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Overview: The history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages

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ABSTRACT

Rebaudioside A is a sweet tasting steviol glycoside extracted and purified from *Stevia rebaudiana* (Bertoni). Steviol glycosides can currently be used as a food ingredient in only a handful of countries. Questions on specifications, safety and special population effects have prevented steviol glycosides from obtaining a legal status permitting their use as a sweetener in most countries. A set of papers reporting results of research studies and reviews has been compiled in this Supplement to definitively answer unresolved questions. Specifically, recently completed studies on the general and reproductive toxicity of rebaudioside A corroborate studies carried out with purified steviol glycosides demonstrating safety at high dietary intake levels. Comparative metabolism studies provide further affirmation of the common metabolic pathway for all steviol glycosides and the common metabolism between rats and humans. Finally, clinical studies provide further evidence that purified rebaudioside A has no effect on either blood pressure or glucose homeostasis. This paper summarizes the information used to conclude that high purity rebaudioside A (rebiana) produced to food-grade specifications and according to Good Manufacturing Practices is safe for human consumption under its intended conditions of use as a general purpose sweetener.

1. Introduction

Stevia is the generic term used for food ingredients derived from the herb *Stevia rebaudiana* (Bertoni). Steviol glycoside is a more precise term for a group of intensely sweet compounds extracted and purified from *S. rebaudiana*. Stevioside and rebaudioside A are the predominant steviol glycosides found in *S. rebaudiana*. Commercial interest in steviol glycoside sweeteners has been high for a long time. Technical problems reducing a bitter or licorice aftertaste, coupled with regulatory barriers caused by inadequate specifications and unresolved safety questions, previously prevented steviol glycoside sweeteners from becoming more widely marketed. Although steviol glycosides cannot be patented, increasing concerns about managing appropriate caloric intake as well as consumer demand for more sugar-substitute options provided the commercial motivation to overcome the technical and regulatory hurdles for commercializing steviol glycosides as a food ingredient.

In a few countries stevia has been consumed as a food and medicine (ethnobotanical) for many years, including most notably Japan and Paraguay. Currently, stevia in leaf or extracted forms is permitted as a dietary supplement in the US, and under similar classifications in several other countries. Erroneous reports that stevia is widely used in western food and beverage products sold in Japan or South America has created a public impression that the sweetener has been held off the market in the US and Europe for arbitrary reasons. However, a number of prominent food safety and regulatory agencies from around the world have made their concerns with stevia-based ingredients precisely known for many years (JECFA, 1999; SCF, 1985; FDA, 2007). This review paper, and the studies presented in this Supplement, aim to address the safety and specification-related questions that have historically prevented regulatory acceptance of steviol glycoside sweeteners.

Accurate and consistent specifications are important for any globally marketed food ingredient for safety, commercial and regulatory reasons. However, consistent specifications may not be easy to establish for a naturally-derived ingredient, especially

Abbreviations: ADI, acceptable daily intake; bw, body weight; DBP, diastolic blood pressure; DNA, deoxyribonucleic acid; DSHEA, Dietary Supplement Health and Education Act; FDA, Food and Drug Administration; GRAS, generally recognized as safe; Hb, hemoglobin; HDL, high-density lipoprotein; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LDL, low-density lipoprotein; MAP, mean arterial pressure; mm Hg, millimeters of mercury; NOEL, no observed effect level; NOAEL, no observed adverse effect level; SCF, Scientific Committee on Food; SBP, systolic blood pressure; US, United States of America; WHO, World Health Organization.

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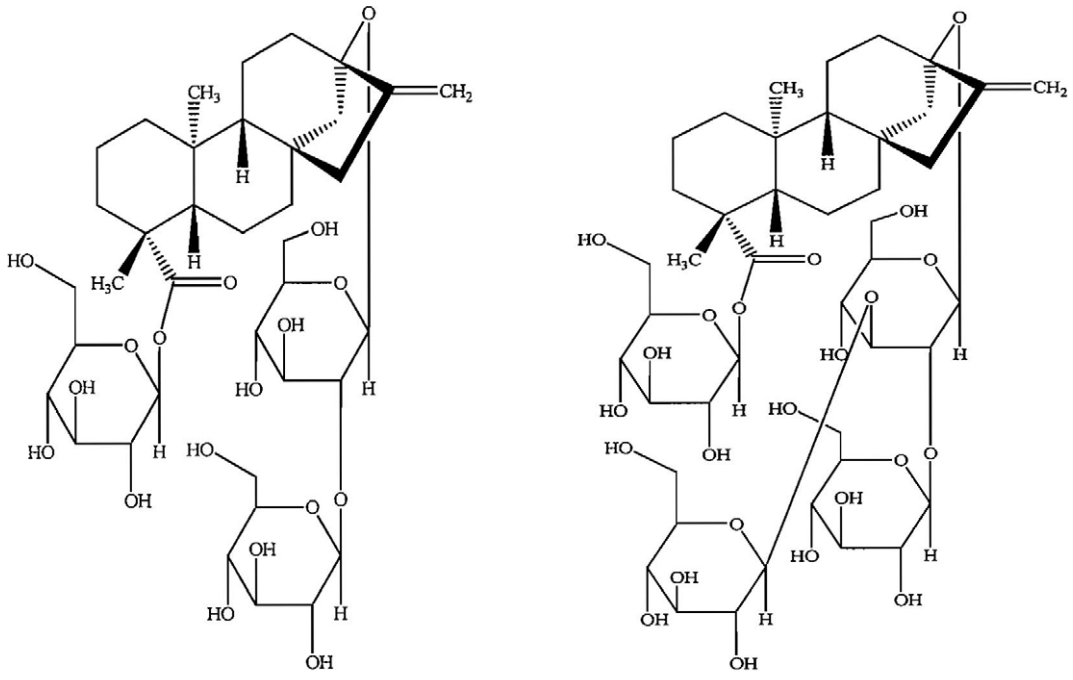
one having a history of use in various forms. The development of steviol glycosides has been impeded by a confusing array of published studies conducted with crude or semi-purified test articles having poorly defined or variable specifications. In 2007, JECFA established specifications for steviol glycoside sweeteners calling for them to consist of at least 95% of seven named steviol glycosides (JECFA, 2007). Most steviol glycoside products sold today consist primarily of stevioside or rebaudioside A. Products containing a high level of rebaudioside A are also known as rebiana. Rebiana is a "common or usual" name for steviol glycoside ingredients comprised predominantly of rebaudioside A.

