of Identity), is "Generally Recognized as Safe" (GRAS), based on scientific procedures.

The Panel consisted of the below-signed qualified scientific experts: Henry Black, M.D. (New York University School of Medicine), Samuel Cohen, Ph.D., M.D. (University of Nebraska Medical Center), Morey Haymond, M.D. (Baylor College of Medicine), Glenn Sipes, Ph.D. (University of Arizona), and William Waddell, M.D. (University of Louisville).

The Panel, independently and collectively, critically examined a comprehensive package of publicly available scientific information and data compiled from the literature by Cantox Health Sciences International and other published sources based on searches of the published scientific literature conducted through March 2008. In addition, the Panel evaluated other information deemed appropriate or necessary, including data and information provided by Cargill. The data evaluated by the Panel included information pertaining to the method of manufacture and product specifications, analytical determinations, stability, intended uses in food, consumption estimates, and comprehensive literature and studies concerning the safety of rebiana, other related steviol glycosides, *Stevia* extracts, metabolites including steviol, and various degradation products.

Following independent, critical evaluation of such data and information, the Panel unanimously concluded that under the conditions of intended use as a general purpose sweetening agent in conventional foods (when otherwise not precluded by Standards of Identity), rebiana, meeting appropriate food-grade specifications, and manufactured and used in accordance with current good manufacturing practice, is GRAS based on scientific procedures. A summary of the basis for the Panel's conclusion, excluding confidential data and information, is provided below.

CURRENT GLOBAL REGULATORY POSITION OF STEVIOL GLYCOSIDES

Rebaudioside A is one of several naturally occurring steviol glycoside constituents of the *Stevia rebaudiana* (Bertoni) plant. Currently, steviol glycosides, including rebaudioside A and stevioside (a closely related structural analogue of rebaudioside A) are permitted for use in foods and beverages in South Korea, Japan, Argentina, Paraguay, and Brazil and in dietary supplements in the U.S. (Madley, 2002; O'Brien Nabors, 2002; Geuns, 2003; Lipinski, 2003; Schoenhals, 2003). The Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) allocated a temporary Acceptable Daily Intake (ADI) of 0-2 mg/kg body weight/day, expressed as steviol, for steviol glycosides based on a no-observed-adverse-effect level (NOAEL) of 970 mg/kg body weight from a 2-year study in rats (Toyoda *et al.*, 1997). This corresponds to an ADI of 0 to 6 mg/kg body weight/day for rebiana. JECFA requested further information regarding potential pharmacological effects of steviol glycosides. Steviol glycosides are scheduled for re-evaluation by JECFA in June 2008.

IDENTITY AND CHARACTERIZATION

The substance that is the subject of this GRAS determination is rebiana, a highly purified form of rebaudioside A (CAS No. 58543-16-1). Rebaudioside A (13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid β-D-glucopyranosyl ester) is identified by the chemical formula $C_{44}H_{70}O_{23}$ and has a molecular weight of 967.014 g/mol. The structural formula of rebaudioside A is presented in Figure 1. Rebiana is produced as a white to off-white powder, with a characteristic sweet taste.

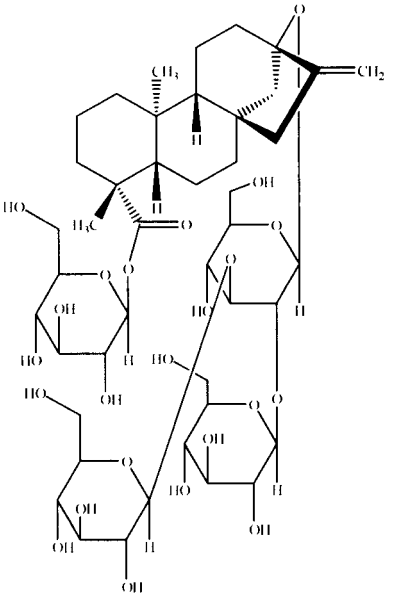

Figure 1 Structural Formula of Rebaudioside A

COMPOSITION, PRODUCTION PROCESS, AND SPECIFICATIONS

The process begins with a hot water extraction of leaves from the *Stevia rebaudiana* plant. The process can be divided into 2 phases, with the first phase involving the extraction of the leaves and preliminary purification to yield steviol glycoside primary extract. The second phase involves re-crystallization of the steviol glycoside primary extract from a water/alcohol mixture resulting in a final product of not less than 97% rebaudioside A (*i.e.*, rebiana).

Rebiana is produced in accordance with current good manufacturing practices and meets appropriate food grade specifications. The chemical and microbiological specifications for rebiana are presented in Table A1-1 (see Attachment 1). Analyses of three representative lots demonstrated consistent compliance with final product chemical and microbiological specifications. Multi-residue pesticide screens revealed no pesticide residues in the steviol glycoside primary extract or rebiana. Rebiana is routinely screened for arsenic, cadmium, and mercury.

Several related steviol glycosides have been identified at low levels in rebiana. The related steviol glycosides included rebaudioside B, rebaudioside F, IMP-2, IMP-4, DAQ1, DAQ3, DAQ4, DAQ5, and DAQ7. Many of the related steviol glycosides have the same aglycone structure as rebaudioside A, while a few others are characterized by slight structural differences of the terpene ring structure. The structural characteristics of the related steviol glycosides are summarized in Table A1-2 (see Attachment 1).

STABILITY

Studies assessed the bulk stability of rebiana (dry) under various storage conditions. Studies also were conducted in a number of food matrices (food/beverages at both room temperature and elevated temperature conditions) to determine the stability of rebiana in foods and beverages over a range of pH values and processing conditions. The photostability of rebiana was examined under dry and aqueous conditions.

In a 5-year study designed to evaluate the bulk stability of rebiana, slight reductions in rebaudioside A content were observed at interim analyses at 52 and 104 weeks when stored in glass bottles (in the range of less than 1% to not more than 1.5%) or in polyethylene bags (ranging from 1.5 to 2.7%) at 25°C and 60% relative humidity. The levels of rebaudioside A were shown to decrease by not more than 6.4% over the course of a 65-week period under accelerated storage conditions (40°C and 75% relative humidity) in sealed glass bottles. Rebiana (dry) was demonstrated to be photostable after exposure to fluorescent (1.2 million lux hours; equivalent to approximately 2 weeks) and near-UV (not less than 200 watt hours/m^2) light in a light chamber set at 25°C and 60% relative humidity. Two (2) degradation products were identified in the bulk material: DS-1 and rebaudioside B. The structural characteristics of these degradation products are summarized in Table A1-2 (see Attachment 1).

The general stability of aqueous rebiana solutions (500 mg/L; Lot No. 1001) was assessed in 0.1 M phosphate buffer at temperatures ranging from 5 to 80°C and at pH values from 2 to 8. The extent and rate of rebiana degradation were shown to be dependent on pH, temperature, and time. In general, rebiana was more stable at pH between 4 and 6 and at temperatures between 5 and 25°C than under other pH and temperature conditions.

A 3-dimensional model was developed to evaluate rebiana stability in all proposed food uses. This model was previously validated for aspartame stability determinations (Pariza *et al.*, 1998). The extremes of the 3-dimensional model were represented by the following food types: table-top product (low moisture, low heat, medium pH), mock beverages (high moisture, low heat, low pH), thermally processed beverages (high moisture, medium to high heat, low pH), yogurt (high moisture, medium heat, low pH), and white cake (moderate moisture, high heat, medium pH).

Rebiana was demonstrated to remain stable under normal (25°C and 60% relative humidity) and accelerated storage conditions (40°C and 75% relative humidity) for up to 26 weeks when evaluated in a table-top product at a rebiana concentration of 1% (10,000 mg/kg).

The stability of rebiana in mock beverages (500 mg/mL), assessed under various pH conditions reflective of a range of potential end food-uses [pH 2.8 (cola drinks), pH 3.2 (cola drinks), pH 3.8 (lemon-lime soft drinks), and pH 4.2 (root beer soft drinks)], and at different storage temperatures (5, 20, 30, and 40°C) for periods of up to 26 weeks, was demonstrated to be pH-, temperature-, and time-dependent. Solutions with a pH of 3.2 were stored at 20°C for 8 weeks, conditions considered by the United States Food and Drug Administration (FDA) as representative for the evaluation of non-nutritive sweetener stability in carbonated soft drinks (U.S. FDA, 1998). At 8 weeks and either 5 or 20°C, rebaudioside A loss was <3.5% at all 4 pH values. At 20°C, rebaudioside A loss was slightly increased from 2.2% at week 8 to 4.1% at week 26 in the pH 3.2-beverage. At the higher temperatures (30 or 40°C) and the lower pH values (2.8 and 3.2) degradation ranged from >4.5% to <27% at week 8 and from >11% to <62% at Week 26. DAQ1 and DAQ3 were identified as the major degradation products. Levels of rebaudioside B, which was detected in the mock beverage samples at the start of the storage period, also increased in all samples stored at 30 or 40°C. Other degradation products detected in mock beverages at 8 and 26 weeks included DAQ2, DAQ4, DAQ5, DAQ6, and DAQ7, which were detected only at higher temperatures (30 or 40°C) at all pH values. The structural characteristics of the degradation products of rebiana identified in the mock beverage stability study are presented in Table A1-2 (see Attachment 1).

Rebiana was demonstrated to be photostable in mock beverages (pH 3.8) exposed to fluorescent (minimum of 1.2 million lux hours; equivalent to approximately 2 weeks) and near-UV (minimum 200 watt hours/m^2) light. Short-term thermal processing as used in pasteurization of rebiana in a mock beverage at low (3.2) and high (6.5) pH conditions resulted in less than 1% loss of rebaudioside A. A minimal decrease in rebaudioside A content in the mock beverage at

pH 3.2 was accompanied by a small increase in the levels of DAQ3. A small increase in the level of rebaudioside B was noted in the mock beverage at pH 6.5.

Rebiana was demonstrated to be stable when added to plain yogurt at a rebaudioside A target concentration of 0.034% (340 mg/kg), pasteurized (190°F for 5 minutes), fermented, and then stored for 6 weeks at 5±3°C. Rebiana also was demonstrated to be stable following addition to cake batter at a rebaudioside A target concentration of 0.0673% (0.2491 g rebaudioside A/370 g batter or 673 mg/kg), and baked for 20 to 25 minutes in a conventional oven at 360°F or in a convection oven at 335°F and stored for 5 days at 25°C and 60% relative humidity no loss of rebaudioside A was measured during baking or storage.

INTENDED USE AND ESTIMATED EXPOSURE

Stevia rebaudiana was officially discovered in the West in 1887 by Antonio Bertoni (a South American natural scientist). *Stevia rebaudiana* and stevioside have been consumed for hundreds of years by humans, in various countries, as sweeteners in foods and beverages (Geuns, 2003). There have been no reports of adverse effects following the use of these natural sweeteners (Lee, 1979; Ferlow, 2005). The native peoples of Brazil and Paraguay have used the leaves of *Stevia rebaudiana* for hundreds of years as both a food ingredient and as a tea (Blumenthal, 1995). The native Indians of the Guarani Tribe also have been documented to use *Stevia* leaves as a sweetener since pre-Columbian times (Ferlow, 2005). *Stevia* became a popular herbal tea ingredient in the United States in the 1980s (Blumenthal, 1995; Ferlow, 2005).

In 1995, the use of stevioside in Asia was reported to be approximately 160,000 metric tons sucrose equivalents (SE), while in 1999, such use reportedly increased to approximately 200,000 metric tons SE (Anonymous, 2001). Stevioside has been used as a sweetener in Japan for more than 30 years, and its use has been reported to be safe, without the occurrence of adverse effects (Ferlow, 2005).

Rebiana is intended for use as a general purpose sweetening agent, in accordance with current good manufacturing practices. Considering that rebiana is characterized by a sweetness profile that is, for the most part, comparable to that of aspartame, the uses and use levels for rebiana are likely to reflect those currently permitted for aspartame in the United States with a few minor exceptions. These small differences will have no impact on the estimated consumption of rebiana.

Based on production data, the *per capita* consumption of caloric sweeteners in the United States (U.S.) is 216.5 g/day (USDA, 2007). Assuming that rebiana would replace all sugar consumption, and assuming that rebiana is 200 times as sweet as sugar, this corresponds to a rebiana intake expressed as steviol equivalents of 5.9 mg/kg body weight/day (average body

weight of 60 kg assumed). However, these estimated intakes are highly conservative since it is unlikely that rebiana would entirely replace sugar consumption.

Numerous studies in the U.S., Canada, Australia/New Zealand, and countries in the European Union (EU) identify consumer intakes of aspartame and other high intensity sweeteners (HIS) through post-market surveillance data. These studies allow for a more realistic, but conservative approach to estimate rebiana intake based on published intake figures. The intake of rebaudioside A was estimated by Renwick (2008a) using the published data on dietary exposures to approved intense sweeteners with adjustment for their relative sweetness intensities, assuming a relative sweetness for rebiana of 200 times that of sucrose. For the purposes of this assessment, it was assumed that the composition of rebiana is 100% rebaudioside A. Using this approach, the mean intake of rebiana was predicted to range from 1.3 mg/kg body weight/day (0.43 mg/kg body weight/day as steviol equivalents) for non-diabetic adults to 3.4 mg/kg body weight/day (1.12 mg/kg body weight/day as steviol equivalents) for diabetic children. Predicted intakes for heavy consumers ranged from 3.4 mg/kg body weight/day (1.12 mg/kg body weight/day as steviol equivalents) for non-diabetic adults to 5.0 mg/kg body weight/day (1.64 mg/kg body weight/day as steviol equivalents) for non-diabetic children. The predicted intakes of rebiana are all below the current temporary ADI defined by the JECFA for steviol glycosides (JECFA, 2005) of 0-2 mg/kg body weight/day as steviol equivalents.

DATA PERTAINING TO SAFETY

The safety of rebiana for its intended use as a general purpose sweetening agent is based on scientific procedures as described in Title 21 of the Code of Federal Regulations (21CFR§170.30) (U.S. FDA, 2007) and includes the evaluation of data from safety studies carried out on steviol glycosides, including *in vitro* and *in vivo* metabolism and pharmacokinetic studies; short- and long-term animal feeding studies; reproductive and developmental toxicology studies; *in vitro* and in *vivo* mutagenicity/genotoxicity; and human studies.

Absorption, Distribution, Excretion and Metabolism

Following oral administration, steviol glycosides are not readily absorbed from the upper small intestine. Human digestive enzymes are not capable of hydrolyzing β-glycosidic bonds and thus steviol glycosides are expected to escape digestion in the upper gastrointestinal tract. Steviol glycosides therefore pass undigested through the upper portion of the gastrointestinal tract and enter the colon intact where they are subject to microbial degradation by microbes of the *Bacteroidaceae* family. Rebaudioside A is first converted to either stevioside (major pathway) or rebaudioside B (minor pathway) prior to being ultimately degraded to steviol (Koyama *et al.*, 2003a). Stevioside is further degraded to steviolbioside, steviolmonosides, and finally steviol, with glucose released with each sequential hydrolysis (Nakayama *et al.*, 1986; Koyama *et al.*, 2003a). Since glucose is released in the lower segment of the gut (colon in humans/cecum in

rodents), it is not expected to be a significant source of energy. Steviol is rapidly absorbed from the gastrointestinal tract and is transported to the liver for further metabolism (Koyama *et al.*, 2003b), with low distribution to other organs (Nakayama *et al.*, 1986). In the liver, steviol has been shown to primarily undergo conjugation with glucuronic acid to form steviol glucuronide.

A comparable pharmacokinetic profile was observed for the occurrence of radioactivity in plasma following gavage treatment of male and female Sprague-Dawley rats with similarly radiolabeled rebaudioside A or stevioside (Roberts and Renwick, 2008). With either steviol glycoside, the T_{max} for radioactivity occurred within 2 to 8 hours of dosing for both rebaudioside A and stevioside.

In rats, steviol, administered as steviol or available following cleavage of glycosides in the gut, has been shown to be primarily excreted in the feces *via* the bile (generally within 48 hours), with smaller amounts in the urine (less than 3%) (Wingard *et al.*, 1980; Nakayama *et al.*, 1986; Sung, 2002; Roberts and Renwick, 2008). Following elimination in the bile, steviol is available to be released again from its conjugated form by the action of the microflora and may enter enterohepatic circulation. Consequently, free and conjugated steviol are excreted in the feces, along with any unhydrolyzed fraction of the administered glycosides.

Overall, the data demonstrate that rebaudioside A and stevioside have similar metabolism and pharmacokinetics in the rat. Therefore, the results of toxicology studies on either stevioside or rebaudioside A are applicable to both compounds.

In humans, steviol glucuronide is the primary metabolite in plasma following ingestion of stevioside or rebaudioside A (Geuns and Pietta, 2004; Simonetti *et al.*, 2004; Geuns *et al.*, 2007). Human data indicate that in contrast to rats, steviol glycosides are excreted primarily as steviol glucuronide in the urine (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Geuns *et al.*, 2006, 2007; Wheeler *et al.*, 2008). The inter-species difference in the route of elimination of systemically absorbed steviol as steviol glucuronide (*via* the bile in rats and in the urine in humans) occurs as a result of the lower molecular weight threshold for biliary excretion in rats (325) as compared to humans (500 to 600) (Renwick, 2008b).

Acute Toxicity

Rebaudioside A, administered as a single gavage dose of 2 g/kg body weight to male Swiss-Webster mice, was reported to produce no toxic effects (Medon *et al.,* 1982). Similarly, stevioside (96% purity) was not associated with any adverse effects following intragastric administration at dose levels of up to 15 g/kg body weight in mice, rats, and hamsters (Medon *et al.*, 1982; Toskulkao *et al.*, 1997). Nephrotoxicity observed in acute toxicity studies conducted with hamsters and rats using non-oral routes of administration and/or without purity information (Panichkul *et al.*, 1988; Toskulkao *et al.*, 1994, 1997) is not relevant to human safety assessment. However, the nephrotoxic effects observed in hamsters after oral administration of

steviol may be due to differences in species sensitivity, metabolism, and pharmacokinetics (Toskulkao *et al.*, 1997).