Rebiana evaluated in studies reported in this Supplement met all current JECFA specifications for steviol glycosides. Most of the published literature on the physiological and toxicological effects of steviol glycosides used stevioside rather than rebaudioside A. However, the two glycosides are structurally very similar with rebaudioside A having one more glucose moiety as compared to stevioside (Fig. 1).

All steviol glycosides are metabolized to steviol and it is the safety evaluation of steviol that is important for risk assessment (Roberts and Renwick, 2008). For the purposes of comparing intake and safety limits, all steviol glycosides are converted to their *steviol equivalents*. This results in potentially confusing terminology for those unfamiliar with the conversion. Based on their relative molecular weights, stevioside quantities are multiplied by 0.40 and rebaudioside A quantities by 0.33 to convert both to steviol equivalents. The current JECFA temporary acceptable daily intake (ADI) of 0–2 mg/kg body weight (bw)/day on a steviol equivalent basis corresponds to 0–6 mg of rebaudioside A/kg bw/day using this molecular weight conversion. The projected permanent ADI for rebaudioside A is 0–12 mg/kg bw/day based on an anticipated permanent JECFA ADI for steviol equivalents of 0–4 mg/kg bw/day. While the terminology throughout this Supplement is clear on the use of the conversion, readers are cautioned that the general scientific literature can be quite confusing on this point.

2. Historical use and regulation

Stevia leaves were used by indigenous peoples in Paraguay and Brazil since before recorded history (Lee, 1979; Soejarto, 2002).



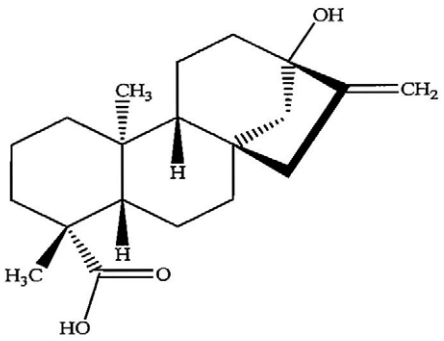


Fig. 1. Structures of steviol glycosides stevioside and rebaudioside A and their similar core and metabolite, steviol.

Stevia became more widely known outside central South America following the 1887 "discovery" of stevia by botanist, Antonio Bertoni. Due to its sweetness, stevia has been given many names including honey leaf, sweet leaf of Paraguay, sweetleaf, sweet herb, candyleaf and honey yerba.

Stevia sweeteners are probably best known from their use in Japan. Used in place of saccharin after it was banned in the 1970s, stevia sweeteners have been consumed in Japan more widely than in any other country, although not as much as has been reported. Stevia sweeteners were investigated but never commercialized for diet Coke® in Japan as has been reported. Early stevia formulations had a well-known licorice off-taste and lingering sweet aftertaste that always limited their commercial development, especially in beverages.

In the 1970s and 1980s stevia began to appear in herbal and "health-food" stores in North America and Europe. The US FDA has denied several attempts to market stevia as a food additive. Stevia leaves or extracts have only been allowed for use as a dietary supplement in the US since the passage of the Dietary Supplement Health and Education Act (DSHEA) in 1994 (FDA, 1995). On several occasions since the early 1990s, FDA has issued warning letters to food manufacturers questioning the legality of products containing stevia-derived ingredients that appear to market them as a sweetener, which is not permitted under DSHEA. In some of these letters FDA has also expressed concerns about gaps in the safety assessment of steviol glycosides specifically related to reproductive effects and glycemic control (FDA, 2007).

The European Commission's Scientific Committee on Food (SCF) reviewed the safety of stevia-derived sweeteners on several occasions (SCF, 1985, 1999a,b). Their reviews concluded that gaps and uncertainties remained in the available safety database. Several recently-submitted petitions for stevia extracts are again pending at the new European Food Safety Agency. Steviol glycosides remain approved for use as a sweetener in only a handful of countries, most notably Japan, Brazil, China, Korea, and Paraguay. Stevia leaves are approved for sale as a food in a few other countries (e.g., Israel and Thailand), but not as purified extracts.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed steviol glycosides at its 58th, 63rd, and 68th meetings. In the earlier meetings, JECFA established both temporary specifications and a temporary ADI for steviol glycosides of 0–2 mg/kg bw/day expressed as steviol using a safety factor of 200. In addition to detailed information on specifications, JECFA requested human studies conducted in normotensive and hypotensive subjects to answer questions about potential blood pressure lowering effects, and in subjects with insulin-dependent and insulin-independent diabetes to answer questions about effects on glucose homeostasis. When sufficient information was submitted and reviewed, the Committee stated that it would reduce the safety factor to 100 and make the ADI permanent. While information supplied to the 68th JECFA meeting allowed the establishment of final specifications, the data submitted was considered insufficient for answering JECFA's questions regarding the potential effects on blood pressure and glucose homeostasis and the temporary ADI of 0–2 mg/kg bw/day was extended.

The studies and reviews included in this Supplement were designed to answer JECFA's questions as well as those raised by other national and international food safety authorities concerning the safety of purified steviol glycosides for use in food and beverages. This review considers the historical database of safety studies on crude stevia and purified steviol glycosides, and addresses uncertainties that have previously prevented a definitive safety assessment for rebaudioside A. The areas of focus and objectives for this scientific discussion include the following:

Specifications – provide specifications for rebiana that are consistent with the current level of technical detail required by JECFA and various national and international regulatory agencies.
Comparative Metabolism – provide further substantiation that metabolism of rebaudioside A and stevioside (and by extension all steviol glycosides) do not differ, and substantiate that metabolic disposition of steviol glycosides in rats is similar to humans and allows risk assessment based on the results of available rodent toxicity studies.
Subchronic and Chronic Toxicity – resolve uncertainties about previously reported renal toxicity and provide a solid basis for answering any questions about general toxicity.
Genotoxicity – review and validate the previously established lack of *in vivo* mutagenicity of steviol glycosides and their common metabolite steviol.
Carcinogenicity – review and validate the previously established lack of carcinogenicity of steviol glycosides and their common metabolite steviol.
Reproduction and Developmental Safety – resolve the previously reported uncertainty about adverse effects on reproductive organs and reproduction.
Cardiovascular effects – resolve the uncertainties previously reported about the effects of steviol glycosides on blood pressure, particularly in healthy subjects with blood pressure measurements in the lower population percentiles.
Effects on glucose homeostasis – resolve the uncertainties previously reported about the effects of steviol glycosides on glucose homeostasis, particularly in subjects with type 2 diabetes.
Intake assessment – establish and document the potential intake of steviol glycosides across a wide spectrum of food and beverage uses and validate a margin of safety between the projected consumption by high-intake consumers (e.g., children with diabetes) and the ADI.

3. Specifications and technical function

Rebiana is a purified form of the major glycoside rebaudioside A that meets JECFA specifications along with strict sensory criteria established by the manufacturer (Table 1).

To be considered suitable for commercial use, rebiana must adhere to the following: (a) technical, purity and safety specifications set forth by JECFA, (b) manufacture by a validated and reproducible method, (c) production under current Good Manufacturing Practices, (d) sensory criteria established from studies using trained panelists, and (e) rebaudioside A purity levels determined by the validated analytical method.

Production of rebiana begins with hot water (50–60 °C) extraction of rebaudioside A from *S. rebaudiana* leaves followed by filtration. The sweet water is passed through an adsorption resin, where the steviol glycosides are retained, separating them from other components. The steviol glycosides are eluted from the resin using food grade methanol or ethanol. The product is then dried, typically by spray or vacuum drying. The end product is packaged into sealed, food-grade bags (Prakash et al., 2008).

Rebiana is very stable when stored as a powder. Storage for 24 months in polyethylene bags resulted in loss of only 1–2% of rebaudioside A (Prakash et al., 2008). Degradation products were primarily other steviol glycosides and related steviol compounds produced via hydrolysis that present no safety issues. Rebiana also has very good thermal stability, with no measurable degradation following pasteurization of dairy products. In the laboratory, rebiana has proven stable in baking applications to at least 390 °F. While rebiana does show some degradation over time at pH less than 3.0 in very high environmental temperatures (e.g., 40 °C), it is more stable in acidic beverages than other widely used intense

Table 1
Specifications for rebiana

Specification parameter	Specification	Method
Identity	Conforms to IR standard	FCC V[a]
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

HPLC, high performance liquid chromatography; ICP, inductively coupled plasma mass spectrometry; IR, infrared; wt, weight.
[a] FCC (2003).

sweeteners. Stability in acid beverages is more than sufficient for the commercial production of soft drinks. Degradation products observed in stored beverages were the same as those found in dry powder. Contrary to previous reports on steviol glycosides (Chang and Cook, 1983), rebiana is photo-stable (Clos et al., 2008).

4. Comparative metabolism

The SCF (1999a) indicated in its review that comparative metabolism studies were important in the interpretation of safety data and the establishment of an ADI. As noted previously, JECFA concluded that all steviol glycosides are metabolized *in vivo* to steviol based on studies published in the past several years. The rebiana research program results further establishes this through comparative ADME and pharmacokinetic data for stevioside and rebaudioside A.

Previous work in both rats and humans showed that (a) little or no stevioside is absorbed into the blood; (b) that some enterohepatic circulation of metabolites occurs in rats; and (c) conversion of steviol to its glucuronide is an important elimination pathway (Nakayama et al., 1986; Geuns et al., 2003, 2006, 2007a). The papers by Roberts and Renwick (2008) and Wheeler et al. (2008) in this Supplement confirm the previous work on stevioside and demonstrate that rebaudioside A is metabolized in the same manner as stevioside in both rats and humans (Fig. 2).

Given the previous and separate *in vivo* metabolism research on stevioside and *in vitro* research on rebaudioside A in rats, humans and other species, this result is not surprising. The finding that both rebaudioside A and stevioside are metabolized to steviol, and that only steviol is absorbed, further validates the approach by JECFA in setting an ADI for all steviol glycosides based on steviol content. It also confirms the validity of using stevioside safety study results in the safety assessment of rebiana.

The basics of steviol glycoside metabolism are similar in rats and humans, but rebiana metabolism study results also demonstrated a difference between rat and human steviol excretion that has been previously reported (Guens, 2006), but is perhaps not widely appreciated. Excretion of steviol glucuronide in rats occurs primarily in feces via the biliary tract, while in humans urinary excretion of the glucuronide is predominant. This is due to different molecular weight thresholds for human and rat biliary excretion of organic anions (e.g., glucuronides) and is observed with many ingested substances (Levine, 1978; Kwon, 2002). Organic anions with a molecular weight of less than 325 in the rat and less than 500–600 in humans are excreted in urine instead through a hepatic transport process for organic anions (Renwick, 2008b). Steviol and its glucuronide are therefore subject to some enterohepatic re-circulation in the rat although much of the steviol appears to be quickly eliminated in the feces (Roberts and Renwick, 2008). Therefore, both rats and humans have very little systemic exposure to steviol. In rats very little steviol is found beyond the portal or biliary systems, and in humans absorbed steviol is quickly converted to its glucuronide, a stable detoxification product that is quickly excreted by the kidney.