Subchronic Toxicity

The subchronic toxicity potential of high purity rebaudioside A has been evaluated in feeding studies carried out with the Han Wistar and Sprague-Dawley strains of rat (Curry and Roberts, 2008; Nikiforov and Eapen, 2008). Rebiana (97% rebaudioside A) was evaluated for safety in an FDA Redbook- and Good Laboratory Practices (GLP)-compliant 13-week toxicity in the Han Wistar rat (Curry and Roberts, 2008). In this study, rebiana was reported not to present any evidence of adverse effects when provided to both sexes of Han Wistar rats at dietary concentrations of up to 100,000 ppm (9,938 and 11,728 mg/kg body weight/day for males and females, respectively) for 4 weeks or 50,000 ppm (4,161 and 4,645 mg/kg body weight/day for males and females, respectively) for 13 weeks (Curry and Roberts, 2008). These authors concluded that the decreases in body weight gains during the initial study period were due to acclimation to the intensely sweet diets, as well as the lower caloric density of the rebiana-containing diets and not related to any adverse effects of rebiana. Overall, the effects of treatment on food conversion efficiency were minimal, except early in the study when the rat adjusted to the diet during a period of rapid growth. Importantly, while observations of reduced food consumption and body weight gain were noted, no toxicity was observed over the dose-range in the study.

Based on the World Health Association (WHO, 1987) guidance in regard to the interpretation of lower body weight gain in the absence of other toxicity due to consumption of a test material with known nutritive and palatability effects, the body weight effects observed in both studies were not considered as an adverse effect of rebiana. Similar effects have been reported in safety studies of other HIS dosed at high levels which were likewise determined to be of no toxicological significance.

When clinical chemistry and hematological parameters were measured, only small increases in serum urea and creatinine were noted. However, they were within the normal range, were inconsistent over time and dose, and were not associated with histopathologic alterations. Therefore, they were not considered to be of biological or toxicological significance.

The results of the 13-week toxicity study of rebiana (Curry and Roberts, 2008) closely match those of a similar study on the same test material in Sprague-Dawley rats (Nikiforov and Eapen, 2008). Nikiforov and Eapen (2008) reported that feeding of rebaudioside A in the diet *ad libitum* to produce target doses 500, 1,000, and 2,000 mg/kg body weight/day was without adverse effect on body weight gain, terminal body weights, clinical and functional observational battery observations, or on the results of the hematology, serum chemistry, or urinalysis evaluations. Treatment was reportedly not associated with any organ weight, macroscopic or microscopic tissue changes. Similar to Curry and Roberts (2008), a slight decrease in food conversion

efficiency was noted in the high-dose males, an effect associated with decreased terminal body weight and body weight gain. According to Nikiforov and Eapen (2008), the decrement in food conversion efficiency and in terminal body weights was suggested to possibly be the result of the inclusion of a high concentration of a non-nutritive substance in the diet. Other observations reported in Nikiforov and Eapen (2008) also tend to mirror those of Curry and Roberts (2008), namely, an indication of decreased serum bile acids, a tendency to decreased urine volumes in treated animals, and similar slight changes in serum electrolytes. Nikiforov and Eapen (2008) considered minor decreases in body weight and food conversion efficiency as non-adverse and ascribed a NOAEL of 2,000 mg/kg body weight/day, the highest dose tested.

Overall, the subchronic toxicity study data on rebiana indicating no specific toxicity concerns at concentrations in the diet of up to 100,000 ppm for 4 weeks, providing 9,938 mg rebiana/kg body weight/day for males and 11,728 mg rebiana/kg body weight/day for females, and at concentrations in the diet of up to 50,000 ppm for 13 weeks, providing 4,161 mg rebiana/kg body weight/day for males and 4,645 mg rebiana/kg body weight/day for females corroborate the findings of other studies on related steviol glycosides (Akashi and Yokoyama, 1975; Lee *et al.*, 1979; Aze *et al.*, 1991; Xili *et al.*, 1992).

Developmental and Reproductive Toxicity

Multi-generational reproductive and developmental studies conducted with purified rebaudioside A and stevioside have shown a lack of reproductive or developmental toxicity in Han Wistar rats and hamsters. In a dose-finding reproduction study, female rats administered rebiana (97% rebaudioside A) in the diet during lactation displayed no toxic effects related to rebiana consumption. The offspring were reported to have decreased body weight gains at concentrations in the diet of higher than 37,500 ppm; however, the decreases in body weight gains in the offspring were accompanied by a concomitant decrease in food consumption (Curry *et al.*, 2008). The authors attributed the decreased food consumption to the palatability of the diet. Likewise, in the main multi-generational reproductive and developmental study, decreased body weight and body weight gains were reported in all generations of Han Wistar rats administered rebiana in the diet at concentrations of 12,500 and 25,000 ppm (Curry *et al.*, 2008). The authors noted that food consumption decreases occurred within the first 2 weeks of administration of the diet in the immediate post-weaning period when the weanlings were first exposed to the intensely sweet diet.

Findings of the 2 reproductive toxicity studies conducted on rebiana (Curry *et al.*, 2008) demonstrate no adverse effects on reproductive function or reproductive organs at dietary concentrations of up to 25,000 ppm, or approximately 2,048 (males) and 2,273 (females) mg/kg body weight/day based on pre-mating intakes and body weights. These studies corroborate the results of a number of reproductive and developmental studies conducted with purified stevioside (purity 90 to 96%) administered in the diet or by gavage to rats or hamsters (Akashi

and Yokoyama, 1975; Mori *et al.*, 1981; Yodyingyuad and Bunyawong; 1991; Usami *et al.*, 1995), where a lack of reproductive or developmental toxicity was observed.

Carcinogenicity/Chronic Toxicity

The available long-term toxicity/carcinogenicity data on steviol glycosides (Xili *et al.*, 1992; Toyoda *et al.*, 1997) and *Stevia* extract (Yamada *et al.*, 1985) show no evidence of carcinogenic potential. Moreover, these studies show no indication of toxicity associated with prolonged high-dose dietary exposure. A NOAEL of 970 mg/kg body weight/day was established for stevioside based on the results of the 2-year dietary rat study conducted by Toyoda *et al.* (1997) which formed the basis for the temporary ADI of 0 to 2 mg/kg body weight (expressed as steviol) set by JECFA for steviol glycosides.

Genotoxicity

Rebaudioside A consistently demonstrated absence of mutagenic or genotoxic activity *in vitro* and *in vivo*. The genotoxic potential of stevioside and steviol also has been assessed extensively *in vitro* and *in vivo*. The results of such studies provide a weight of evidence that these agents are not genotoxic. Importantly, stevioside and steviol were not genotoxic in 13 of 14 *in vivo* studies. The single positive *in vivo* finding was considered inadequate because of methodological and purity concerns (Geuns, 2007; Williams, 2007) and was not replicated in another study (Sekihashi *et al.*, 2002).

Special Studies

Glucose Homeostasis and Insulin Secretion

Numerous *in vitro* and *in vivo* studies in both normal and diabetic animals have been conducted to assess the effects of rebaudioside A, stevioside, and extracts of *Stevia* on glucose metabolism parameters. The *in vitro* studies and the *in vivo* studies that used non-oral routes of exposure are not relevant to the oral intake of rebiana based on metabolism data.

High oral doses of *Stevia* extract (Suzuki *et al.*, 1977; von Schmeling *et al.*, 1977) and stevioside (Jeppesen *et al.*, 2002, 2003; Dyrskog *et al.*, 2005a) administered to rats have been associated with decreased plasma glucose levels and/or plasma glucose area under the curve (AUC) values, although statistical significance was not achieved in all cases. However, Dyrskog *et al.* (2005b) could not confirm antihyperglycemic effects of rebaudioside A. Oral dosing of rats with stevioside (Jeppesen *et al.*, 2003; Lailerd *et al.*, 2004; Chang *et al.*, 2005; Chen *et al.*, 2005) has been associated with decreased glucose levels and improved insulin response following glucose tolerance tests.

Antihyperglycemic and increased insulin sensitivity effects of stevioside have been reported in diabetic/insulin resistant and genetically obese rats (von Schmeling *et al.*, 1977; Hübler *et al.*,

1994; Jeppesen *et al.*, 2003; Lailerd *et al.*, 2004; Chang *et al.*, 2005; Chen *et al.*, 2005). The mechanism(s) for these changes are unclear.

Effects on Blood Pressure

A number of *in vitro* and *in vivo* studies have investigated the antihypertensive/vasorelaxation effects of stevioside. These studies have been previously reviewed by JECFA at their 51st and 63rd meetings.

Oral studies report that stevioside may have a mild antihypertensive effect in rats. The mechanism by which stevioside, or more likely, its metabolite steviol, exerts its antihypertensive effect has not been elucidated. *In vivo* studies, including those that utilize non-oral routes of administration and high-dose exposures, while showing an antihypertensive effect of stevioside (steviol), reported no toxicologically significant effects or any evidence that the antihypertensive effect was adverse.

Effects on Renal Function

Effects on renal function have been observed with stevioside in the studies conducted by Melis and others. It should be noted that stevioside was administered parenterally in all of these studies. This route of administration by-passes first-pass metabolism of the glycoside that would occur with oral exposure and also eliminates removal of the glucose moieties by intestinal flora. As such, the significance of theses studies in an assessment of oral safety is questionable. Crude extracts of *Stevia*, which were administered to rats orally over extended periods of time (up to 60 days) also were shown to exert hypotensive properties accompanied by changes in renal function parameters, similar to those observed with intravenously administered stevioside (Melis, 1995, 1996). These studies are irrelevant given the uncharacterized nature of the test material.

In dogs, no changes were observed in blood pressure or renal function parameters following intravenous administration of stevioside at dose levels of up to 26 mg/kg body weight or following oral administration at 6 mg/kg body weight/day for 10 days (Krejci and Koechel, 1992). Likewise, neither single intravenous nor repeat oral administration of a *Stevia* extract to dogs was associated with any blood pressure or renal function variations (Chagas *et al.*, 1990).

Clinical Trials

A number of studies have been identified that were conducted in humans to assess the safety and tolerability of purified steviol glycosides and *Stevia* extracts following single- and repeat-dose administration. As a result of the putative effects of steviol glycosides on blood pressure regulation and glucose homeostasis, many of these studies included endpoints to specifically assess these effects.

No changes in blood glucose or insulin levels were observed in normal healthy subjects following treatment with stevioside at dose levels of 750 mg/day for a period of 3 days (Temme *et al.*, 2004; Geuns *et al.*, 2007) or in hypertensive subjects treated with stevioside at daily dose levels of 750 or 1,500 mg for 1- or 2-year periods, respectively (Chan *et al.*, 2000; Hsieh *et al.*, 2003). Likewise, Jeppesen *et al.* (2006) demonstrated no effects on glucose or insulin levels in Type-2 diabetic subjects consuming stevioside (1,500 mg/day) for up to 3 months. Barriocanal *et al.* (2008) reported no effects of steviol glycosides on HbA_{1C} in subjects with Type-1 and 2 diabetes mellitus.

A number of studies also evaluated effect on blood pressure following treatment with stevioside in 3 different cohorts of healthy, hypertensive, and diabetic subjects. Single doses of stevioside (1 g) exerted no effects on blood pressure in Type-2 diabetic subjects (Gregersen *et al.*, 2004). Likewise, no changes in blood pressure were observed in a 3-day study in which normal, healthy subjects received 750 mg of stevioside (Temme *et al.*, 2004; Geuns *et al.*, 2007). Jeppesen *et al.* (2006) also reported no clinically significant effects on blood pressure in subjects with Type-2 diabetes consuming stevioside at daily dose levels of 1,500 mg for 3 months. However, following administration of stevioside at doses of 750 or 1,500 mg/day for 1 and 2 years, respectively, decreased blood pressure in hypertensive patients was reported (Chan *et al.*, 2000; Hsieh *et al.*, 2003). Two further studies conducted with either rebiana or steviol glycosides used ambulatory blood pressure monitoring (Barriocanal *et al.*, 2008; Maki *et al.*, 2008a). Both studies showed no clinically significant change in average 24-hour blood pressure. One study also showed no effect on either daytime or nighttime average blood pressure (Maki *et al.*, 2008a).

Apart from parameters related specifically to blood pressure and glucose regulation, many of the studies also assessed other standard urinalysis and clinical chemistry parameters, including liver enzyme levels, as well as tolerability of the steviol glycosides and extracts. In none of the studies were any adverse effects reported in test group subjects following consumption of up to 1,500 mg stevioside per day for up to 2 years (Alvarez *et al.*, 1981; Chan *et al.*, 2000; Gregersen *et al.*, 2001, 2004; Hsieh *et al.*, 2003; Temme *et al.*, 2004; Ferri *et al.*, 2006; Cavalcante da Silva *et al.*, 2006; Jeppesen *et al.*, 2006; Geuns *et al.*, 2007).

Two (2) longer term studies were conducted with rebiana (>97% rebaudioside A) in light of questions raised by JECFA at the 63rd and 68th meetings regarding the potential for pharmacological effects of steviol glycosides in subjects with Type-2 diabetes and in subjects with low to normal blood pressure. These studies demonstrated that oral administration of rebiana had no pharmacological or clinically significant effects in healthy adults or in subjects with Type-2 diabetes (Maki *et al.*, 2008a,b).

Collectively, the results of the human clinical trials conducted with rebiana or stevioside demonstrate that steviol glycosides are safe and well-tolerated in groups of normotensive or hypotensive individuals and Type-2 diabetics following long-term consumption at doses of up to

1.5 g/day, or about 25 mg/kg body weight/day. Clinical trials specifically on rebiana showed no effects on glucose homeostasis or blood pressure at doses of up to 1,000 mg/day (about 16 mg/kg body weight/day), a dose more than 10-fold greater than the predicted intake of rebaudioside A in children with diabetes (~1.5 mg/kg body weight/day).

CONCLUSION

We, the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that the proposed use of rebiana as a general purpose sweetener in foods and beverages (when not otherwise precluded by a Standard of Identity), produced consistently with current Good Manufacturing Practices (cGMP) and meeting appropriate food grade specifications described herein, is safe. We further conclude that the proposed use of rebiana as a general purpose sweetening agent is Generally Recognized As Safe (GRAS) based on scientific procedures.

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REFERENCES

Akashi, H.; Yokoyama, Y. 1975. [Security of dried-leaves extracts of stevia—report of toxicological test]. Shokuhin Kogyo 18(20):34-43.

Alvarez, M.; Bazzote, R.B.; Godoy, G.L.; Cury, R.; Espinoza, A.D. 1981. Efeito do extrato aquoso de Stevia rebaudiana Bertoni sobre parametros bioquimicos de pesoas adultas normais = [Hypoglycemic effect of Stevia rebaudiana Bertoni]. Arq Biol Tecnol 24(1):178 [Abstract No. H-35].

Anonymous. 2001. Developments in the high-intensity sweeteners markets. Zuckerindustrie 126(12):970.

Aze, Y.; Toyoda, K.; Imaida, K.; Hayashi, S.; Imazawa, T.; Hayashi, Y.; Takahashi, M. 1991. Subchronic oral toxicity study of stevioside in F344 rats. Eisei Shikenjo Hokoku (109):48-54 [In Japanese].

Barriocanal, L.; Palacios, M.; Benitez, G.; Benitez, S.; Jimenez, J.T.; Jimenez, N.; Rojas, V. 2008. Apparent lack of pharmacological effect of steviol glycosides used as a sweetener in humans. A pilot study of repeated exposures in some normotensive and hypotensive individuals and Type 1 and Type 2 diabetics. Regul Toxicol Pharmacol. [In Press Accepted Manuscript].

Blumenthal, M. 1995. FDA lifts import alert on stevia. herb can be imported only as dietary supplement; future use as a sweetener is still unclear. HerbalGram(35 - Fall):17-18.

Cavalcante da Silva, G.E.; Assef, A.H.; Cordeiro Albino, C.; de Araujo Funari Ferri, L.; Tasin, G.; Takahashi, M.H.; Filho, W.E.; Barbosa Bazotte, R. 2006. Investigation of the tolerability of oral stevioside in Brazilian hyperlipidemic patients. Braz Arch Biol Technol 49(4):583-587.

Chagas, A.M.; Tabarelli, Z.; Simoes, S.R.M.; Azzolin, E.L.C. 1990. Effects of total aqueous extract of Stevia-rebaudiana and its stevioside upon renal parameters in vagrant dogs and dogs with water overload. Rev Cinc Biomed 11:1-12.

Chan, P.; Tomlinson, B.; Chen, Y.; Liu, J.; Hsieh, M.; Cheng, J. 2000. A double-blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in human hypertension. Br J Clin Pharmacol 50(3):215-220.

Chang, J.-C.; Wu, M.C.; Liu, I.-M.; Cheng, J.-T. 2005. Increase of insulin sensitivity by stevioside in fructose-rich chow-fed rats. Horm Metab Res 37(10):610-616.

Chen, T.-H.; Chen, S.-C.; Chan, P.; Chu, Y.-L.; Yang, H.-Y.; Cheng, J.-T. 2005. Mechanism of the hypoglycemic effect of stevioside, a glycoside of *Stevia rebaudiana*. Planta Med 71(2):108-113.

Curry, L.; Roberts, A. 2008. Acute and Subchronic Toxicity of Rebaudioside A. ***[In Press]***

Curry, L.; Roberts, A.; Brown, N. 2008. Rebaudioside A: Two-Generation Reproductive Toxicity Study in Rats. ***[In Press]***

Dyrskog, S.E.U.; Jeppesen, P.B.; Colombo, M.; Abudula, R.; Hermansen, K. 2005a. Preventive effects of a soy-based diet supplemented with stevioside on the development of the metabolic syndrome and type 2 diabetes in Zucker diabetic fatty rats. Metabolism 54(9):1181-1188.]