Finally, Renwick and Tarka's (2008) review of the microbial metabolism of stevioside and rebaudioside A in this Supplement concludes that both substances are metabolized to steviol in the gut via the same mechanisms. Both rebaudioside A and stevioside undergo hydrolysis by intestinal microflora to steviol, which is not further metabolized by the intestinal flora. *Bacteroides* sp. have been found to be almost entirely responsible for the conversion of steviol glycosides to steviol in the lower intestinal tract of both rats and humans. Fecal incubation studies with both human and animal mixed intestinal flora provide similar results, supporting the use of the rat as an appropriate model for studies on steviol glycosides.

5. Intake assessment

An intake assessment method using actual intake data for existing low-calorie sweeteners with a high level of consumption provides a more accurate process compared to models that assume complete caloric and non-caloric sweetener replacement (Renwick, 2006). Renwick (2008a) evaluated the intake of existing low-calorie sweeteners from published surveys conducted in multiple countries, and equilibrated the sweetener comparisons by expressing their intake in sucrose equivalents. Following conversion to sucrose equivalents, this approach averaged 90th or higher percentile intakes from multiple surveys resulting in a conservative overall 90th percentile rebiana intake estimate based on real low-calorie sweetener data. Intakes from all sweeteners were considered, but data from sweeteners with relatively low consumption in a particular database were often excluded when their inclusion would inappropriately reduce the overall intake estimate. Importantly, the process specifically includes results for children and consumers with diabetes thus allowing for dietary exposure estimation in special populations with potentially high intakes of a new sweetener.

Using this approach, Renwick (2008a) determined the average and high-percentile consumer intakes of rebiana at 1.3 and 3.4 mg/kg bw/day, respectively, for the general population. Daily consumption in children is predicted to be 2.1 and 5.0 mg/kg bw/day for average and high-percentile consumers, respectively. Intake estimates for children with diabetes range from 3.4 to 4.5 mg/kg bw/day for average and high-percentile consumers. It is important to note that these estimates significantly overestimate realistic rebiana intakes, especially for the near-term, as they con-

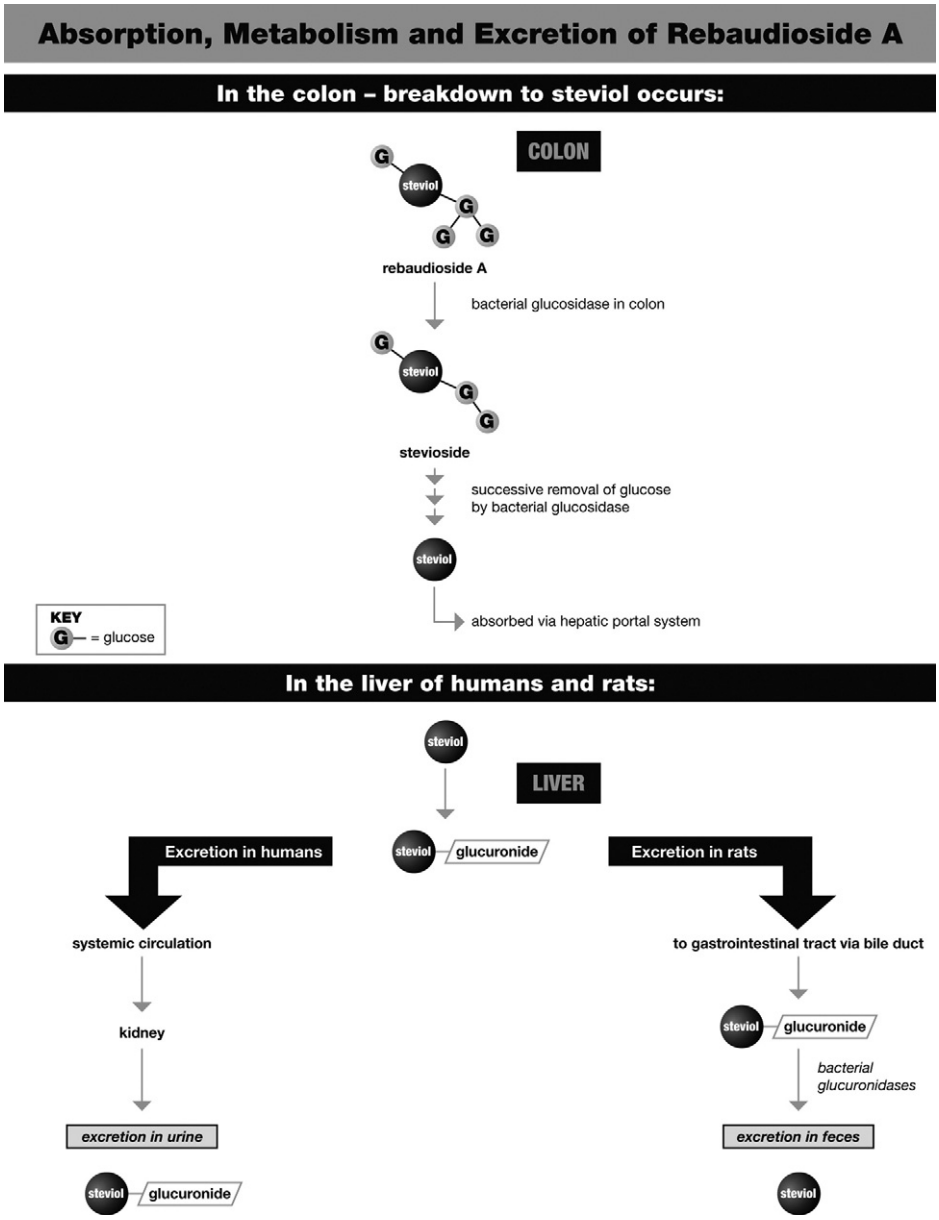


Fig. 2. Metabolism of rebaudioside A in humans and rats.

servatively assume significant market penetration and total consumer substitution for many of the currently available intense sweeteners. In addition, most intense sweeteners, including rebiana, will be used as a blend with other sweeteners so that the overall intake for each individual sweetener will be lower.