Dyrskog, S.E.; Jeppesen, P.B.; Chen, J.; Christensen, L.P.; Hermansen, K. 2005b. The diterpene glycoside, rebaudioside A, does not improve glycemic control or affect blood pressure after eight weeks treatment in the Goto-Kakizaki rat. Rev Diabet Stud 2(2):84-91.

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

Ferri, L.A.F.; Alves-Do-Prado, W.; Yamada, S.S.; Gazola, S.; Batista, M.R.; Bazotte, R.B. 2006. Investigation of the antihypertensive effect of oral crude stevioside in patients with mild essential hypertension. Phytother Res 20(9):732-736.

Geuns, J.M.C. 2003. Stevioside. Phytochemistry 64(5):913-921.

Geuns, J.M.C. 2007. Comments to the paper by Nunes *et al.* (2007), Analysis of genotoxic potentiality of stevioside by comet assay, Food and Chemical Toxicology 45 (2007) 662-666 [Including response from A.P.M. Nunes *et al.*]. Food Chem Toxicol 45(12):2601-2604.

Geuns, J.M.C.; Pietta, P. 2004. Stevioside metabolism by human volunteers. Unpublished report from Laboratory Functional Biology, Kuleuven, Leuven Belgium and ITB-CNR, Segrate (MI); Italy. Submitted to WHO by the Federal Ministry of Social Affairs, Public Health and the Environment; Belgium. Cited In: JECFA, 2006.

Geuns, J.M.C.; Buyse, J.; Vankeirsbilck, A.; Temme, E.H.M.; Compernolle, F.; Toppet, S. 2006. Identification of steviol glucuronide in human urine. J Agric Food Chem 54(7):2794-2798.

Geuns, J.M.C.; Buyse, J.; Vankeirsbilck, A.; Temme, E.H.M. 2007. Metabolism of stevioside by healthy subjects. Exp Biol Med 232(1):164-173.

Gregersen, S.; Hermansen, K.; Jeppesen, P.B.; Holst, J.J. 2001. Acute effects of the diterpene glycoside stevioside in type II diabetic patients. Diabetologia 44(Suppl. 1):A236 [Abstract No. 905].

Gregersen, S.; Jeppesen, P.B.; Holst, J.J.; Hermansen, K. 2004. Antihyperglycemic effects of stevioside in type 2 diabetic subjects. Metabolism 53(1):73-76.

Hsieh, M.H.; Chan, P.; Sue, Y.M.; Liu, J.C.; Liang, T.H.; Huang, T.Y.; Tomlinson, B.; Chow, M.S.; Kao, P.F.; Chen, Y.J. 2003. Efficacy and tolerability of oral stevioside in patients with mild essential hypertension: a two-year, randomized, placebo-controlled study. Clin Ther 25(11):2797-2808.

Hübler, M.O.; Bracht, A.; Kelmer-Bracht, A.M. 1994. Influence of stevioside on hepatic glycogen levels in fasted rats. Res Commun Chem Pathol Pharmacol 84(1):111-118.

JECFA. 2005. Steviol glycosides. In: Evaluation of Certain Food Additives. 63rd Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 8-17, 2004, Geneva, Switz. World Health Organization (WHO); Geneva, Switz., WHO Technical Report Series, No. 928, pp. 34-39 & 138. Available from: http://whqlibdoc.who.int/trs/WHO_TRS_928.pdf.

JECFA. 2006. Steviol glycosides. In: Safety Evaluation of Certain Food Additives. Sixty-third Meeting of the Joint FAO/WHO Expert Committee on Food Additives, June 8-17, 2004, Geneva, Switz. World Health Organization (WHO), International Programme on Chemical Safety (IPCS); Geneva, Switz., Switz., WHO Food Additives Series, No. 54, pp. 117-144 & 638. Available from: http://whqlibdoc.who.int/publications/2006/9241660546_eng.pdf.

Jeppesen, P.B.; Gregersen, S.; Alstrup, K.K.; Hermansen, K. 2002. Stevioside induces antihyperglycaemic, insulinotropic and glucagonostatic effects in vivo: Studies in the diabetic Goto-Kakizaki (GK) rats. Phytomedicine 9(1):9-14.

Jeppesen, P.B.; Gregersen, S.; Rolfsen, S.E.; Jepsen, M.; Colombo, M.; Agger, A.; Xiao, J.; Kruhoffer, M.; Orntoft, T.; Hermansen, K. 2003. Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. Metabolism 52(3):372-378.

Jeppesen, P.B.; Barriocanal, L.; Meyer, M.T.; Palacios, M.; Canete, F.; Benitez, S.; Logwin, S.; Schupmann, Y.; Benitez, G.; Jimenez, J.T. 2006. Efficacy and tolerability of oral stevioside in patients with type 2 diabetes: a long-term, randomized, double-blinded, placebo-controlled study. Diabetologia 49(Suppl. 1):511-512 [Abstract No. 0843].

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

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

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

Krejci, M.E.; Koechel, D.A. 1992. Acute effects of carboxyatractyloside and stevioside, inhibitors of mitochondrial ADP/ATP translocation, on renal function and ultrastructure in pentobarbital-anesthetized dogs. Toxicology 72(3):299-313.

Lailerd, N.; Saengsirisuwan, V.; Sloniger, J.A.; Toskulkao, C.; Henriksen, E.J. 2004. Effects of stevioside on glucose transport activity in insulin-sensitive and insulin-resistant rat skeletal muscle. Metabolism 53(1):101-107.

Lee, C.K. 1979. Carbohydrate sweeteners: Structural requirements for taste [Includes Stevioside]. In: Bourne, G.H. (Ed.). Some Special Aspects of Nutrition. Karger AG; Basel, Switz., World Review of Nutrition and Dietetics, Vol. 33, pp. 142-197 (Stevioside on pp. 184-185).

Lee, S.J.; Lee, K.R.; Park, J.R.; Kim, K.S.; Tchai, B.S. 1979. A study on the safety of stevioside as a new sweetening source. Hanguk Sikpum Kwahakhoe Chi 11(4):224-231.

Lipinski, G.W.R. 2003. Intense sweeteners - status and new developments. Int Sugar J 105(1255):308-312.

Madley, R.H. 2002. How sweet it is(n't) [Stevia, *etc.*]. Nutraceuticals World 5(1):54-60, 62-63 & 65.

Maki, K.C.; Curry, L.L.; Carakostas, M.; Tarka, S.; Reeves, M.S.; Farmer, M.V.; McKenney, J.M.; Toth, P.D.; Schwartz, S.L.; Lubin, B.C.; Dicklin, M.R.;Boileau, A.; Bisognano, J.D. 2008a. The Hemodynamic Effects of Rebaudioside A in Healthy Adults with Normal and Low-Normal Blood Pressure. **[*In Press*]**

Maki, K.C.; Curry, L.L.; Reeves, M.S.; Toth, P.D.; McKenney, J.M.; Farmer, M.V.; Schwartz, S.L.; Lubin, B.C.; Boileau, A.; Dicklin, M.R.; Carakostas, M.; Tarka, S. 2008b. Chronic Consumption of Rebaudioside A, a Steviol Glycoside, in Men and Women with Type 2 Diabetes Mellitus. **[*In Press*]**

Medon, P.J.; Pezzuto, J.M.; Hovanec-Brown, J.M.; Nanayakkara, N.P.; Soejarto, D.D.; Kamath, S.K.; Kinghorn, A.D. 1982. Safety assessment of some Stevia rebaudiana sweet principles. Fed Proc 41(5):1568 [Abstract No. 7587].

Melis, M.S. 1995. Chronic administration of aqueous extract of *Stevia rebaudiana* in rats: Renal effects. J Ethnopharmacol 47(3):129-134.

Melis, M.S. 1996. A crude extract of *Stevia rebaudiana* increases the renal plasma flow of normal and hypertensive rats. Braz J Med Biol Res 29(5):669-675.

Mori, N.; Sakanoue, M.; Takeuchi, M.; Simpo, K.; Tanabe, T. 1981. Effect of Stevioside on fertility in rats. Shokuhin Eiseigaku Zasshi 22(5):409-414 [In Japanese].

Nakayama, K.; Kasahara, D.; Yamamoto, F. 1986. Absorption, distribution, metabolism and excretion of stevioside in rats. Shokuhin Eiseigaku Zasshi 27(1):1-8.

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

Nunes, A.P.M.; Ferreira-Machado, S.C.; Nunes, R.M.; Dantas, F.J.S.; De Mattos, J.C.P.; Caldeira-de-Araújo, A. 2007. Analysis of genotoxic potentiality of stevioside by comet assay. Food Chem Toxicol 45(4):662-666.

O'Brien Nabors, L. 2002. Sweet choices: Sugar replacements for foods and beverages. Food Technol 56(7):28, 30, 32, 34 & 45.

Panichkul, T.; Glinsukon, T.; Buddasukh, D.; Cheuvchit, P.; Pimolsri, U. 1988. The plasma levels of urea nitrogen, creatinine and uric acid and urine volume in rats and hamsters treated with stevioside. Thai J Toxicol 4:47-52.

Pariza, M.W.; Ponakala, S.V.; Gerlat, P.A.; Andress, S. 1998. Predicting the functionality of direct food additives. Food Technol 52(11):56-60.

Renwick, A.G. 2008a. The use of a sweetener substitution method to predict dietary exposures for the intense sweetener rebaudioside A. **[*In Press*]**

Renwick, A.G. 2008b. Toxicokinetics [section on elimination: excretion via the gut]. In: Hayes, W. (Ed.). Principles and Methods of Toxicology (5th Ed.). Taylor and Francis/CRC Press; Philadelphia, Penn., p. 188.

Roberts, A.; Renwick, A.G. 2008. Comparative Toxicokinetics and Metabolism of Rebaudioside A, Stevioside, and Steviol in Rats. **[*In Press*]**

Schoenhals, K. 2003. How sweet it is: Functional sweeteners. Nat Prod Insider (Mar. 3, 2003).

Sekihashi, K.; Saitoh, H.; Sasaki, Y.F. 2002. Genotoxicity studies of stevia extract and steviol by the comet assay. J Toxicol Sci 27(Suppl. 1):1-8.

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

Sung, L.H. 2002. Report on pharmacokinetic (PK) studies of T100 sunstevia 95% stevioside in rats. Unpublished report from Sunlabel Pte Ltd.; Singapore. Submitted to WHO by the Ministry of Health and Welfare; Japan. Cited In: JECFA, 2006.

Suzuki, H.; Kasai, T.; Sumiiara, M.; Sugisawa, H. 1977. Influence of oral administration of stevioside on levels of blood glucose and liver glycogen of intact rats. Nippon Nogeikagaku Kaishi 51(3):171-173.

Temme, E.H.; Vankeirsblick, A.; Buyse, J. 2004. A short term study of stevioside in healthy subjects. In: Geuns, J.M.C.; Buyse, J. (Eds.). "Safety of Stevioside": Proceedings of the First Symposium Sponsored by KULeuven, April 16, 2004, Leuven, Belgium. Euprint ed.; Heverlee, Belgium, pp. 63-74.

Toskulkao, C.; Deechakawan, W.; Leardkamolkarn, V.; Glinsukon, T.; Buddhasukh, D. 1994. The low calorie natural sweetener stevioside: Nephrotoxicity and its relationship to urinary enzyme excretion in the rat. Phytother Res 8(5):281-286.

Toskulkao, C.; Chaturat, L.; Temcharoen, P.; Glinsukon, T. 1997. Acute toxicity of stevioside, a natural sweetener, and its metabolite, steviol, in several animal species. Drug Chem Toxicol 20(1&2):31-44.

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

U.S. FDA. 1998. Food additives permitted for direct addition to food for human consumption; acesulfame potassium. Fed Regist 63(128):36344-36362.

U.S. FDA. 2007. Part 170–Food additives. Section §170.30–Eligibility for classification as generally recognized as safe (GRAS). In: U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs (U.S. Food and Drug Administration). U.S. Food and Drug Administration (U.S. FDA), U.S. Government Printing Office (GPO); Washington (DC), pp. 13-15. Available from: http://a257.g.akamaitech.net/7/257/2422/26mar20071500/edocket.access.gpo.gov/cfr_2007/aprqtr/pdf/21cfr170.30.pdf

Usami, M.; Sakemo, K.; Kawashima, K.; Tsuda, M.; Ohno, Y. 1995. Teratogenicity study of stevioside in rats. Eisei Shikenjo Hokoku 113:31-35 [In Japanese].

USDA. 2007. What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2003-2004. U.S. Department of Agriculture (USDA); Riverdale, Maryland. Available from: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release.

von Schmeling, G.A.; Carvalho, F.N.; Espinoza, A.D. 1977. Stevia rebaudiana Bertoni. evaluation of the hypoglycemic effect on alloxanized rabbits. Cienc Cult 29(5):599-601.

Wheeler, A.; Boileau, A.; Winkler, P.; Compton, J.; Prakash, I.; Jiang, X.; Mandarino, D.; Jenkins, J. 2008. Pharmacokinetics of Rebaudioside A and Stevioside after Single Oral Doses in Healthy Men. **[*In Press*]**

WHO. 1987. Test procedures and evaluations. 5.5.3. Toxicological versus physiological responses. In: Principles for the Safety Assessment of Food Additives and Contaminants in Food. World Health Organization (WHO), International Programme on Chemical Safety (IPCS); Geneva, Switz., Environmental Health Criteria, No. 70, p. 82. Available from: http://www.inchem.org/documents/ehc/ehc/ehc70.htm.

Williams, G.M. 2007. Letter to the editor [including response from Nunes *et al.*]. Food Chem Toxicol 45(12):2597-27600.

Wingard, R.E. (Jr.); Brown, J.P.; Enderlin, F.E.; Dale, J.A.; Hale, R.L.; Seitz, C.T. 1980. Intestinal degradation and absorption of the glycosidic sweeteners stevioside and rebaudioside A. Experientia 36(5):519-520.

Xili, L.; Chengjiany, B.; Eryi, X.; Reiming, S.; Yuengming, W.; Haodong, S.; Zhiyian, H. 1992. Chronic oral toxicity and carcinogenicity study of stevioside in rats. Food Chem Toxicol 30(11):957-965.

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

Yodyingyuad, V.; Bunyawong, S. 1991. Effect of stevioside on growth and reproduction. Hum Reprod 6(1):158-165.

ATTACHMENT 1

Chemical and Microbiological Specifications for Rebiana and Structural Characteristics of Related Steviol Glycosides

Table A1-1 Chemical and Microbiological Specifications for Rebiana		
Specification Parameter	**Specification**	**Method**
Chemical Parameters		
Identity	Conforms to IR standard	FCC V[1]
Solubility	Freely soluble in water	FCC V
pH	Between 4.5 and 7.0 (1% solution; wt/v)	FCC V
Assay (Rebaudioside A content)	Not less than 97.0% and not more than 102.0% (wt/wt) (on an anhydrous basis)	HPLC method (Cargill method No. STV-001-01)
Other related steviol glycosides	Not more than 3.0% (wt/wt)	HPLC method (Cargill method No. STV-001-01)
Loss on drying	Not more than 6.0% (105 °C)	FCC V
Residue on ignition (ash)	Not more than 1.0% (wt/wt)	FCC V, Method I
Specific rotation (water 0.5 wt%) $[\alpha]_D^{25}$	Between -29 and -31° (on anhydrous basis)	FCC V
Lead (Pb)	Not more than 1 ppm	ICP (AOAC method 993.14)
Solvent residues		
Ethanol	Not more than 0.5% (wt/wt)	FCC V
Methanol	Not more than 0.02% (wt/wt)	FCC V
Microbiological Parameters		
Standard plate count	Not more than 1,000 CFU	AOAC (1998)[2] Chapter 4
Total coliforms	Not more than 3 CFU	AOAC (1998) Chapter 4
Fecal coliforms	Not more than 3 CFU	AOAC (1998) Chapter 4
Escherichia coli	Not more than 10 CFU	AOAC (1998) Chapter 4
Listeria	Negative (in 11 grams)	AOAC (2001)[3] Chapter 36
Salmonella	Negative (in 25 grams)	AOAC (2001) Chapter 36
Staphylococcus	Not more than 10 CFU	AOAC (2001) Chapter 39
Yeast	Not more than 100 CFU	AOAC (2001) Chapter 20
Mold	Not more than 100 CFU	AOAC (2001) Chapter 20

CFU = Colony Forming Unit; HPLC = High Performance Liquid Chromatography; ICP-MS = Inductively Coupled Plasma Mass Spectrometry; IR = Infrared.

[1] FCC. 2003. Food Chemicals Codex (5th Ed.). National Academy Press (NAP); Washington, DC.

[2] AOAC. 1998. Official Methods of Analysis of the Association of Official Analytical Chemists (16th Ed.). Association of Official Analytical Chemists (AOAC), Inc.; Arlington, VA.

[3] AOAC. 2001. Official Methods of Analysis of the Association of Official Analytical Chemists (17th Ed.). Association of Official Analytical Chemists (AOAC); Arlington, Virginia. Vols. 1&2. (2002, Revision 1).