In order to compare rebiana intake estimates from Renwick with the JECFA temporary and projected permanent ADIs of 0–2 mg/kg bw/day and 0–4 mg/kg bw/day respectively, each rebiana intake estimate must be multiplied by 0.33 to convert to steviol equivalents. Therefore, the highest projected intakes in children and children with diabetes of 5.0 mg/kg bw/day and 4.5 mg/kg bw/day, respectively, become 1.6 mg/kg bw/day and 1.5 mg/kg bw/day, respectively. Projected 90th percentile intakes of rebiana for children and adults, even those with diabetes, are well below the anticipated permanent ADI for steviol glycosides.

These rebiana intake estimates compare quite favorably with intake estimates for steviol glycosides established by substituting for sugar and honey consumption as reported in the WHO Global Environment Monitoring System–Food Contamination Monitoring and Assessment Programme (GEMS/Food) database (WHO, 2007). Steviol glycoside intake estimates were determined from consumption figures assuming complete replacement of sugar and honey in or as food in distinct major geographic areas and assuming the lowest estimate of steviol glycoside sweetness intensity compared to sugar of 200:1. Using this approach steviol-equivalent intake estimates ranged from 0.9 mg/kg bw/day for Africa to 7.1 mg/kg bw/day for North America. However, replacement of all sugar was considered by the World Health Organization (WHO) to be a highly conservative assumption. Steviol glycoside intakes were thought likely to be only 20–30% of those estimated from total sugar replacement making the WHO estimates nearly identical to those obtained by Renwick in this Supplement.

6. Subchronic and chronic toxicity

As would be expected from their structure and history of use, steviol glycosides are classified as non-toxic under acute oral

Table 2
Summary of published subchronic dietary toxicity study results with rebaudioside a and stevioside

Species (Strain)	Study duration	Test article purity	Reported NOEL or NOAEL	Reference
Rat (HsdBR1 Han:Wist;)	4 weeks	97% rebaudioside A	9,938 mg/kg bw/day in males, and 11,728 mg/kg bw/day in females	Curry and Roberts (2008)
Rat (HsdBR1 Han:Wist;)	13 weeks	97% rebaudioside A	4,161 mg/kg bw/day males and 4,645 mg/kg bw/day in females	Curry and Roberts (2008)
Rat (Crl:CD(SD))	13 weeks	99.5 rebaudioside A	2050 mg/kg bw/day in males and 2055 mg/kg bw/day females	Nikiforov and Eapen (2008)
Rat (F344)	13 weeks	95.6% stevioside	2,500 mg/kg bw/day in males and females	Aze et al. (1991)
Rat (Wistar)	90 days	85% stevioside	2,125 mg/kg bw/day in males and females	Xili et al. (1992)
Cobb broiler chickens	2 weeks	96% stevioside	Approx. 137 mg/kg bw/day	Geuns et al. (2003)
Hisex brown laying hens	10 days	96% stevioside	78 mg/hen/day	Geuns et al. (2003)

toxicity study conditions (Medon et al., 1982; Toskulkao et al., 1997). In subchronic studies up to 13 weeks in length, both stevioside and rebaudioside A have virtually no oral toxicity (Table 2). Multiple-dose stevioside toxicity studies in rats with dosing periods ranging from 90 days to 56 weeks without any observed toxicity have also been reported (Akashi and Yokoyama, 1975; Lee et al., 1979).

The predominant finding in 13 weeks and longer oral toxicity studies has been a reduction in body weight in rats given very high doses of steviol glycosides. Curry and Roberts (2008) report that Wistar rats given rebaudioside A as 5% of their diet for 90-days had reduced food intake early in the study due to taste aversion caused by the high concentrations of the sweetener and the lower caloric density of the diets. Likewise, Nikiforov and Eapen (2008) report body weight gain decrements at the highest dietary concentrations of rebaudioside A administered to male Sprague–Dawley rats in their study. No evidence of systemic toxicity was reported in either of these studies. Curry and Roberts (2008) reported a NOAEL in their 13-week study of over 4000 mg/kg bw/day, the highest dose tested.

Decreases in body weight gain during rodent feeding studies have previously been described for high intensity sweeteners such as neotame, sucralose and Luo Han fruit (Flamm et al., 2003; Mayhew et al., 2003; Marone et al., 2008). These investigators report the impact of palatability, caloric density and subtle decrements in food consumption early in a study on body weight gains throughout the study. Taste aversion at higher dietary concentrations in food additive safety studies is a common finding, especially for high-intensity sweeteners. As a consequence, early changes in body weight and rates of body weight gain are typically considered not toxicologically significant even if they persist to the end of the study as this effect would have no practical extrapolation to human consumption patterns.