Table A1-2 Comparison of Impurities and Degradation Product Structural Properties to Rebaudioside A Structure

Steviol Glycoside	Classification	Aglycone (Backbone Structure)	No. of Mono-Saccharide Units
Rebaudioside A	--	Steviol	4 Glu
Rebaudioside B	Impurity	Steviol	3 Glu
Rebaudioside F	Impurity	Steviol	3 Glu, 1 Xyl
IMP-2	Impurity	Endocyclic double bond and a hydroxyl group at C-17	4 Glu
IMP-4	Impurity	Steviol	5 Glu
DAQ1	Impurity/ Degradation Product	Hydroxyl group at C-16	4 Glu
DAQ3	Impurity/Degradation Product	Endocyclic double bond	4 Glu
DAQ4	Impurity/ Degradation Product	Endocyclic double bond	3 Glu
DAQ5	Impurity/ Degradation Product	Isosteviol	1 Glu
DAQ7	Impurity/ Degradation Product	Steviol	1 Glu
DAQ2	Degradation Product	Hydroxyl group at C-16	3 Glu
DS-1	Degradation Product	Steviol	5 Glu
DAQ6	Degradation Product	Steviol	2 Glu

Glu = Glucose; Xyl = Xylose.

Appendix 11

APPENDIX II

SUMMARY OF STUDIES CONDUCTED WITH REBAUDIOSIDE A, OTHER RELATED STEVIOL GLYCOSIDES, *STEVIA* EXTRACTS, OR STEVIOL

Table A.II-1 Summary of Acute LD_{50} Values for Rebaudioside A, Related Steviol Glycosides, *Stevia* Extracts, Steviol, and Isosteviol

Test Article (purity)	Species (Strain)	Route of Administration	LD_{50} (g/kg bw)	Reference
Rebaudioside A (not reported)	Mouse (Swiss-Webster)	Gavage	>2.0	Medon *et al.* (1982)
Stevioside (not reported)	Mouse (Swiss-Webster)	Gavage	>2.0	
Stevioside (96%)	Mouse (Swiss albino)	Gavage	>15	Toskulkao *et al.* (1997)
Stevioside (96%)	Rat (Wistar)	Gavage	>15	
Stevioside (96%)	Hamster (Syrian golden)	Gavage	>15	
Rebaudioside B (not reported)	Mouse (Swiss-Webster)	Gavage	>2.0	Medon *et al.* (1982)
Steviolbioside (not reported)	Mouse (Swiss-Webster)	Gavage	>2.0	
Stevia extract, crude (20.4% stevioside)	Mouse (DDY-N)	Oral	~17	Akashi and Yokoyama (1975)
Stevia extract, refined (41.4% stevioside)	Mouse (DDY-N)	Oral	>42	
Stevia extract (50% stevioside)	Rat	Gavage	3.4	Lee *et al.* (1979)
Steviol (90%)	Mouse (Swiss albino)	Gavage	>15	Toskulkao *et al.* (1997)
Steviol (90%)	Rat (Wistar)	Gavage	>15	
Steviol (90%)	Hamster (Syrian golden)	Gavage	5.2 (males)	
			6.1 (females)	
Isosteviol (not reported)	Mouse (male Swiss)	Oral	>0.5	Bazotte *et al.* (1986)
Isosteviol (not reported)	Rat (male Holtzman)	Oral	>0.5	
Isosteviol (not reported)	Dog	Oral	>0.5	

Table A.II-2 Summary of Acute Toxicity Studies on Stevioside or Steviol

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
Stevioside						
Mouse (male and female Balb/c; 6/group, number/sex/group NR)	0, 6.25, 12.5, or 25 mg/kg bw (oral; presumably gavage)	Single dose	NR	No changes in general behavior or mortality	-lack of information regarding steviol glycoside composition of test article	Sehar *et al.* (2008)
Rat (male Wistar; 5 to 10/group)	0 or 4.1 g/kg bw (gavage)	Single dose	90%	↑ blood uric acid; AST; ALT; urine osmolarity; BUN ↓ urine volume NSD urinary protein; pH	-reported steviol glycoside composition is below 95% -sensitive test animal (hamster) -very high dose levels	Panichkul *et al.* (1988)
Hamster (male Syrian golden; 5 to 10/group)				↑ blood uric acid; AST; ALT; hematocrit; BUN; creatinine; urine osmolarity ↓ urine volume Dilation of the convoluted tubules with tubular necrosis in the cortex and corticomedullary zone; coagulative pyknotic nuclei, cell debris, and proteinaceous casts or cloudy swelling and hydropic degeneration in necrotic convoluted tubules and cells		
Rat (male Wistar; 10/group)	0 or 1.5 g/kg bw (s.c.)	Single dose (BUN determined up to 96 h post-administration) 2 additional groups killed 9 h after administration)	90%	↑ BUN	-reported steviol glycoside composition is below 95% -non-oral route of exposure	Toskulkao *et al.* (1994a)

Table A.II-2 Summary of Acute Toxicity Studies on Stevioside or Steviol

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
		Single dose (killed 9 h after administration)		↑ BUN; plasma creatinine; urine glucose; ALP; γ-GTP NSD urine protein; NAG; GST Histopathological changes in cytoplasm of PCT cells (swollen, pale, and with marked hydropic degeneration); nuclei described as pyknotic, swollen, and evacuated; cell debris and proteinaceous material detected in DCT and glomeruli Electron microscopy revealed degenerative changes of the proximal tubules and disruption of the cristae in mitochondria of PCT		
		Single dose (Killed 0, 1, 3, 6, 7.5, and 9 h after administration)		↑ BUN; plasma creatinine; urinary γ-GTP time-dependently NSD lipid peroxide levels in plasma and renal cortex		
Dog (strain not specified)	0 or 26.1 mg/kg bw (i.v.)	Single dose (followed by 6 h of monitoring)	NR	No microscopic changes of the renal tissues or liver enzyme variations	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure	Krejci and Koechel (1992)
Steviol						
Hamster (male Syrian golden; 6 to 8/group)	0 or 5 g/kg bw (gavage)	Single dose	90%	↑ BUN; plasma creatinine; total protein NSD AST; ALT Swollen and pale renal proximal tubules with marked vacuolar degeneration and cell debris in distal tubules; swollen hepatocytes and fine vacuoles in liver	-doses of steviol available for systemic absorption much greater than what would be available if a steviol glycoside was administered	Toskulkao *et al.* (1997)

↓ = decreased; ↑ = increased; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; DCT = distal convoluted tubules; GST = glutathione-S-transferase; γ-GTP = γ-glutamyl transpeptidase; i.v. = intravenous; NAG = N-acetyl-β-D-glucuronidase; NSD = no significant differences; PCT = proximal convoluted tubules; s.c. = subcutaneous.
[a] unless stated otherwise, all reported effects are relative to control group(s).

Table A.II-3 Summary of Subchronic Toxicity Studies on Steviol Glycosides and *Stevia* Extracts						
Species (Strain)	**Dose (route)**	**Study Duration**	**Purity**	**Reported Effects[a]**	**Comments**	**Reference**
Rebaudioside A						
Rat (HsdBR1 Han:Wist; 10/sex/group)	0 , 25,000, 50,000, 75,000, or 100,000 ppm [0, 2,367, 4,842, 7,143, and 9,938 mg/kg bw/d for males, respectively and 0, 2,616, 5,422, 8,190, and 11,728 mg/kg bw/d for females, respectively] (diet)	4 weeks	97%	↓ bw gains in both sexes at concentration of 50,000 ppm or higher during first 4 days of treatment; . Over entire study period, only 100,000 ppm females had ↓ bw gains. ↓ food consumption at 3 highest concentration in males and 2 highest concentrations in females; however, over entire study period, 100,000 ppm males had ↑ food consumption. ↑ plasma creatinine in all treated males and 75,000 and 100,000 ppm females; urine specific gravity in 75,000 and 100,000 ppm males and all treated females. ↓ total bile acid levels in 75,000 and 100,000 ppm males; relative adrenal weights in 50,000, 75,000, and 100,000 ppm females; relative heart weights in 75,000 and 100,000 males; absolute testes weights in 100,000 ppm males. NSD: food conversion efficiency; macroscopy and microscopy of the testes, epididymides, and seminiferous tubules. NBSD: hematological parameters.	-test article meets JECFA specifications	Curry and Roberts (2008)

Table A.II-3 Summary of Subchronic Toxicity Studies on Steviol Glycosides and *Stevia* Extracts						
Species (Strain)	**Dose (route)**	**Study Duration**	**Purity**	**Reported Effects[a]**	**Comments**	**Reference**
Rat (HsdBR1 Han:Wist; 20/sex/group)	0, 12,500, 25,000, or 50,000 ppm [0, 1,506, 3,040, and 5,828 mg/kg bw/d in week 1 and 0, 698, 1,473, and 3,147 mg/kg bw/d in week 13 for males, respectively and 0, 1,410, 2,841, and 5,512 mg/kg bw/d in week 1 and 0, 980, 1,914, and 3,704 mg/kg bw/d in week 13 for females, respectively] (diet)	13 weeks	97%	NBSD: general appearance; behavior; sensory reactivity; fore- and hindlimb grip strength; opthamological examination; hematological parameters; clinical chemistry parameters except for total bile acids; urinalysis; and relative and absolute organ weights. ↓ bw gains in males and females receiving 25,000 or 50,000 ppm; food intake during first 3 days of study in all treated males and females receiving 25,000 and 50,000 ppm. ↓ total bile acids on days 10, 46, and 89 in all treated males and day 46 in all treated females.	-test article meets JECFA specifications	Curry and Roberts (2008)
Rat (Crl:CD(SD); 20/sex/group)	0, 517, 1,035, 2,055 mg/kg bw/d for males and 0, 511, 1,019, or 2,050 mg/kg bw/d for females (diet)	13 weeks	99.5%	NBSD: hematological, clinical chemistry, or urinalysis parameters; organ weights. No compound-related alterations observed upon macroscopic and microscopic examination.	-test article meets JECFA specifications	Nikiforov and Eapen (2008)
Stevioside						
Rat (F344; 10/sex/group)	0, 0.31, 0.62, 1.25, 2.5, or 5% [0, 155, 310, 625, 1,250, or 2,500 mg/kg bw/d] (diet)	13 weeks	95.6%	NSD: body weight gains; and food intakes. NBSD: hematology, serum biochemistry, and urinalysis parameters; organ weights.	-test article meets JECFA specifications -study article in Japanese	Aze *et al.* (1991)
Rat (Wistar; 10/sex/group)	0, 3, or 5% [0, 1,500, or 2,500 mg/kg bw/d] (diet)	90 days	85%	NSD: body weight gain; food conversion efficiency; or behavior.	-reported steviol glycoside composition is below 95%	Xili *et al.* (1992)

Table A.II-3 Summary of Subchronic Toxicity Studies on Steviol Glycosides and *Stevia* Extracts

Species (Strain)	Dose (route)	Study Duration	Purity	Reported Effects[a]	Comments	Reference
Rabbit (strain NR; 1 male)	2.22 g [total, over entire study] (i.v. or s.c.)	15 days	NR	No signs of toxicity reported.	-lack of information regarding steviol glycoside composition of test article -study article in French -lack of a control group -single test animal	Pomaret and Lavieille (1931)
Chicken (strain not reported; 1 male)	4 g (oral capsules)	2 days				
Chicken (Hisex brown; 4/group)	667 mg/kg diet [78 mg/hen/day, respectively, body weight of hens not reported] (diet)	10 days	96% stevioside; 3% steviolbioside; 0.5% rebaudioside A	NSD: feed consumption; body weight gains; or weights of the eggs produced.	-test article meets JECFA specifications	Geuns *et al.* (2003a)
Chicken (Cobb broiler; 8/group)	667 mg/kg diet [137 mg stevioside/kg bw/day] (diet)	2 weeks				
Stevia Extract						
Rat (SLC-Wistar; 15/sex/group)	Male 0, 0.28, 1.4, or 7% [0, 112, 590, and 2,988 mg pure stevioside/kg bw/d] (diet) Female 0, 0.28, 1.4, or 7% [0, 115, 629, and 3,026 mg pure stevioside/kg bw/d] (diet)	13 weeks	53.1% stevioside	NSD: in food intake; feed efficiency; serum biochemistry; urinalysis; and histopathology. Various increases in decreases in organ weights and body weights were considered to be biologically irrelevant due to lack of clear changes in hematology, serum biochemistry, or histopathological examinations.	-reported steviol glycoside composition is below 95% -study article in Japanese	Akashi and Yokoyama (1975)

Table A.II-3 Summary of Subchronic Toxicity Studies on Steviol Glycosides and *Stevia* Extracts

Species (Strain)	Dose (route)	Study Duration	Purity	Reported Effects[a]	Comments	Reference
Rat (strain not reported; 8 to 10/sex/group)	0, 0.5, or 1 g [approximately 0, 1,250, or 2,500 mg/kg bw/d, based on a 0.4 kg body weight] (diet)	56 days	50% stevioside	↑ creatinine levels in 0.5 g males. ↓ total protein in 0.5 g females; β-globulin in 0.5 g females; P in 1 g females; and LDH activity in all treated females and in 1 g males. NSD: body weight gain; RBC counts; WBC counts; Hb concentration; hematocrit; total serum protein; albumin; α_1-globulin; α_2-globulin; γ-globulin; glucose; triglycerides; total cholesterol; creatinine; urea; Ca^{2+} levels; albumin/globulin ratios; ALP; AST; hepatocytes; Kupffer cells; and areas of portal fibrosis.	-reported steviol glycoside composition is below 95% -study article in Korean	Lee *et al.* (1979)
Chicken (male broiler; 250/group)	0, 0.0085, 0.0425, or 0.085% [approximately 0, 3.8, 19.8, and 38.0 µg/kg bw/d of *Stevia* extract] (diet)	41 days	NR	↓: bw in 0.085% dose group on day 42. NSD: feed intake; feed conversion efficiency; or body weights until day 21.	-lack of information regarding steviol glycoside composition of test article	Wood *et al.* (1996)

↓ = decreased; ↑ = increased; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; bw = body weight; Ca^{2+} = calcium; Cl^- = chloride; Hb = hemoglobin; K^+ = potassium; LDH = lactate dehydrogenase; MCHC = mean cell hemoglobin concentration; Na^+ = sodium; NOAEL = no-observed-adverse-effect level; NR = not reported; NBSD = no biologically significant differences; NSD = no significant differences P = phosphorus; RBC = red blood cell; TP = total protein; WBC = white blood cell.
[a] unless stated otherwise, all reported effects are relative to control group(s).

Table A.II-4 Summary of Developmental and Reproductive Toxicity Studies on Stevioside, *Stevia* Extracts, and Steviol

Species (Strain)	Dose (route)	Study Duration	Purity	Reported Effects[a]	Comments	Reference
Rebaudioside A						
Rat (female HsdRcc: Han Wistar; F_0=6/group, F_1=10/sex/group)	0, 25,000, 37,500, or 50,000 ppm [0, 4,711, 8,021, or 9,484 mg/kg bw/d during first 4 days of treatment; and 0, 6,291, 10,045, or 11,386 mg/kg bw/d during day 17 to 20 of lactation] (diet)	F_0 females: Day 14 to 21 of lactation	97%	NSD: general condition; body weight; body weight gains; food consumption; or incidence of macroscopic abnormalities.	-test article meets JECFA specifications	Curry *et al.* (2008)
	0, 25,000, 37,500, or 50,000 ppm [0, 5,814, 9,849, or 14,076 mg/kg bw/d, males and 0, 5,679, 9,338, or 13,088 mg/kg bw/d, females] (diet)	F_1 : Day 0 to 14 (nursing), day 14 to 35 post-partum (diet)		↓: bw gains and food consumption in groups administered 37,000 or 50,000 ppm NSD: body weight gains or food consumption in 25,000 ppm males and females; testicular morphology (males only), or spermatogenesis (males only).		
Rat (HsdRcc: Han Wistar; 30/sex/group)	0, 7,500, 12,500, or 25,000 ppm [0, 586, 975, or 2,048 mg/kg bw/d, males; 0, 669, 1,115, or 2,273 mg/kg bw/d, pre-mating females; 0, 648-713, 1,119-1,169, or 2,263-2,381 mg/kg bw/d, gestation; and 0, 715-1,379, 1,204-2,388, and 2,602-5,019, lactation] (diet)	F_0 males: 17 weeks F_0 females: Pre-mating, 10 weeks; Mating, up to 3 weeks; gestation, days 1 to 20 after conception; Lactation, days 1 to 21 after parturition (total of approximately 20 weeks)	97%	No adverse effects on bw, bw gains, food consumption, mating performance, fertility, gestation lengths, estrous cycles, spermatogenesis, weight of testis, seminal vesicle, or epididymis, male reproductive system, organ weights. Litter size, pre- and post-natal survival of the offspring, or offspring sex ratio in all generations	-test article meets JECFA specifications	Curry *et al.* (2008)
		F_1 offspring (all offspring): Post-partum day 1 to 21 (nursing). Unselected F_1 offspring: killed at 30 days of age				

Table A.II-4 Summary of Developmental and Reproductive Toxicity Studies on Stevioside, *Stevia* Extracts, and Steviol						
Species (Strain)	**Dose (route)**	**Study Duration**	**Purity**	**Reported Effects[a]**	**Comments**	**Reference**
	0, 7,500, 12,500, or 25,000 ppm [0, 734, 1,254, or 2,567 mg/kg bw/d, males; 0, 798, 1,364, or 2,768 mg/kg bw/d, pre-mating females; 0, 562-625, 900-1,058, or 2,036-2,212 mg/kg bw/d, gestation; and 0, 976-1,406, 1,752-2,394, and 3,289-4,893, lactation]	F_1 parental males: 17 weeks F_1 parental females: Pre-mating, 10 weeks; Mating, up to 3 weeks; Gestation, days 1 to 20 after conception; Lactation, days 1 to 21 after parturition (total of approximately 20 weeks) F_2 generation: Post-partum day 1 to 21 (nursing). Killed at 30 days of age				
Stevioside						
Rat (female Wistar; 21 to 24/group)	0, 250, 500, or 1,000 mg/kg bw/d (drinking water)	Day 6 to 15 of gestation	95.6%	NSD: body weight, food consumption, fetal malformations; or toxicity signs in pregnant rats and fetuses	- test article meets JECFA specifications	Usami *et al.* (1995)
Rat (Sprague-Dawley; 5/sex/group)	0, 0.69, 0.35, or 0.15% [0, 83.4, 80.0, or 84.9 mg/kg bw/d, males; 96.2, 101.7, or 101.2 mg/kg bw/d, females] (diet)	21 days prior to mating	20 to 95%	NSD: behavior, bw, feed intake, pregnancy rate, or fetal condition.	-study article in Japanese	Akashi and Yokoyama (1975)
Rat (Wistar; 22/sex/group)	0, 0.15, 0.75, or 3% [0, 100, 480, and 2,100 mg/kg bw/d for males and 0, 120, 530, 2,100 mg/kg bw/d for females] (diet)	Male 60 days before and during mating Female 14 days before mating and 7 days during gestation	95.98%	Delayed increase in body weight in early period of administration in highest dose NSD: food and water consumption	-test article meets JECFA specifications	Mori *et al.* (1981)

Table A.II-4 Summary of Developmental and Reproductive Toxicity Studies on Stevioside, *Stevia* Extracts, and Steviol						
Species (Strain)	**Dose (route)**	**Study Duration**	**Purity**	**Reported Effects[a]**	**Comments**	**Reference**
Hamster (golden; *Mesocricetus auratus*; 10/sex/group)	0, 500, 1,000, or 2,500 mg/kg bw/d (gavage, except drinking water for period of late pregnancy and lactation)	3 generations	90%	NSD: growth, fertility, or reproductive tissue of parents; duration of pregnancy; or number of fetuses.	-reported steviol glycoside composition is below 95%	Yodyingyuad and Bunyawong (1991)
Chicken (broiler embryo, 7 days of incubation)	0, 0.08, 0.8, or 4.0 mg/egg (injection)	Single dose	>96%	NSD: egg weight, embryonic mortality, body weight, or organ weights. No malformations observed.	-non-oral route of exposure	Geuns *et al.* (2003c)
Stevia Extract						
Mouse (female HaM/ICR; 6 to 7/group)	Study 1 and 2 0 or 200 to 250 mg/kg bw/d (gavage) Study 3 0, 200 to 250, or 1,000 to 1,250 mg/kg bw/d (gavage)	Study 1 8 days during copulation period Study 2 6 days prior to copulation Study 3 12 days prior to copulation (after first pregnancy)	NR	Study 1 ↑: resorption. ↓: ratio of the number of implants per pregnancy NSD: in number of implantation Study 2 ↓: ratio of the number of implant per pregnant mouse NSD: in fertility rate and number of total implants. Study 3 ↑: number of offspring per pregnant mouse during second mating ↓: Incidence of pregnancy after first mating; number of offspring/pregnancy in low dose group in third pregnancy NSD: number of offspring/pregnancy in high group in third pregnancy	-lack of information regarding the steviol glycoside composition of the test article -study article in Portuguese	Nunes and Pereira (1988)
Rat (Wistar; number/group NR)	0 or 5% [0 or 5,000 mg/kg bw/d] (drinking water)	Female before and during mating period (exact length NR)	NR	NSD: birth index; or average litter size.	-lack of information regarding the steviol glycoside composition of the test article	Shiotsu (1996)

Table A.II-4 Summary of Developmental and Reproductive Toxicity Studies on Stevioside, *Stevia* Extracts, and Steviol

Species (Strain)	Dose (route)	Study Duration	Purity	Reported Effects[a]	Comments	Reference
		Male during mating period (exact length NR)			-study article in Japanese	
Rat (female Albino; 14/group)	0, or 5% [0 or 2,000 mg/kg bw/d] (drinking water)	18 days	NR	↓ fertility. NSD: food intake, or health of rats.	-lack of information regarding the steviol glycoside composition of the test article	Mazzei-Planas and Kuc (1968)
Rat (male Wistar; 30/treatment group; 20/control group)	0 or 14.58 g of dried leaves/kg bw/d (gavage)	60 days	NR	↓ seminal vesicle weights. NSD: body weights; testis, submandibular gland, or adrenal weights; serum levels of T_3; zinc levels in the prostate, testis, submandibular gland, and pancreas; and water content of the testis and prostate.	-lack of information regarding the steviol glycoside composition of the test article	Oliveira-Filho *et al.* (1989)
Rat (female Wistar; 8/group)	0, 0.2, 1, or 10% [0, 8.5, 41, or 433 mg/kg bw/d] (oral; presumed to be gavage)	60 days before mating	NR	NSD: number of corpus lutea, implantations, dead fetuses, size of fetus; and female rat reproduction.	-lack of information regarding the steviol glycoside composition of the test article	Saenphet *et al.* (2006)
Rat (prepubertal male Wistar; 10/group)	0 or 6,778 mg/kg bw/d (gavage)	60 days	NR	↓: relative weights of cauda epididymides, seminal vesicle, and testis; concentration of spermatozoa stored in the cauda epididymides, and plasma testosterone levels. ↓: fructose content of prostate and seminal vesicle plus coagulating gland. NSD: histological and morphometric analysis of testis, seminal vesicle, prostate; and cauda epididymidis.	-lack of information regarding the steviol glycoside composition of the test article	Melis (1999a)

Table A.II-4 Summary of Developmental and Reproductive Toxicity Studies on Stevioside, *Stevia* Extracts, and Steviol						
Species (Strain)	**Dose (route)**	**Study Duration**	**Purity**	**Reported Effects[a]**	**Comments**	**Reference**
Steviol						
Hamster (female golden Syrian; *Mesocricetus auratus*; 20/group; highest-dose and positive control groups, 12 and 6/group, respectively)	0, 250, 500, 750, or 1,000 mg/kg bw/d (gavage)	Days 6 to 10 of gestation	90%	↑: maternal mortality rate; incidence of dilation and hyaline formation on the convoluted tubules of maternal kidneys in 3 highest dose groups. ↓: maternal body weight gain; mean fetal weight; and number of live fetuses/litter in 3 highest dose groups. NSD skeletal and visceral development of offspring.	-doses of steviol available for systemic absorption are much greater than what would be available if a steviol glycoside was administered in the diet	Wasuntarawat *et al.* (1998)
Chicken (broiler embryo, 7 days of incubation)	0.025, 0.25. 1.25 mg/egg (injection)	Once	98%	NSD egg weight, embryonic mortality, bw; or organ weight. No malformations observed.	-non-oral route of exposure	Geuns *et al.* (2003c)

↓ = decreased; ↑ = increased; bw = body weight; F_0 = first generation; F_1 = second generation; F_2 = third generation; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NOAEL = *no observable adverse effect level*; NR = *not reported*; NSD = *no significant difference*; T_3 = triiodothyronin; T_4 = thyroxine.
[a] unless stated otherwise, all reported effects are relative to control group(s)

Table A.II-5 *In vitro* Studies of the Genotoxicity of Steviol Glycosides and *Stevia* Extracts

Endpoint	Test object	Test material (purity)	Concentration/dose	Results	Reference
Forward mutation	*Salmonella typhimurium* TM677	Rebaudioside A (NS)	0.1-10.0 mg/plate[a]	Negative	Pezzuto *et al.* (1985)
Forward mutation	*S. typhimurium* TM677	Rebaudioside A (NS)	NS[a]	Negative	Medon *et al.* (1982)
Chromosome aberration	Chinese hamster lung fibroblasts (CHL/IU)	Rebaudioside A (NS)	1.25-5 mg/mL[a]	Negative	Nakajima, (2000a)
Reverse mutation	*S. typhimurium* TA98, TA100	Stevioside (99%)	12.5-50 mg/plate[a]	Negative[b]	Suttajit *et al.* (1993)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104	Stevioside (83%)	0.05-5 mg/plate[c]	Negative	Matsui *et al.* (1996a)
			0.05-1 mg/plate[d]		
	S. typhimurium TA1535, TA1537		0.05-1 mg/plate[c]		
	Escherichia coli WP2*uvrA*/pkM101				
Reverse mutation	*S. typhimurium* TA98, TA100	Stevioside (NS)	12.5-50 mg/plate[a]	Negative	Klongpanichpak *et al.* (1997)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Stevioside (18%)	0.01-10 mg/plate[a]	Negative	Okumura *et al.* (1978)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Stevioside (55%)	0.01-10 mg/plate[a]	Negative	Okumura *et al.* (1978)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Stevioside (95-98%)	0.01-10 mg/plate[a]	Negative	Okumura *et al.* (1978)
Forward mutation	*S. typhimurium* TM677	Stevioside (83%)	0.1-10 mg/plate[a]	Negative	Matsui *et al.* (1996a)
Forward mutation	*S. typhimurium* TM677	Stevioside (NS)	0.1-10 mg/plate[a]	Negative	Pezzuto *et al.* (1985)
Forward mutation	*S. typhimurium* TM677	Stevioside (NS)	NS[a]	Negative	Pezzuto *et al.* (1983)
Forward mutation	*S. typhimurium* TM677	Stevioside (NS)	NS[a]	Negative	Medon *et al.* (1982)
umu gene mutation	*S. typhimurium* TA1535/pSK1002	Stevioside (83%)	1.25-5 mg/mL[a]	Negative	Matsui *et al.* (1996a)
Gene mutation	*Bacillus subtilis* H17 rec$^+$, M45 rec$^-$	Stevioside (83%)	10 mg/disc[a]	Negative	Matsui *et al.* (1996a)
Gene mutation	*B. subtilis* H17 rec$^+$, M45 rec$^-$	Stevioside (18%)	0.02-2 mg/disc[e]	Negative	Okumura *et al.* (1978)
Gene mutation	*B. subtilis* H17 rec$^+$, M45 rec$^-$	Stevioside (55%)	0.02-2 mg/disc[e]	Negative	Okumura *et al.* (1978)

Table A.II-5 *In vitro* Studies of the Genotoxicity of Steviol Glycosides and *Stevia* Extracts

Endpoint	Test object	Test material (purity)	Concentration/dose	Results	Reference
Gene mutation	*B. subtilis* H17 rec^{+}, M45 rec^{-}	Stevioside (95-98%)	0.02-2 mg/disc[e]	Negative	Okumura *et al.* (1978)
Gene mutation	Mouse lymphoma L5178Y cells, Tk$^{+/-}$ locus	Stevioside (96.8%)	1.25-5 mg/mL[a,e]	Negative	Oh *et al.* (1999a,b)
Chromosome aberration	Chinese hamster lung fibroblasts	Stevioside (85%)	12 mg/mL[c]	Negative	Ishidate *et al.* (1984)
Chromosome aberration	Chinese hamster lung fibroblasts	Stevioside (83%)	2-12 mg/mL[a]	Negative	Matsui *et al.* (1996a)
Chromosome aberration	Chinese hamster D-6 cells	Stevioside (NS)	0.5-1%[e] (5-10 g/mL)[f]	Negative	Nadamitsu *et al.* (1985)
			2-4%[e] (20-40 g/mL)[f]	Positive	
Chromosome aberration	Human lymphocytes	Stevioside (NS)	10 mg/mL	Negative	Suttajit *et al.* (1993)
Chromosome aberration	Human lymphocytes	Stevioside (NS)	0.008-8 µg/mL[e]	Negative	Höhn and Zankl, (1990)
Sister chromatid exchange	Chinese hamster D-6 cells	Stevioside (NS)	0.5-1%[e] (5-10 g/mL)[f]	Negative	Nadamitsu *et al.* (1985)
			2-4%[e] (20-40 g/mL)[f]	Positive	
Sister chromatid exchange	Human lymphocytes	Stevioside (NS)	0.008-8 µg/mL[e]	Negative	Höhn and Zankl, (1990)
Sister chromatid exchange	Human lymphocytes	Stevioside (NS)	7.5 µg/mL	Negative	Flores *et al.* (1987)
Micronucleus formation	Human lymphocytes	Stevioside (NS)	0.008-8 µg/mL[e]	Positive	Höhn and Zankl, (1990)
Forward mutation	*S. typhimurium* TM677	Rebaudioside B (NS)	NS[a]	Negative	Medon *et al.* (1982)
Forward mutation	*S. typhimurium* TM677	Rebaudioside C (NS)	0.1-10 mg/mL[a]	Negative	Pezzuto *et al.* (1985)
Forward mutation	*S. typhimurium* TM677	Rebaudioside C (NS)	NS[a]	Negative	Medon *et al.* (1982)
Forward mutation	*S. typhimurium* TM677	Dulcoside A (NS)	1.0-10 mg/mL[a]	Negative	Pezzuto *et al.* (1985)
Forward mutation	*S. typhimurium* TM677	Dulcoside A (NS)	NS[a]	Negative	Medon *et al.* (1982)
Forward mutation	*S. typhimurium* TM677	Steviolbioside (NS)	0.1-10 mg/mL[a]	Negative	Pezzuto *et al.* (1985)

Table A.II-5 *In vitro* Studies of the Genotoxicity of Steviol Glycosides and *Stevia* Extracts					
Endpoint	**Test object**	**Test material (purity)**	**Concentration/dose**	**Results**	**Reference**
Forward mutation	*S. typhimurium* TM677	Steviolbioside (NS)	NS[a]	Negative	Medon *et al.* (1982)
Micronucleus formation	Human buccal mucosal epithelium	*Stevia* extract – *Stevia* sweet (NS)	NS	Positive	Höhn and Zankl, (1990)

NS = not specified
[a] With and without metabolic activation.
[b] A positive response in TA98 without metabolic activation at 50 mg/plate was reported, but not at lower concentrations of up to 20 mg/plate.
[c] Without metabolic activation.
[d] With metabolic activation.
[e] Inadequate detail provided.
[f] Based on an assumed density of 1 g/mL.

Table A.II-6 *In vitro* Studies on the Genotoxicity of Steviol and Steviol Derivatives

Endpoint	Test object	Test material (purity)	Concentration/ dose	Results	References
Reverse mutation	*Salmonella typhimurium* TA98, TA100	Steviol (NS)	20 mg/plate[a]	Negative	Suttajit *et al.* (1993)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104	Steviol (99%)	0.05-5 mg/plate[a]	Negative	Matsui *et al.* (1996a)
Reverse mutation	*S. typhimurium* TA1535, TA1537,	Steviol (99%)	0.05-5 mg/plate[b]	Negative	Matsui *et al.* (1996a)
	Escherichia coli WP2 *uvrA*/pKM101				
Reverse mutation	*S. typhimurium* TA98, TA100	Steviol (NS)	0.25-2 mg/plate[a]	Negative	Klongpanichpak *et al.* (1997)
Forward mutation	*S. typhimurium* TM677	Steviol (99%)	0.1-10 mg/mL[b]	Negative	Matsui *et al.* (1996a)
			0.1-10 mg/plate[c]	Positive	
Forward mutation	*S. typhimurium* TM677	Steviol (NS)	10 mg/plate[d]	Positive	Pezzuto *et al.* (1986)
Forward mutation	*S. typhimurium* TM677	Steviol (NS)	0.1-10 mg/mL[b]	Negative	Pezzuto *et al.* (1985)
			0.1-10 mg/mL[c,e]	Positive	
Forward mutation	*S. typhimurium* TM677	Steviol (NS)	NS[b]	Negative	Pezzuto *et al.* (1983)
			NS[c]	Positive	
Forward mutation	*S. typhimurium* TM677	Steviol (NS)	NS[c]	Positive	Terai *et al.* (2002)
umu gene mutation	*S. typhimurium* TA1535/pSK1002	Steviol (99%)	625-1,250 µg/plate[a]	Weakly positive	Matsui *et al.* (1996a)
			1,250-2,500 µg/plate[c]		
Gene mutation	*Bacillus subtilis H17 rec*$^{+}$*, M45 rec*$^{-}$	Steviol (99%)	10 mg/disc[a]	Negative	Matsui *et al.* (1996a)
Gene mutation	Chinese hamster lung fibroblasts	Steviol (99%)	0.4 mg/mL[c]	Positive[f]	Matsui *et al.* (1996a)
Gene mutation	Mouse lymphoma L5178Y Tk$^{+/-}$ locus	Steviol (NS)	340 µg/mL[a]	Negative	Oh *et al.* (1999a,b)
Chromosomal aberration	Chinese hamster lung fibroblasts	Steviol (99%)	0.125-5 mg/mL[b] 0.5 mg/mL[c]	Negative	Matsui *et al.* (1996a)
			1.0-1.5 mg/mL[c]	Positive	

Table A.II-6 *In vitro* Studies on the Genotoxicity of Steviol and Steviol Derivatives