There are three studies in the literature that investigated chronic toxicity of stevioside over a 2-year period in rats. Yamada et al. (1985) and Xili et al. (1992) reported NOELs of 550 mg/kg bw/day and 600 mg/kg bw/day, respectively, the highest doses tested in each study. Toyoda et al. (1997) reported dose-related body weight reductions that were considered toxicologically significant in high-dose males (2000 mg/kg bw/day) and females (2,400 mg/kg bw/day), along with a significantly reduced survival rate in high-dose males. The authors considered the NOEL in this study to be 970 mg/kg bw/day based on the body weight gain and survival effects observed in males. The body weight gain effects are not surprising and have been considered toxicologically irrelevant by others investigating the safety of high intensity sweeteners or other food additives with the potential for taste aversion. The authors noted that the reduced survival in males appeared to be due to large granular cell leukemia, a tumor that commonly occurs at a high rate in aged F344 control rats. This study was conducted with a high-purity stevioside preparation (95.6%) that met the JECFA specifications and has been used by JECFA to set the current ADI.

7. Genotoxicity

In 2005, a report from JECFA concluded that stevioside and rebaudioside A have not shown evidence of genotoxicity *in vitro* or *in vivo* (JECFA, 2005). The report also concluded that steviol (or some of its metabolites) show signs of genotoxic activity *in vitro*, but produce no significant genotoxicity *in vivo* up to doses greater than 2000 mg/kg bw/day. However, since all steviol glycosides are metabolized to steviol, early reports of positive genotoxicity tests with steviol, albeit only *in vitro*, led to safety concerns about stevia that have now been resolved.

Stevia extracts and steviol glycosides, particularly stevioside, have been subjected to a broad array of both *in vitro* and *in vivo* assays that detect damage to DNA (Brusick, 2008). The tests include those measuring mutation, chromosome alterations and simple DNA breakage. With the exception of a single positive trial in strain TA98 of the Ames test (stevioside at 50 mg/plate which exceeded the recommended upper concentration limits for that assay), all *in vitro* test results for steviol glycosides, including rebaudioside A, produced no evidence that steviol glycosides induce DNA damage. *In vivo* studies of steviol glycosides included those assessing the ability of stevioside to induce DNA strand breakage in mice and rats and a micronucleus test for chromosome damage in the mouse. The *in vivo* assays conducted in mice and rats failed to show any genotoxicity up to dose levels of 2000 mg/kg bw/day. One recently published study assessing DNA strand breakage in Wistar rats exposed to stevioside at 4 mg/ml in drinking water for 45 days produced what appeared to be positive results after 5 weeks of exposure (Nunes et al., 2007). Several genetic toxicology experts have evaluated this study and have identified a number of methodological and data interpretation problems, including the lack of adequate controls (Geuns, 2007b; Williams, 2007). The fact that stevioside failed to induce DNA strand breaks *in vitro*, or in other *in vivo* studies employing higher dose levels, strongly suggests that the data from the Wistar rat study was technically compromised.

The steviol glycoside metabolite, steviol, has also been evaluated for genetic activity *in vitro* and *in vivo* with predominantly negative results in most conventional tests (Brusick, 2008). Positive results in two mammalian cell assays for chromosome aberrations and gene mutation were attributed to secondary cytotoxic effects resulting from excessively high treatment levels and not the results of direct DNA interaction. Steviol is not mutagenic in the Ames assay; however steviol was reported mutagenic in a forward mutation assay using *S. typhimurium* TM677, a histidine independent revertant of strain TA1535 that is repair deficient and contains both the plasmid pKM101 and rfa mutation. In order to be mutagenic in TM677, steviol must be metabolized to a reactive intermediate by S9 mix from rats induced by polychlorinated biphenyls. Steviol, therefore, appears to have a highly specific mutagenic mechanism for a single bacterial strain. The unique specificity of steviol for TM677 can be demonstrated by its lack of mutagenic activity in a second forward mutation assay in a bacteria strain that does not carry the *rfa* mutation and is not repair

deficient. Several known and hypothesized steviol metabolites were tested for mutagenic activity in TM677, but no definitive conclusions were reached regarding identification of the reactive intermediate. *In vivo* studies of steviol included tests for DNA strand breakage in mice and micronucleus studies in mice, rats and hamsters at dose levels up to 2000 mg/kg bw/day. None of the *in vivo* studies reported evidence of genetic toxicity.

8. Carcinogenicity

One of the earliest studies assessing the carcinogenic potential of stevioside was a study of urinary bladder initiation and promotion conducted by Hagiwara et al. (1984). Fischer 344 rats were administered stevioside (5% in the diet) for 36 weeks. Results showed that stevioside did not enhance the development of pre-neoplastic or neoplastic lesions in the urinary bladders when administered alone or when administered after an initiating dose of the bladder carcinogen of *N*-nitrosobutyl-*N*-(4-hydroxybutyl) amine. Studies of this type, however, are not adequate for assessing carcinogenic risk.

Xili et al. (1992) published the results of a combined oral 24-month chronic toxicity and carcinogenicity study of stevioside (purity, 85%) in Wistar rats, and no neoplastic or pre-neoplastic lesions were reported in any tissue. However, the highest dose of this study was 600 mg/kg bw/day which is relatively low considering the lack of toxicity observed in subchronic studies performed at much higher dose levels.

A 13-week pre-oncogenicity dietary study in Fischer 344 rats exposed to a range of dietary doses up to 5% stevioside (2500 mg/kg bw/day) produced no severe adverse effects (Aze et al., 1991). The results of this study were used to set dose levels for a 24-month carcinogenicity study conducted in this rat strain with stevioside (purity, 95.6%). The dietary dose levels used in the carcinogenicity study were 0, 2.5, and 5% (equal to 0, 970, and 2000 mg/kg bw/day for males and 0, 1100, and 2400 mg/kg bw/day for females). The animals were exposed *ad libitum* for 104 weeks and all surviving animals were killed at 108 weeks. The high dose was determined to be an adequate maximum-tolerated dose based on a slight depression in body weight gain. Food consumption was not changed, but a significant decrease in survival rate in the male rats was seen at the high dose (discussed in Section 6). No evidence of increased non-neoplastic or neoplastic lesions was reported and the authors concluded that stevioside was non-carcinogenic (Toyoda et al., 1997). This study was considered to be clear evidence of non-carcinogenicity by JECFA in its evaluations published in 1999 and 2006 (JECFA, 1999, 2006). JECFA used the 970 mg/kg bw/day dose to establish a temporary ADI of 2 mg/kg bw/day. The comparative metabolism study results by Roberts and Renwick (2008) demonstrate that this carcinogenicity study result also applies to rebaudioside A.