Endpoint	Test object	Test material (purity)	Concentration/ dose	Results	References
Chromosomal aberration	Human lymphocytes	Steviol (NS)	0.1-0.2 mg/mL [a]	Negative	Suttajit *et al.* (1993)
DNA damage (comet assay)	Human lymphoblastoid TK6 and WTK1 cells	Steviol (NS)	62.5-500 µg/mL [a]	Negative	Sekihashi *et al.* (2002)
Forward mutation	*S. typhimurium* TM677	Isosteviol (NS)	1.0-10 mg/mL [a]	Negative	Pezzuto *et al.* (1985)
Forward mutation	*S. typhimurium* TM677	Isosteviol (NS)	NS [a]	Negative	Pezzuto *et al.* (1983)
Forward mutation	*S. typhimurium* TM677	Dihydrosteviol A (NS)	1.0-10 mg/mL [a]	Negative	Pezzuto *et al.* (1985)
Forward mutation	*S. typhimurium* TM677	Dihydrosteviol B (NS)	1.0-10 mg/mL [a]	Negative	Pezzuto *et al.* (1985)
Forward mutation	*S. typhimurium* TM677	Steviol-16α,17-epoxide (NS)	1.0-10 mg/mL [a]	Negative	Pezzuto *et al.* (1985)
Forward mutation	*S. typhimurium* TM677	Steviol-16α,17-epoxide (NS)	NS [d]	Positive	Terai *et al.* (2002)
Forward mutation	*S. typhimurium* TM677	Steviol-16β,17β-epoxide, *trans* (NS)	NS [g]	Positive	Pezzuto *et al.* (1983)
Forward mutation	*S. typhimurium* TM677	Steviol-16β,17β-epoxide, *cis* (NS)	NS [h]	Negative	Pezzuto *et al.* (1983)
Forward mutation	*S. typhimurium* TM677	15α-hydroxysteviol (NS)	0.31-7.5 mg/mL [c]	Negative	Compadre *et al.* (1988)
Forward mutation	*S. typhimurium* TM677	15α-hydroxysteviol (NS)	0.31-7.5 mg/mL [a]	Negative	Pezzuto (1986)
Forward mutation	*S. typhimurium* TM677	15α-hydroxysteviol (NS)	NS [a]	Negative	Terai *et al.* (2002)
Forward mutation	*S. typhimurium* TM677	15-oxosteviol (NS)	25-200 µg/mL [b]	Positive	Compadre *et al.* (1988)
Forward mutation	*S. typhimurium* TM677	15-oxosteviol (NS)	25-200 µg/mL [g]	Positive	Pezzuto (1986)
Forward mutation	*S. typhimurium* TM677	15-oxosteviol (NS)	25-200 µg/mL [b]	Negative	Procinska *et al.* (1991)
Forward mutation	*S. typhimurium* TM677	15-oxosteviol (NS)	NS [c]	Weakly Positive	Terai *et al.* (2002)
Forward mutation	*S. typhimurium* TM677	Steviol methylester (NS)	NS [c]	Positive	Terai *et al.* (2002)
Forward mutation	*S. typhimurium* TM677	16-oxo-steviol methylester (NS)	NS [a]	Positive	Terai *et al.* (2002)
Forward mutation	*S. typhimurium* TM677	13,16-seco-13-oxo-steviol methylester (NS)	NS [c]	Positive	Terai *et al.* (2002)

Table A.II-6 *In vitro* Studies on the Genotoxicity of Steviol and Steviol Derivatives					
Endpoint	**Test object**	**Test material (purity)**	**Concentration/ dose**	**Results**	**References**
Forward mutation	*S. typhimurium* TM677	13,16-seco-13α-hydroxy-steviol methylester (NS)	NS[a]	Positive	Terai *et al.* (2002)
Forward mutation	*S. typhimurium* TM677	Steviol methylester-8, 13-lactone (NS)	NS[a]	Positive[i]	Terai *et al.* (2002)
Forward mutation	*S. typhimurium* TM677	*ent*-Kaurenoic acid (NS)	0.625-15 mg/mL[c]	Negative	Pezzuto *et al.* (1986)
Forward mutation	*S. typhimurium* TM677	Steviol acetate (NS)	0.625-15 mg/mL[c]	Negative	Pezzuto *et al.* (1986)

NS = not specified
[a] With and without metabolic activation.
[b] Without metabolic activation.
[c] With metabolic activation.
[d] With metabolic activation derived from human liver microsomes.
[e] Exhibited a positive dose-response relationship.
[f] Diphtheria toxin-resistant colonies.
[g] Authors described test material as a direct-acting mutagen, presumed that metabolic activation was not present.
[h] Inadequate detail provided.
[i] The presence of metabolic activation decreased the mutagenicity

Table A.II-7 *In vivo* Studies on the Genotoxicity of Steviol Glycosides and *Stevia* Extracts

Endpoint	Test object	Test material (purity)	Concentration/dose	Results	References
Micronucleus formation	Male BDF_1 mouse bone marrow	Rebaudioside A (NS)	500-2,000 mg/kg bw once daily for 2 days by gavage[a]	Negative	Nakajima, (2000b)
Mutation	*Drosophila melanogaster* Muller 5 strain	Stevioside (NS)	2% in feed	Negative	Kerr *et al.* (1983)
Chromosome aberration	Wistar rat bone marrow cells	Stevioside (NS)	7.2 mg/kg bw/d for 60 days in the drinking water	Negative	Flores *et al.* (1987)
DNA damage (comet assay)	Male Wistar rat blood cells, liver, brain, spleen cells	Stevioside (88.6%)	4 mg/L in drinking water *ad libitum* for 45 days (~400 to 500 mg/kg bw/d)	Positive	Nunes *et al.* (2007)
Micronucleus formation	ddY[b] mouse bone marrow and regenerating liver cells[c]	Stevioside (96.8%)	62.5-250 mg/kg bw as a single oral administration[d]	Negative	Oh *et al.* (1999a,b)
Micronucleus formation	Wistar rat[e]	Stevioside (NS)	150 mg/kg bw in the drinking water for 60 days	Negative	Flores *et al.* (1987)
DNA damage (comet assay)	Male BDF_1 mouse stomach, liver, colon, kidney, bladder, lung, brain, and bone marrow cells	*Stevia* extract (stevioside, 52%, rebaudioside A, 22%)	250, 500, 1,000 or 2,000 mg/kg bw as a single dose by gavage	Negative	Sekihashi *et al.* (2002)
DNA damage (comet assay)	Male ddY mouse stomach, colon, liver, kidney, bladder, lung, brain, bone marrow cells[f]	*Stevia* (NS)	2,000 mg/kg bw as a single oral dose[d]	Negative	Sasaki *et al.* (2002)
Dominant lethal test	Male Norwegian rat	*Stevia* leaf extract (NS)	0.2, 1, or 10% (14.29-714.29 mg/kg bw/d) for 8 weeks orally[d]	Negative	Aritajat *et al.* (2000)

NS = not specified
[a] Killed 30 hours after second administration
[b] From English abstract; ICR mice described in study.
[c] Animals killed 24 hours after administration.
[d] Route not specified
[e] Animals killed 48, 72, and 96 hours after administration.
[f] Animals killed 3 and 24 hours after administration.

Table A.II-8 *In vivo* Studies on the Genotoxicity of Steviol

Endpoint	Test object	Test material (purity)	Concentration/dose	Results	References
DNA damage (comet assay)	Male BDF_1 mouse stomach, colon, liver cells	Steviol (>99%)	250-2,000 mg/kg bw as a single oral dose[a,b]	Negative	Sekihashi *et al.* (2002)
	Male CRJ:CD1 mouse liver, kidney, colon, and testes cells				
Micronucleus formation	MS/Ae mice	Steviol (99%)	125-1,000 mg/kg bw as a single intraperitoneal injection[c]	Negative	Matsui *et al.* (1996a)
Micronucleus formation	Swiss mouse bone marrow cells	Steviol (approximately 90%)	8,000 mg/kg bw as a single dose by gavage[d]	Negative	Temcharoen *et al.* (2000)
Micronucleus formation	ddY mouse[e] regenerating liver cells	Steviol (NS)	50-200 mg/kg bw as a single oral dose[b]	Negative	Oh *et al.* (1999a,b)
Micronucleus formation	Wistar rat bone marrow cells	Steviol (approximately 90%)	8,000 mg/kg bw as a single dose by gavage[d]	Negative	Temcharoen *et al.* (2000)
Micronucleus formation	Syrian golden hamster bone marrow cells	Steviol (approximately 90%)	4,000 mg/kg bw as a single dose by gavage[d]	Negative	Temcharoen *et al.* (2000)

NS = not specified
[a] Killed at 3 hours and 24 hours.
[b] Exact route not specified.
[c] 4/6 mice at highest dose given intraperitoneally died.
[d] Killed at 24, 30, 48, or 72 hours. Ratio of polychromatic to normochromatic erythrocytes was decreased at later time-point(s) in females.
[e] From English abstract; ICR mice described in study.

Table A.II-9 Summary of Chronic Toxicity and Carcinogenicity Studies on Stevioside and *Stevia* Extract						
Species (Strain)	**Dose (route)**	**Duration**	**Purity**	**Results[a]**	**Comments**	**Reference**
Stevioside						
Rat (male and female F344/DuCrj; 50/sex/group)	0, 2.5 (low), or 5.0% (high) [0, 969, and 1,997 mg/kg bw/d in males; 0, 1,120, and 2,387 mg/kg bw/d in females] (diet)	104 weeks	95.6%	↓ final survival time of high-dose males, bw of high-dose males and females, absolute kidney weights of high-dose males, absolute left ovary weights in high-dose females. ↑ relative brain weights of high-dose females. NSD in mean survival times, food consumption, hematological examinations, or incidences of tumors.	-test article meets JECFA specifications -study article in Japanese	Toyoda *et al.* (1997)
Rat (Weanling male and female Wistar; 45/sex/group)	0, 0.2 (low), 0.6 (mid), or 1.2% (high) [0, 128.5, 367.6, and 748.6 mg/kg bw/d in males; 0, 146.3, 416.2, and 838.9 mg/kg bw/d in females; or 0, 51.4, 147.0, and 299.4 mg steviol equivalents/kg bw/d in males, and 0, 58.5, 166.5, and 335.6 mg steviol equivalents/kg bw/d in females] (diet)	24 months	85%	NSD in bw gain, food consumption, food utilization during first 3 months, or bw or clinical signs of toxicity over the entire study period. NBD in hematology or clinical chemistry parameters.	-reported steviol glycoside composition is below 95% -test article does not meet JECFA specifications	Xili *et al.* (1992)

Table A.II-9 Summary of Chronic Toxicity and Carcinogenicity Studies on Stevioside and *Stevia* Extract

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
Stevia Extract						
Rat (4-week-old male and female F344/DuCrj; 30 or 70/sex/group)	0, 0.1, 0.3, or 1% [0, 50, 150, and 550 mg/kg bw/d, or 0, 18, 136, 194 mg steviol equivalents/kg bw/d] (diet)	22 or 24 months	74.54% stevioside, 16.27% rebaudioside A	↓ growth after 69 weeks, hematological and biochemical parameters after 6 months, but not after 12 months or at study completion. Changes in various absolute and relative organ weights after 6 months of treatment were not accompanied by pathological findings. Enlarged spleens with a pale-yellow appearance and a high leukocyte count observed in control and test groups; animals diagnosed with leukemia (common disorder in aged rats). NSD in general appearance, mortality rates, or pathology.	-reported steviol glycoside composition is below 95% -test article does not meet JECFA specifications	Yamada *et al.* (1985)

↓ = decreased; ↑ = increased; bw= body weight; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NSD = no significant differences
[a] unless stated otherwise, all results compared to control group(s)

Table A.II-10 Summary of Two-Stage Carcinogenicity Studies on Stevioside or *Stevia* Extracts						
Species (Strain)	**Dose (route)**	**Duration**	**Purity**	**Results[a]**	**Comments**	**Reference**
Rebaudioside A						
Rat (6-week-old male F344; 11/group)	0 or 200 ppm [0 or 20 mg/kg bw/d] (diet)	5 weeks	>99.5%	No induction or promotion of colonic ACF with or without treatment of AOM (15 mg/kg bw; injection). ↓ food consumption (statistical significance NR). ↓ mean bw in AOM+Reb A group and absolute and relative liver weights in AOM+Reb A and Reb A-only groups	-test-article meets JECFA specifications	Kawamori *et al.* (1995)
Stevioside						
Mouse (6-week-old female ICR or SENCAR;15/group)	0 or 85 nmol [0 or 68 µg] (topical)	Single application	NR	Inhibition of skin tumor formation after initiation with DMBA or peroxynitrite, followed by promotion with TPA	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure	Konoshima and Takasaki (2002)
Rat (6-week-old male F344/DuCrj and F344)	0 or 5% [0 or 2,500 mg/kg bw/d] (diet)	34 weeks	NR	No promotion of urinary bladder tumors following a 4-week initiation period with 0, 0.01, or 0.05% N-butyl-N-(hydroxybutyl)nitrosamine provided in the drinking water.	-lack of information regarding steviol glycoside composition of test article	Hagiwara *et al.*, (1984); Ito *et al.*, (1984)

Table A.II-10 Summary of Two-Stage Carcinogenicity Studies on Stevioside or *Stevia* Extracts

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
Stevia Extract						
Mouse (female ICR)	0, 0.1 or 1 mg (topical)	Single application	48.9% stevioside, 24.4% rebaudioside A, 9.8% rebaudioside C, 5.6% dulcoside A, and 11.3% unidentified components	↓ average number of skin tumors per mouse following initiation with DMBA and promotion with TPA.	-reported steviol glycoside composition is below 95% -non-oral route of exposure	Yasukawa *et al.* (2002)
	0, 0.008, 0.04, 0.2, or 1 mg (topical)		NR (stevioside)	ID_{50} of inflammation = 291.6 µg/ear	-lack of information regarding purity -non-oral route of exposure	
			NR (rebaudioside A)	ID_{50} of inflammation = 92.2 µg/ear		
			NR (rebaudioside C)	ID_{50} of inflammation = 54.1 µg/ear		
			NR (dulcoside A)	ID_{50} of inflammation = 92.5 µg/ear		
			48.9% stevioside, 24.4% rebaudioside A, 9.8% rebaudioside C, 5.6% dulcoside A, and 11.3% unidentified components	ID_{50} of inflammation = 239.9 µg/ear	-reported steviol glycoside composition is below 95% -non-oral route of exposure	

↓ = decreased; ↑ = increased; ACF = aberrant crypt foci; AOM = azoxymethane; bw= body weight; DMBA = 7,12-dimethylben[a]anthracene; ID_{50} = median inhibitory dose; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NR = not reported; NSD = no significant differences; TPA = 12-O-tetradecanoylphorbol 13-acetate

[a] unless stated otherwise, all results compared to control group(s)

Table A.II-11 Summary of the Effects of Steviol Glycosides, *Stevia* Extracts, and Isosteviol on Glucose Parameters in Animal Studies

Species (Strain)	Dose (route)	Study Duration	Purity	Results[a]	Comments	References
Rebaudioside A						
Rat (GK, 11-week-old male; 12/group)	0 or 25 mg/kg bw/d (diet)	8 weeks; IAGTT performed at end of treatment period	97.8%	↓: plasma glucose at weeks 2, 3, and 5; and blood pressure at week 6. NSD: plasma insulin; plasma glucagon; food consumption; body weights; plasma glucose $AUC_{(0-9)}$; FFA; TG; or total cholesterol. During IAGTT, NSD: plasma glucose; plasma insulin; or plasma glucacon.	-test article meets JECFA specifications	Dyrskog *et al.* (2005b)
Stevioside						
Rat (Zucker, female lean and obese; 5/group)	0, 200 or 500 mg/kg bw/d (gavage)	Single dose; glucose administered 2 hours after, followed by GTT	NR	↓. insulin in lean, high-dose group at 15 and 30 minutes; insulin and glucose-insulin AUCs in high-dose groups; and blood glucose in obese high-dose group at 30 minutes . NSD: plasma glucose in low- and lean, high-dose groups; and insulin responses in low dose groups and obese high dose group.	-lack of information regarding steviol glycoside composition of test article	Lailerd *et al.* (2004)
Rat (male Wistar; normal; 10/group)	0, 0.5, 1, or 5 mg/kg bw/d (gavage)	Single dose; glucose administered 1 hour after, followed by GTT	99%	↓ glucose levels in all groups Began to normalize at 30 minutes, normalization continued up to 90 minutes.	-test article meets JECFA specifications	Chen *et al.* (2005)
Rat (male Wistar; normal and IRI; 8/group)	0, 0.5, 1, or 5 (oral, presumed gavage)	Single dose; glucose administered 90 minutes after, followed by GTT	98.6%	↓: plasma glucose levels in normal high-dose level groups compared to IRI; insulin levels in normal mid- and high-dose groups compared to IRI; and plasma glucose AUCs and glucose-insulin index in all stevioside-treated groups.	-test article meets JECFA specifications	Chang *et al.* (2005)
Rat (male GK and Wistar)	0 or 200 mg/kg bw/d (intravenous)	Single dose	99.6%	↑: blood glucose at 15, 30, and 45 minutes and plasma insulin levels at 30 minutes in Wistar rats compared to control. NSD: blood glucose or plasma insulin responses in GK or Wistar rats.	-non-oral route of exposure	Jeppesen *et al.* (2003)

Table A.II-11 Summary of the Effects of Steviol Glycosides, *Stevia* Extracts, and Isosteviol on Glucose Parameters in Animal Studies

Species (Strain)	Dose (route)	Study Duration	Purity	Results[a]	Comments	References
Rat (male Wistar: #/group not reported)	0 or 200 µmol stevioside or steviol (alone or with fructose) [748.7 mg stevioside/kg bw and 296.2 mg steviol/kg bw] (oral; presumed drinking water)	Single dose (after a 24-hour fast)	NR	↑: glycogen accumulation in stevioside+fructose and stevioside+fructose groups, but more pronounced with stevioside+fructose group.	-lack of information regarding steviol glycoside composition of test article	Hübler *et al.* (1994)
	0, 1.0 or 2.0 mmol/L of stevioside [112.3 to 300 mg/kg bw] or 1.0 mmol/L steviol [44.4 to 118.5 mg/kg bw] (drinking water)	Single dose (during a 24 - or 48- hour fast)		↑: glycogen levels for 24-hour, 2.0 mmol/L and 48- hour 1.0 mmol/L stevioside groups. NSD: glycogen levels in aqueous steviol groups.		
Rat (Wistar; 8/group)	0, 100, 150, or 200 mg/kg bw/h (intravenous)	2 hours	95%	↑: blood glucose levels in lowest dose group at 30 minutes and in mid and highest dose group at all time periods measured over the 2 hours.	-non-oral route of exposure	Suanarunsawat and Chaiyabutr (1997)
Rat (Wistar; 8/group)	0 or 200 mg/kg bw/h (intravenous)	2 hours	95%	↓: glucose carbon recycling and glucose clearance rates. ↑: plasma glucose levels. NSD: plasma glucose turnover rates and plasma insulin level.	-non-oral route of exposure	Suanarunsawat and Chaiyabutr (1997)
Rat (Wistar; 8/group)	0 or 200 mg/kg bw/h (intravenous[b])	2 hours	95%	↑: blood glucose in all groups except the insulin dosed group.	-non-oral route of exposure	Suanarunsawat and Chaiyabutr (1997)