A report of the SCF (1999a) raised a concern that the high incidence of interstitial cell tumors in the control and treated male animals (common in this rat strain) in the Toyoda et al. (1997) study precluded their ability to evaluate the long-term effects on testes. They suggested that another chronic oral toxicity study be conducted in a different strain of rats. However, it appears SCF concerns about testicular effects were driven by unresolved questions about reproductive toxicity, in general, along with unresolved questions about the genotoxicity of steviol (SCF, 1999a). New information, supported by the subchronic toxicity studies and the 2-generation reproductive safety study reported in this Supplement, has resolved those concerns. Both subchronic studies and the reproductive safety study reported in this Supplement demonstrated the lack of male and female reproductive toxicity at extraordinarily high exposure levels. Additionally, previously published human metabolism studies along with the metabolism study reported in this Supplement demonstrate an efficient phase II detoxification process is present in humans that quickly convert steviol to its glucuronide with rapid urinary excretion.

This set of studies, combined with the absence of any genotoxic activity, strongly supports the JECFA conclusion that steviol glycosides do not pose a carcinogenic risk and that a permanent ADI can be set without additional chronic/carcinogenicity studies.

9. Reproduction and developmental safety

Stevia has been reportedly used as an oral contraceptive by women from Paraguayan Matto Grosso Indian tribes (Kinghorn, 2002). This ethnobotanical use has led to significant interest in the effect of stevia extracts on reproductive performance and to a number of investigations exploring the fertility effects of stevia extracts in rodent species.

The most oft-cited study regarding female fertility effects of stevia was conducted by Mazzei-Planas and Kuc (1968). This study reports significantly reduced fertility rates in female rats following administration of a stevia "weed" decoction at a rate of 10 ml/kg body weight per day. The study may be of historical interest, but its scientific usefulness is extremely limited because of the crude nature (ground up leaves) of the test material and the limitations of the study design.

Other investigators, including Oliveira-Filho et al. (1989) and Melis (1999) have reported the outcomes from studies conducted with crude or semi-purified stevia extracts to evaluate fertility effects in both male and female rats. These investigators report reduced weights of one or more male reproductive organs, but no specific macroscopic or microscopic lesions. As with the Mazei-Planas and Kuc study, the utility of these studies is limited because of the poor characterization of the test materials administered to the animals. There is no evidence collectively from published subchronic toxicity (Aze et al., 1991), chronic toxicity (Xili et al., 1992; Toyoda et al., 1997), and developmental and reproductive toxicity (Mori et al., 1981; Yodyingyuad and Bunyawong, 1991; Usami et al., 1995) studies that *purified* stevioside or rebaudioside A have an adverse affect on the male or female reproductive systems. Reproductive toxicity studies on stevioside of known purity (90.0% or 96.5%) with doses of up to 2500 mg/kg bw/day in hamsters (Yodyingyuad and Bunyawong, 1991), and up to 3000 mg/kg bw/day in rats (Mori et al., 1981), showed no effect on indices of developmental toxicity. In the hamster study reported by Yodyingyuad and Bunyawong (1991), both males and females were treated with stevioside during 3 rounds of mating. There was no reported effect on fertility, number of offspring, or on reproductive tissues of either sex. No developmental toxicity was reported in rats administered stevioside (95.6% purity) up to 1000 mg/kg bw/day in the diet (Usami et al., 1995).

Because of the historical database and continued debate over the reproductive effects of stevia extracts, further investigations were conducted to confirm the reproductive safety of high purity rebaudioside A The first phase of the assessment included histopathological examination of the testes in high-dose males from both 28- and 90-day feeding studies (Curry and Roberts, 2008). Macroscopic and microscopic examinations of the testes from the 100,000 ppm group from the 28-day study and all reproductive organs from males in the 50,000 ppm group from the 90-day study were unremarkable. The conclusion from both studies was that there were no treatment-related adverse effects observed on male or female reproductive systems.

Subsequent to the 90-day feeding study, a palatability study was conducted in juvenile rats to discern appropriate dose levels for a multi-generational reproductive study. Histopathological examination of the testes from the high dose group (50,000 ppm) in this

study indicated no effects on testicular morphology or spermatogenesis. To complete the reproductive safety assessment, a two-generation reproductive safety study was conducted at dietary levels up to 25,000 ppm in Han Wistar rats (Curry et al., 2008). In this study, no treatment-related effects of rebaudioside A were observed in either the F_0 or F_1 generations on reproductive performance parameters including mating performance, fertility, gestation lengths, estrus cycles, or sperm motility, concentration, or morphology. Likewise, no developmental effects were noted in the offspring. The NOAEL from this study was determined to be 2048–2273 mg/kg bw/day, the highest dose tested. This is consistent with results from a multigenerational study carried out in hamsters with purified stevioside (Yodyingyuad and Bunyawong, 1991). These studies corroborate the existing body of evidence on the reproductive safety of purified steviol glycosides and address any outstanding concerns about steviol glycoside-related reproductive toxicity questions resulting from early studies with methodological problems.