Table A.II-11 Summary of the Effects of Steviol Glycosides, *Stevia* Extracts, and Isosteviol on Glucose Parameters in Animal Studies

Species (Strain)	Dose (route)	Study Duration	Purity	Results[a]	Comments	References
Rat (GK and Wistar; 12 to 14/group)	0 or 200 mg/kg bw/d (intravenous)	2 hours	96%	↓: glucagon levels, response to glucose and IAUC in GK rats. ↑: blood insulin levels in GK rats; insulin levels in Wistar rats at 15 minutes but returned to normal at 90 minutes. NSD: blood glucose and glucagon levels in Wistar rats.	-non-oral route of exposure	Jeppesen *et al.* (2002)
Rat (Wistar; 8/group)	0 or 4,000 mg/kg bw/d in 2 doses (oral)	6 hours	95%	NSD: blood glucose levels.	-test article meets JECFA specifications	Suanarunsawat and Chaiyabutr (1997)
Rat (male Wistar; normal and STZ-induced IDDM; 10/group)	0 or 1, 2, or 10 in 2 doses (gavage)	1 day	99%	↓: blood glucose in normal and STZ-induced IDDM rats. ↑: blood insulin in normal rats.	-test article meets JECFA specifications	Chen *et al.* (2005)
Rat (male Wistar; STZ-induced diabetes; 8/group)	0 or 5 mg/kg bw/d (oral, presumed gavage)	10 days	98.6%	↑: plasma glucose lowering activity of insulin in stevioside treated rats.	-test article meets JECFA specifications	Chang *et al.* (2005)
Rat (male Wistar; STZ-induced IDDM and NIDDM; 10/group)	0, 1, 2, or 10 in 2 doses (gavage)	15 days	99%	↓: blood glucose within 1 day for STZ-induced IDDM at all dose levels and NIDDM at high-dose level, within 5 days for NIDDM at all other dose levels. ↑: plasma glucose lowering activity of tolbutamide in all stevioside NIDDM groups.	-test article meets JECFA specifications	Chen *et al.* (2005)
Rat (Wistar; #/group not reported)	0 or 20 mg/kg bw/d (subcutaneous, alone or combined with MKC)	15 days	NR	↓: blood glucose levels beginning on day 4 in MKC only and stevioside only group. Greater hypoglycemic effects observed in MKC and Stevioside combined group compared to individual administration.	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure	Raskovic *et al.* (2005)

Table A.II-11 Summary of the Effects of Steviol Glycosides, *Stevia* Extracts, and Isosteviol on Glucose Parameters in Animal Studies

Species (Strain)	Dose (route)	Study Duration	Purity	Results[a]	Comments	References
Rat (male Wistar; normal and IRI; 8/group)	0 or 5 mg/kg bw/d (oral, presumed gavage)	28 days	98.6%	↓: body weight and plasma glucose in IRI; and ability of tolbutamide to decrease plasma glucose in IRI rats by day 6 and normal rats by day 12.	-test article meets JECFA specifications	Chang *et al.* (2005)
Rat (male; strain and #/group not reported)	0, 50, or 100 mg/kg bw/d (high-fat or high-carbohydrate diet)	4 weeks	NR	↓: absolute and relative liver glycogen content in high- carbohydrate diet groups (both dose levels). ↑: relative thyroid gland weight in high-carbohydrate diet group (low-dose level) at 4 weeks. NSD: food efficiency, blood glucose levels, relative liver, thyroid or adrenal weights in high-fat and high-carbohydrate diet groups at high-dose level and high-carbohydrate diet group at low-dose level at 2 weeks.	-lack of information regarding steviol glycoside composition of test article -study article in Japanese	Suzuki *et al.* (1977)
Rat (20-week-old male GK; 12/group)	0 or 30 mg/kg bw/d (high-carbohydrate chow diet or 20% chow + 80% soy protein isolate diet)	5 weeks; IAGTT performed at 4 weeks	NR	↓: $IAUC_{(0\text{-}240\ min)}$ for glucose and $AUC_{(0\text{-}240\ min)}$ plasma glucagon in both diet groups; total plasma cholesterol and plasma TGs at 5 weeks in both diet groups; and systolic blood pressure at 4 weeks in both diet groups. ↑: the $IAUC_{(0\text{-}30\ min)}$ for first-phase plasma insulin. NSD: $IAUC_{(0\text{-}240\ min)}$ for plasma insulin; body weight.	-lack of information regarding steviol glycoside composition of test article	Jeppesen *et al.* (2006b)
Rat (male GK; 20/test group, 10/control group)	0 or 25 mg/kg bw/d (drinking water)	6 weeks	99.6%	↓ IAUC plasma glucose and TAUC glucagon compared to control after GTT. ↑ IAUC plasma insulin from 30 to 60 minutes after GTT compared to control.	-test article meets JECFA specifications	Jeppesen *et al.* (2003)
Rat (male ZDF; 8-week-old; 12/group)	0 or 30 mg/kg bw/d (diet)	10 weeks	91% stevioside, 4% rebaudioside A, 5% other glycosides	↓ BP 2 weeks after study initiation. NSD: fasting plasma glucose, insulin, or glucagon levels. After IAGTT, plasma glucose, insulin, or glucagon.	-test article meets JECFA specifications	Dyrskog *et al.* (2005a)

Table A.II-11 Summary of the Effects of Steviol Glycosides, *Stevia* Extracts, and Isosteviol on Glucose Parameters in Animal Studies

Species (Strain)	Dose (route)	Study Duration	Purity	Results[a]	Comments	References
Rabbit (male chinchilla, diabetes induced; #/group not reported)	0 or 200 mg/kg bw/d (oral)	1 month; GTT performed at end of treatment period	NR	↓: glucose levels; AUC for glucose; or NEFA, TG, total and LDL-C or plasma TBARS. ↑: HDL-C and TAA.	-lack of information regarding steviol glycoside composition of test article	Gorbenko *et al.* (2005)
Stevioside and Stevia Extracts						
Mouse (NMRI-Haan; normal and AIDM; 6/group)	0, 20 mg stevioside/kg bw/d, or 200 mg *Stevia* extract/kg bw/d (intraperitoneal)	4 days; GTT and adrenaline tests performed at end of treatment period	NR	↑: blood glucose in groups treated with adrenaline and *Stevia*, and 1 or 2 doses of alloxan and adrenaline and *Stevia* compared to the *Stevia* alone group; and blood glucose levels in stevioside plus alloxan and adrenaline group.	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure	Raskovic *et al.* (2004a)
Mouse (NMRI-Haan; 6/group)	0, 20 mg stevioside/ kg bw/d, or 200 mg *Stevia* extract/kg bw/d (intraperitoneal)	4 days	NR	↑: blood glucose levels only in MKC and stevioside-treated group.	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure	Raskovic *et al.* (2004b)
Mouse (NMRI-Haan; 18/group)	20 mg stevioside/ kg bw/d or 200 mg *Stevia* extract/kg bw/d (intraperitoneal)	5 treatments over 4 days; GTT and adrenaline tests performed at end of treatment period	NR	↓: blood glucose levels in MKC and *Stevia* combined group compared to *Stevia*-only. ↑: blood glucose levels in *Stevia*-only group administered with adrenaline. NSD: blood glucose levels in MKC and *Stevia* combined group administered with adrenaline or stevioside groups administered with glucose or adrenaline.	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure	Raskovic *et al.* (2004b)

Table A.II-11 Summary of the Effects of Steviol Glycosides, *Stevia* Extracts, and Isosteviol on Glucose Parameters in Animal Studies

Species (Strain)	Dose (route)	Study Duration	Purity	Results[a]	Comments	References
Mouse (strain and #/group not reported)	0, 20 mg stevioside/ kg bw/d, or 200 mg *Stevia* extract/kg bw/d (not reported)	4 days; GTT and adrenaline tests performed at end of treatment period	NR	↓: blood glucose levels before GTT and adrenaline tests performed. ↑: blood glucose levels after GTT and adrenaline tests in *Stevia* treatment group. NSD: blood glucose levels in stevioside treatment group.	-lack of information regarding steviol glycoside composition of test article -lack of information regarding route of exposure	Raskovic *et al.* (2006)
Rat (male Wistar; #/group not reported)	0, 5.5 mg stevioside/kg bw/d or 20 mg *Stevia* extract/kg bw/d (gavage)	15 days; followed by a 15-hour fasting period	NR	↓: glucose levels in rats treated with *Stevia* (compared to controls); and glucose AUC values when livers from *Stevia*-treated rats were perfused with L-alanine, L-lactate, and L-glutamine. NSD: glucose levels in rats treated with stevioside (compared to controls); glucose AUC values when livers from *Stevia*-treated rats were perfused with glycerol; and glucose production or glucose AUC values when livers from stevioside-treated rats where perfused.	-lack of information regarding steviol glycoside composition of test article	Ferreira *et al.* (2006)
Stevia Extract						
Rabbit (7-month-old chinchilla; normal and AIDM; 6/group)	0 or 12 mg/kg bw/d (gavage)	Single dose	NR	↑: plasma glucose levels in AIDM rabbits. NSD: plasma glucose levels in normal rabbits.	-lack of information regarding steviol glycoside composition of test article -study article in Portuguese	von Schmeling *et al.* (1977)

Table A.II-11 Summary of the Effects of Steviol Glycosides, *Stevia* Extracts, and Isosteviol on Glucose Parameters in Animal Studies

Species (Strain)	Dose (route)	Study Duration	Purity	Results[a]	Comments	References
Isosteviol						
Rat (male Wistar; 7 to 8/group; and male and female ZDF; 7 to 9/group)	0 or 10 mg/kg bw/d (intravenous; Wistar rats) 0, 1, 5, or 10 mg/kg bw/d (intravenous; ZDF rats)	Single dose; GTT performed at end of study	99.4%	↓. plasma glucose and AUC in ZDF in 2 highest dose groups between 15 and 60 minutes; plasma glucose in ZDF in lowest dose group at 30 minutes. NSD: AUC in ZDF in lowest dose group at 30 minutes and Wistar; plasma insulin in all ZDF and Wistar; plasma glucose in Wistar.	-steviol glycosides are not metabolized to isosteviol *in vivo*; therefore little to no exposure to isosteviol would be expected in humans -non-oral route of exposure	Ma *et al.* (2007)

↓ = decreased; ↑ = increased; AIDM = alloxan-induced diabetes mellitus; AUC = area under the curve; FFA = free fatty acids; GK = Goto-Kakizaki; GTT = glucose tolerance test; HDL-C = high-density lipoprotein cholesterol; IAGTT = intra-arterial glucose tolerance test; IAUC = incremental area under the curve; IDDM = insulin-dependent diabetic; IRI = insulin-resistance induced; LDL-C = low-density lipoprotein cholesterol; MKC = sodium monoketocholate; mRNA = mitochondrial ribonucleic acid; NEFA = non-esterified free fatty acids; NIDDM = non-insulin dependent diabetes mellitus; NSD: no significant difference; PEPCK = phosphoenol pyruvate carboxykinase; STZ = streptozotocin; TAA = total antioxidant activity; TAUC = total area under the curve; TBARS = thiobarbituric acid reactive substances (lipid peroxidation intermediates); TG = triglyceride; ZDF = Zucker diabetic fatty

[a] all results are statistically significant and compared to the respective control group unless otherwise noted.

[b] infused alone or preceded by various drugs including prazosin, angiotensin II, arginine, indomethacin, insulin

Table A.II-12 Summary of Effects of Stevioside on Blood Pressure in Animals

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
Rat (male WKY, SH, RH, or DH; 6 to 11/group)	100 mg/kg bw (i.p.)	Single dose	NR	↓ SBP in all groups compared to pre-treatment values. NSD in HR.	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure -lack of a control group	De-Yi *et al.* (1990)
Rat (male SH; 13/group)	0.1% [171 mg/kg bw/d] (drinking water)	14 days		↓ BP by 23±4 to 44±6 mmHg compared to pre-treatment values.	-lack of information regarding steviol glycoside composition of test article -lack of a control group	
Rat (conscious male SH; 8/group)	50, 100, or 200 mg/kg bw (i.v.)	Single dose	95%	↓ SBP and DBP in a dose-dependent manner compared to baseline NSD in HR or other abnormalities (no further details provided)	-non-oral route of exposure -lack of a control group	Chan *et al.* (1998)
Rat (anesthetized male SH; 8/group)	100 mg/kg bw (i.v.)			NSD in plasma levels of norepinephrine, epinephrine, and dopamine.		
Rat (SH; sex and strain NR; 8/group)	0 or 25 mg/kg bw (i.p.)	Single dose	95%	Maximal hypotensive effect achieved 60 minutes after administration. ↓ in MAP from 186.2±3.6 to 167±3 mmHg (statistical significance NR)	-non-oral route of exposure	Lee *et al.* (2001)

Table A.II-12 Summary of Effects of Stevioside on Blood Pressure in Animals

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
Rat (male WKY, SH, RH, or DH; 10/group)	0,50, or 100 mg/kg bw (i.p.)	Single dose	NR	↓ SBP between 30 and 90 minutes in WKY rats and between 20 and 120 minutes in hypertensive rats compared to baseline.	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure	Hsu *et al.* (2002)
Rat (male WKY or SH; 10/group)	100 or 200 mg/kg bw (i.p.)			↓ SBP dose-dependently compared to baseline. NSD in HR.		
Rat (male SH; 10/group)	0, 100, 200, or 400 mg/kg bw/d (i.p.)	10 days (treatment period); 4 days (recovery period)		↓ SBP dose-dependently compared to baseline; SPB returned to baseline during recovery period. NSD in HR or bw		
Rat (male SH; 10/group)	0 (control) or 0.1% stevioside [100 mg/kg bw/d] (drinking water)	14 weeks	NR	↓ SBP from 8 to 18 weeks of age and remained ↓ from 20 to 28 weeks of age despite withdrawal during that period of time	-lack of information regarding steviol glycoside composition of test article	
Rat (12-week-old male SH; 13/group)	0 or 0.1% [0 or 200 to 250 mg/kg bw/d] (drinking water)	2 weeks (treatment period); 3 days (recovery period)		↓ SBP throughout treatment period and during first 2 days of recovery period. NSD in HR, or bw throughout treatment or recovery periods or SBP by third day of recovery period		
Rat (male GK; 20/test group, 10/control group)	0 or 25 mg/kg bw/d (drinking water)	6 weeks	99.6%	↓ SBP and DBP throughout study period. ↓ SBP/DBP after 6-week treatment period.	-test article meets JECFA specifications	Jeppesen *et al.* (2003)

Table A.II-12 Summary of Effects of Stevioside on Blood Pressure in Animals

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
Dog (healthy male and female mongrel; number/sex NR; 8/group)	Group 1: 200 mg/kg bw (nasogastric feeding) Group 2: 50 mg/kg bw (i.v.) Group 3: RH; 10, 20, 40, 80, or 160 mg/kg bw (i.v.) Group 4: 5 mg/kg bw (i.v.)	Single dose	NR	↓ SBP, DBP, and MAP between 60 and 120 minutes post-dose administration in Group 1 compared to baseline, but returned to baseline values after 180 minutes. ↓ SBP, DBP, and MAP in Group 2. ↓ SBP, DBP, and MAP dose-dependently in Group 3. NSD in SBP, DBP, or MAP in Group 4	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure -lack of a control group	Liu *et al.* (2003)

↓ = decreased; BP = blood pressure; bw = body weight; DH = deoxycorticosterone acetate hypertensive; DBP= diastolic blood pressure; GK = Goto-Kakizaki; HR = heart rate; i.p. = intraperitoneal; i.v. = intravenous; MAP = mean arterial pressure; NR = not reported; NSD = no significant differences; RH = renal hypertensive; SBP = systolic blood pressure; SH = spontaneously hypertensive; WKY = Wistar-Kyoto

[a] unless stated otherwise, all results compared to control group(s)

Table A.II-13 Summary of Stevioside and *Stevia* Extract Effects on Renal Function in Animal Studies

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
Stevioside						
Normotensive rat (strain not specified)	0.32 or 0.4 mg/mL (infused into renal artery)	Duration of infusion not specified	NR	↑ V and Na^+ excretion No effect on MAP, RPF, GFR	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure -lack of a control group	Chatsudthipong and Thongouppakarn (1995)
Rat (Wistar)	4, 8, 12, or 16 mg/kg bw/h (intravenous)	30 min	NR	C_S higher than C_{in} and lower than C_{PAH} ↑ RPF; ↑ V and Na^+ excretion in 3 highest dose groups compared to control period NSD GFR	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure -lack of a control group	Melis (1992a)
Rat (male Wistar)	4, 8, 12, or 16 mg/kg bw/h (intravenous)	30 min	95%	↓ MAP; ↑ K+ excretion compared to control period ↑ RPF, and V and Na^+ excretion in 3 highest dose groups compared to control period NSD GFR indomethacin reversed all effects	-non-oral route of exposure -lack of a control group	Melis and Sainati (1991a)
Rat (albino, strain not specified)	16 mg/kg bw/h (intravenous)	30 min of stevioside followed by 30 min of verapamil	NR	↓ MAP; ↑ C_{PAH}, and Na^+ and K^+ excretion compared to control period similar effects when order of stevioside and verapamil was reversed	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure -lack of a control group	Sainati *et al.* (1986)

Table A.II-13 Summary of Stevioside and *Stevia* Extract Effects on Renal Function in Animal Studies

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
Rat (male Wistar)	8 or 16 mg/kg bw/h (intravenous)	30 min (2 of 4 groups were then administered verapamil or $CaCl_2$ for 30 min)	NR	↑ V, Na^+, and K^+ excretion and RPF; ↓ MAP in stevioside only groups compared to control period NSD GFR verapamil enhanced effects, $CaCl_2$ inhibited effects	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure -lack of a control group	Melis and Sainati (1991b)
Rat (male Wistar)	0 or 16 mg/kg bw/h (intravenous)	30 min (followed by verapamil or $CaCl_2$)	NR	↓ MAP; ↑ RPF, V, and Na^+ excretion verapamil enhanced effects; $CaCl_2$ inhibited effects of stevioside	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure	Melis (1992b)
Rat (male Wistar; normotensive and hypertensive)	16 mg/kg bw/h (intravenous)	30 min	>90%	↑ C_{PAH}, and V, Na^+, and K^+ excretion; ↓ MAP in both normotensive and hypertensive rat compared to control period ↑ GFR in hypertensive rats compared to control period NSD GFR in normotensive rats	-reported steviol glycoside composition is below 95% -non-oral route of exposure -lack of a control group	Melis (1992c)
Dog (strain not specified)	0 or 26.1 mg/kg bw (intravenous)	Single dose (followed by 6 h of monitoring)	NR	NSD whole blood, plasma, or renal function parameters	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure	Krejci and Koechel (1992)
Dog (strain not specified)	0 or 6 mg/kg bw (intravenous)	Single dose	NR	↑ MAP 5 minutes post-dosing, returned to baseline values 10 minutes post-dosing NSD respiratory function, urine output, blood and plasma pH, osmolality, Na^+, K^+, and creatinine	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure -study article in Portuguese	Chagas *et al.* (1990)
	0 or 6 mg/kg bw/d (oral)	10 days				

Table A.II-13 Summary of Stevioside and *Stevia* Extract Effects on Renal Function in Animal Studies

Species (Strain)	Dose (route)	Duration	Purity	Results[a]	Comments	Reference
Stevia extract						
Rat (male Wistar; antidiuretic and diuretic)	0.5 mg/min/kg bw (intravenous)	30 min	NR	↑ V/GFR, fractional Na^+ and K^+ excretion, solute clearance, and reabsorption of water by the collecting duct in antidiuretic rats compared to control period NSD MAP, GFR, or effective renal plasma flow in antidiuretic rats ↑ clearance of free water in water diuretic rats compared to control period	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure -lack of control group	Melis (1999b)
Rat (male Wistar)	0 or 2 mL/rat (gavage, twice daily)	20, 40, or 60 days	NR	NSD in renal function or MAP following 20 days ↑ V and Na^+ excretion; ↓ MAP following 40 and 60 days ↑ RPF following 60 days NSD RPF following 20 or 40 days NSD GFR in all groups	-lack of information regarding steviol glycoside composition of test article	Melis (1995)
Rat (male Wistar; normotensive and hypertensive)	0 or 2 mL/rat (gavage, twice daily)	30 days	NR	↑ RPF and V, Na^+, and K^+ excretion; ↓ MAP ↑ GFR in hypertensive rats NSD GFR in normotensive rats	-lack of information regarding steviol glycoside composition of test article	Melis (1996)
Dog (strain not specified)	0 or 10% (intravenous)	Single dose	NR	↑ MAP 5 minutes post-dosing, returned to baseline values 10 minutes post-dosing NSD: respiratory function, urine output, blood and plasma pH, osmolality, Na^+, K^+, and creatinine	-lack of information regarding steviol glycoside composition of test article -non-oral route of exposure -study article in Portuguese	Chagas *et al.* (1990)
	0 or 10% (oral; approximately 5,000 mg/kg bw/day)	10 days				

↓ = decreased; ↑ = increased; bw = body weight; C_{in} = clearance of inulin; C_{PAH} = clearance of p-aminohippuric acid; C_s = clearance of stevioside; d = day; GFR = Glomerular filtration rate; MAP = Mean arterial pressure; mon = month; NR = not reported; NSD = No significant difference; RPF = Renal plasma flow; V = Urine flow

[a] unless stated otherwise, all results compared to control group(s)

Table A.II-14 Summary of Other Special Studies

Species (Strain)	Dose (route)	Duration	Purity	Results	Comments	Reference
Potential Effects of Steviol Glycosides on Enzymes						
Mouse (female ICR/Ha; number/group not reported)	0 or 0.01 mmol [159 mg steviol equivalents/kg bw] (oral; specific route not specified)	Single dose	NR (rebaudioside A, B, or C, stevioside, steviolbioside, steviol, or isosteviol)	No effect on GST activity in liver or intestinal mucosa.	-lack of information regarding steviol glycoside composition of test article	Pezzuto *et al.* (1986)
Potential Androgenic Effects of Stevia Extract						
Rat (male Wistar; 5/group)	0, 5, 25, or 100% [10 mL/kg bw] (gavage)	31 days	2.6% stevioside	NSD in bw or absolute organ weights (testicles, prostate, seminal vesicles, and pituitary glands). No signs of intolerance or toxicity or histopatholgical abnormalities.	-reported steviol glycoside composition is below 95%	Sincholle and Marcorelles (1989)
Potential Cariogenic Effects of Steviol Glycosides						
Rat (Sprague-Dawley pups; 15/group)	0, 0.5% stevioside, or 0.5% rebaudioside A (diet)	5 weeks	NR	NSD in *Streptococcus sobrinus* counts, incidence of sulcal caries, food or water intake, or bw gains.	-lack of information regarding steviol glycoside composition of test article	Das *et al.* (1992)
Potential Effects of Stevioside on Monosaccharide Transport						
Hamster (male Syrian golden; 12 to 15/group)	0, 500 [low], 1,000 [mid], or 2,500 [high] mg/kg bw/d (gavage)	12 weeks	90%	↓ bw, *ex vivo* glucose uptake and Na^+/K^+-ATPase activity in highest dose group. ↑ sucrase activity in mid- and high-dose groups. NSD bw in low- and mid-dose groups; and weight, length or ATP content of small intestine or lactase or maltase activity in any group.	-reported steviol glycoside composition is below 95%	Toskulkao and Sutheerawattananon (1994)

ATP = adenosine triphosphate; ATPase = adenosine triphophatase; bw = body weight; K^+ = potassium; Na^+ = sodium; NR = not reported; NSD = no significant differences;

Table A.II-15 Summary of Human Studies on Steviol Glycoside Preparations							
Subjects	**Duration of Treatment**	**Study Design**	**Purity**	**Dose (mg/d)**	**Reported Effects[a]**	**Comments**	**Reference**
Rebaudioside A							
45 subjects with normal glucose tolerance and 49 subjects with type 2 diabetes mellitus	Single oral dose	Randomized, double-blind, placebo controlled crossover clinical trial following 1 week placebo washout phase	>97%	500, 750, or 1,000	NSD: postprandial levels of glucose; insulin; C-peptide; incremental AUC for glucose, insulin, C-peptide, or glucagon; SBP; or DBP	-test article meets JECFA specifications	Maki *et al.* (2007; unpublished)
122 subjects with type 2 diabetes mellitus; Treatment group: 32 men and 28 women aged 59±1 years Placebo group: 30 men and 32 women aged 61±1 years	16 weeks	Randomized double-blind, placebo controlled clinical trial following 2 week placebo phase	>97%	1,000	NSD: HbA1c levels; fasting insulin; C-peptide; glucose; TG; TC; LDL-C; HDL-C; and non-HDL-C levels; body weights; SBP; DBP; index of changes in dosages of diabetes medications; hypoglycemic episodes; clinical chemistry results; hematology results; or urinalysis. ↑: ALT levels; GGT levels; and basophil counts.	-test article meets JECFA specifications	Maki *et al.* (2008a)
100 healthy volunteers with normal or low-normal BP; Treatment group: 12 men and 38 women aged 42±2 years Placebo group: 9 men and 41 women aged 41±2	4 weeks	Randomized, double-blind, placebo controlled clinical trial following 2 week placebo phase	>97%	1,000	NSD: resting, seated SBP, DBP; MAP; HR; or 24h ambulatory BP responses. ↑: post-meal values for supine and standing DBP, and MAP at week 4. ↓: pre-meal values for standing DBP and MAP week 4.	-test article meets JECFA specifications	Maki *et al.* (2008b)

Table A.II-15 Summary of Human Studies on Steviol Glycoside Preparations							
Subjects	**Duration of Treatment**	**Study Design**	**Purity**	**Dose (mg/d)**	**Reported Effects[a]**	**Comments**	**Reference**
Stevioside							
12 subjects with type 2 diabetes mellitus (4 females and 8 males) with a mean age of 65.8±1.6 years with a diabetes duration of 6.0±1.3 years	Single dose	Acute, paired, cross-over study; administered in 1 meal	91% stevioside; 4% rebaudioside A; 5% other derivatives	1,000	↑: insulinogenic index. ↓: postprandial AUC of glucose and glucagon. NSD: postprandial AUC of insulin, glucagon-like peptide, or gastric inhibitory polypeptide; DBP; SBP; TG; or FFA.	-test article meets JECFA specifications	Gregersen *et al.* (2001) [abstract]; Gregersen *et al.* (2004)
10 normal healthy subjects (5 men and 5 women) between ages of 21 and 29 years	3 days	Clinical trial	97%	750 (in 3 doses)	NSD: BP; blood glucose; plasma insulin; ALP; ALT; creatine kinase; LDH; or urine volume.	-test article meets JECFA specifications	Temme *et al.* (2004); Geuns *et al.* (2007)
15 normal, health male and female volunteers between ages 10 and 25 years	4 days	Clinical trial	NR	1,000 (in 2 doses)	↑: glucose tolerance. NSD: plasma lipid; urea; creatinine; cholesterol; or pyruvic lactate levels.	-lack of information regarding steviol glycoside composition of test article -abstract in Portuguese only	Alvarez *et al.* (1981) [abstract]
55 subjects with type 2 diabetes mellitus aged 40 to 70 years (sex/group NR)	3 months	Randomized, placebo-controlled, double-blind parallel trial. An i.v. glucose tolerance test was performed at the end of the study	NR	1,500 (in 3 doses)	NSD: HbA_{1C}; fasting blood glucose; incremental AUC for glucose; insulin response; TG; FFA; cholesterol; or BP.	-lack of information regarding steviol glycoside composition of test article	Jeppesen *et al.* (2006a) [abstract]

Table A.II-15 Summary of Human Studies on Steviol Glycoside Preparations							
Subjects	**Duration of Treatment**	**Study Design**	**Purity**	**Dose (mg/d)**	**Reported Effects[a]**	**Comments**	**Reference**
106 Chinese patients with mild to moderate hypertension Treatment group: 34 men and 26 women, mean age of 54.1±3.8 years Placebo group: 19 men and 27 women, mean age of 53.7±4.1 years	1 year	Trial: Multi-centre, randomized, double-blind, placebo-controlled trial Washout: single-blind placebo washout phase	NR	750 (in 3 doses)	↓: DBP; and SBP. NSD: BMI; HR; serum creatinine; CPK; AST; ALT; Na^+; K^+; Cl^-; plasma glucose; TC; HDL-C; or TG.	-lack of information regarding steviol glycoside composition of test article	Chan *et al.* (2000)
87 Chinese males and 87 Chinese females with mild (stage 1) hypertension Treatment group: 43 men and 42 women, mean age of 51±6 years Placebo group: 44 men and 45 women, mean age of 53±7 years	2 years	Multi-centre, double-blind, placebo-controlled trial	NR	1,500 (in 3 doses)	↓: SBP; DBP; and number of patients with LVH. NSD: serum creatinine; CPK; AST; ALT; Na^+; K^+; Cl^-; plasma glucose; TC; HDL-C; or TG.	-lack of information regarding steviol glycoside composition of test article	Hsieh *et al.* (2003)
Stevia Extract							
25 healthy adults (sex/group and age NR)	Single dose	Acute, double-blind, clinical trial	NR	NR	↓: glucose levels within 6 to 8 hours.	-lack of information regarding steviol glycoside composition of test article	Oviedo *et al.* (1970) [abstract]

Table A.II-15 Summary of Human Studies on Steviol Glycoside Preparations

Subjects	Duration of Treatment	Study Design	Purity	Dose (mg/d)	Reported Effects[a]	Comments	Reference
60 healthy normotensive adults (27 men and 33 women; mean age of 26.5±0.9 years) Group 1: 15 subjects Group 2: 20 subjects Group 3: 25 subjects	1 day	Randomized, double-blind, placebo-controlled pharmacological trial	13.85% stevioside	Group 1: placebo (content NR) Group 2: 50 Group 3: 200	↑: plasma Na^+ and K^+ levels (Group 2); plasma K^+ level (Group 3); plasma FFA in anabolic state (Group 3) during the anabolic state; HR after 5 min; SBP; or sinus arrhythmia. ↓: HR after 15 min; plasma insulin; Ca^{2+}, SBP; DBP, plasma insulin, FFA, and insulin to glucose ratio (Group 2); or plasma glucose levels, plasma Na^+, and QT segment (Group 3). NSD: HR in the supine position (Group 3).	-reported steviol glycoside composition is below 95% -test article does not meet JECFA specifications	Boeckh-Haebisch (1992)
16 healthy male and female adults (sex/group and age NR)	3 days	Oral doses of *S. rebaudiana* extract provided at 6-hour intervals. An oral glucose tolerance test was performed before the first dose of *S. rebaudiana* was provided and after the last dose of *S. rebaudiana*	NR	310 (in 4 doses)	↓: plasma glucose levels.	-lack of information regarding steviol glycoside composition of test article	Curi *et al.* (1986)

Table A.II-15 Summary of Human Studies on Steviol Glycoside Preparations

Subjects	Duration of Treatment	Study Design	Purity	Dose (mg/d)	Reported Effects[a]	Comments	Reference
44 hyperlipidemic volunteers [48 from beginning, 4 subjects (1 from placebo; 3 from treatment) dropped out due to personal reasons; sex/group and age NR]	3 months	Randomized, double-blind placebo controlled clinical trial	73±2% stevioside, 24±2% rebaudioside A, and 3% other plant poly-saccharides	250 (in 2 doses)	↓: serum concentrations of TC, and LDL-C. NSD: serum concentrations of TG, liver-derived enzymes, and glucose.	-test article meets JECFA specifications -unpublished study	Anonymous (2004a)
12 volunteers (sex/group and age NR)	90 days	Follow-up study of Anonymous, 2004a	73±2% stevioside, 24±2% rebaudioside A, and 3% other plant poly-saccharides	195, 450, or 900	NSD: blood; and urine biochemical parameters.	-test article meets JECFA specifications -unpublished study	Anonymous (2004b)
76 subjects (16 type 1 diabetics; 30 type 2 diabetics; 30 non-diabetics between the ages of 20 and 70 years) (sex/group NR)	3 months	Randomized, double-blind, placebo controlled clinical trial	≥92% steviol glycosides (composition NR)	750 (in 3 doses)	↑: SBP and TG in type 1 diabetics at baseline. NSD: SBP; DBP; HbA_{1C}; TC; HDL-C; LDL-C; TG; creatinine; CPK; Na^+, K^+, Cl^-, AST, ALT, GGT.	-reported steviol glycoside composition is below 95% -test article does not meet JECFA specifications	Barriocanal *et al.* (2008)
49 hyperlipidemic patients Treatment group: 25 men and women; ages NR Placebo group: 24 men and women; ages NR	90 days	Randomized, double-blind, placebo-controlled clinical trial	70% stevioside, 20% rebaudioside A, 2% other rebaudiosides	200 (in 4 doses)	↓: TC and LDL-C in both treatment and placebo groups. NSD: body mass index; serum AST, ALT, GGT, glucose, HDL-C, VLDL-C, or TG.	-reported steviol glycoside composition is below 95% -test article does not meet JECFA specifications	Cavalcante da Silva *et al.* (2006)

Table A.II-15 Summary of Human Studies on Steviol Glycoside Preparations

Subjects	Duration of Treatment	Study Design	Purity	Dose (mg/d)	Reported Effects[a]	Comments	Reference
14 farm workers with mild hypertension Treatment group: 6 men and 1 women aged 46.3±8.08 years Placebo group: 6 men and 1 woman aged 43.3±5.64 years	Phase 0: 4 weeks Phase 1: 7 weeks Phase 2: 11 weeks Phase 3: 6 weeks	Randomized double-blind, placebo controlled clinical trial	NR	Phase 0: capsules containing talcum, twice daily Phase 1: 3.75 (in 2 doses) Phase 2: 7.5 (in 2 doses) Phase 3: 15 (in 2 doses)	↓: DBP; HOMA-IR, cholesterol, LDL-C, glucose, insulin levels; and SBP in phase 2. NSD: AST; ALT; GGT; CPK; creatinine; urea; testosterone; free and total PSA; estradiol; blood K^+; blood Cl^-; blood Na^+; BMI; urinary Na^+; HDL-C; HbA_{1C}; or fructosamine.	-lack of information regarding steviol glycoside composition of test article	Ferri *et al.* (2006)

↓ = decreased, ↑ = increased; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; AUC = area under the curve; BMI = body mass index; BP = blood pressure; Ca^{2+} = calcium; Cl^- = chloride; CPK = creatine phosphokinase; DBP = diastolic blood pressure; FFA = free fatty acid; GGT = γ-glutamyl transferase; HbA_{1C} = glycated hemoglobin A_{1C}; HDL-C = high-density lipoprotein-cholesterol; HOMA-IR = homeostasis model of insulin resistance; HR = heart rate; i.v. = intravenous; K^+ = potassium; LDH = lactate dehydrogenase; LDL-C = low-density lipoprotein-cholesterol; LVH = left ventricular hypertrophy; Na^+ = sodium; NR = not reported; NSD = no significant differences; PSA = prostatic specific antigen; QRS = period of depolarization of ventricles; QT = beginning of QRS complex to end of T wave (represent repolarization); SBP = systolic blood pressure; TC = total cholesterol; TG = triglyceride; VLDL-C = very low-density lipoprotein-cholesterol.

[a] unless stated otherwise, all reported effects are relative to control group(s)

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