10. Clinical safety evaluation

Several clinical studies have suggested that stevioside may offer therapeutic benefits for subjects with hypertension and type 2 diabetes mellitus. Two long-term clinical trials have been conducted in hypertensive Chinese subjects (Chan et al., 2000; Hsieh et al., 2003). In the first study, patients with essential hypertension were taken off antihypertensive medications and randomized to either stevioside (750 mg/day) or placebo for 12 months. The same group of investigators conducted a longer follow-up study where patients with newly diagnosed mild essential hypertension were randomized to either stevioside (1500 mg/day) or placebo for 2 years. Both studies reported significant reductions from baseline to the end of treatment for both systolic blood pressure (SBP) and diastolic blood pressure (DBP) in the stevioside treated group. The purity of the stevioside test material used in these studies was not identified by the authors. Beneficial effects of oral stevioside have also been reported for postprandial glucose homeostasis in subjects with type 2 diabetes. Specifically, an acute study by Gregersen et al. (2004) reported reduced area under the curve (AUC) for glucose and glucagon following ingestion of 1 g stevioside administered with a test meal.

The results of these studies raised questions of whether steviol glycosides would have untoward effects in subjects with normal blood pressure, and whether longer term, repeated exposure to steviol glycosides poses any safety concerns for subjects with diabetes. These questions were articulated by JECFA following their 2004 review of steviol glycosides (JECFA, 2005). Not only were these questions raised, but, as discussed previously, the Committee imposed an additional safety factor on the newly established temporary ADI pending availability of additional information pertaining to the potential for pharmacological effects. As a result, two separate randomized, placebo-controlled, double-blind, multi-center clinical trials were included in the rebiana research program to answer the following questions:

(1) Does chronic daily consumption of steviol glycosides have an effect on hemodynamic measurements in subjects with normal or low-normal blood pressure?
(2) Does chronic daily consumption of steviol glycosides have an effect on glucose homeostasis in subjects with type 2 diabetes?

10.1. Cardiovascular effects

The first study (Maki et al., 2008a) included subjects with normal blood pressure, defined as <120 mm Hg SBP and <80 mm Hg DBP. Following a 2-week single-blind placebo lead-in period, subjects were randomized to receive either 1000 mg/day of rebaudioside A or placebo control daily for 4 weeks. The study was designed to provide at least 80% power to detect a 4.5 mm Hg difference in resting, seated SBP response, the primary outcome variable, between rebaudioside A *vs.* placebo. The study procedures also included measurement of resting, seated DBP, mean arterial pressure (MAP), heart rate, 24-hour ambulatory blood pressure, and supine and standing blood pressure responses during a standard meal challenge. In order to provide additional assurance of safety in subjects with low blood pressure, pre-specified subgroup analyses were performed on data for resting, seated and 24-hour blood pressures for subgroups with baseline SBP split at the sex-specific median (< and $\geqslant$108 mm Hg for females and < and $\geqslant$117 mm Hg for males). Compared with placebo, rebaudioside A did not significantly alter resting seated SBP, DBP, MAP, heart rate, or 24-hour ambulatory blood pressure responses. The results of the study indicated that consumption of 1000 mg/day of rebaudioside A was well tolerated and produced no clinically important hemodynamic effects. These results are consistent with those of Ferri and colleagues (2006) who showed no effect of doses up to 15 mg/kg bw/day for 24 weeks of a crude steviol glycoside extract on blood pressure in subjects with mild essential hypertension.

10.2. Effects on glucose homeostasis

In a separate study (Maki et al., 2008b); subjects with type 2 diabetes were randomized to receive 1000 mg/day of rebaudioside A or a placebo for 16 weeks following a 2-week single-blind placebo lead-in period. The investigation did not include subjects with type 1 diabetes since the purported mechanism of action for steviol glycosides involves enhanced secretion of insulin from the pancreas when there is impaired response to glucose stimulation. The primary outcome variable for the study was glycosylated hemoglobin (HbA_{1C}), the standard accepted measure of chronic glycemic control (American Diabetes Association, 2007). The study was designed to provide 90% power to detect a 0.5% difference in HbA_{1C} response between groups. Additional indicators of glycemic control included fasting glucose, insulin, and C-peptide. Adverse events were collected, as was an index of the changes in number and dosages of diabetes medications. The results demonstrated that 1000 mg/day of rebaudioside A for 16 weeks did not affect glucose homeostasis, or the incidence of adverse events. There were also no effects of rebaudioside A on blood pressure or fasting lipid measurements in this population of subjects with type 2 diabetes. The results of this study agree with those reported by Jeppesen et al. (2006) who reported that 3 months of daily stevioside consumption (1500 mg/day) did not affect HbA_{1C}, blood pressure, or blood lipids in subjects with type 2 diabetes mellitus.

Barriocanal and colleagues have recently published similar findings addressing the potential for steviol glycosides to produce pharmacological effects on blood pressure and glucose homeostasis following repeated consumption (Barriocanal et al., 2008). In a randomized, double-blind study, three groups of subjects (those with normal glucose homeostasis, type 1 diabetes and type 2 diabetes) were provided 750 mg/day of steviol glycosides or placebo daily for 3 months. These investigators reported no significant hemodynamic effects in subjects with or without diabetes mellitus. In addition, there was no effect of steviol glycosides on HbA_{1C} or blood lipids (total-, LDL-, HDL-cholesterol). While the test material used in this study did not meet JECFA specifications for steviol glycoside content, the study still supports the overall conclusion of safety for this ingredient.

Maki et al. (2008a,b) studies reported in this Supplement were designed to provide definitive data to fill the knowledge gaps iden-

tified by JECFA and to support the safety of repeated, long term consumption of steviol glycosides in humans. As noted above, other investigators have previously reported a lack of pharmacological effects of steviol glycosides on blood pressure and glucose homeostasis in several chronic studies. Taken together, it is reasonable to conclude that uncertainties pertaining to the potential for steviol glycosides to produce untoward effects on blood pressure in subjects with normal or low blood pressure have been addressed. It is also evident from long-term consumption studies that steviol glycosides do not have an effect on glucose homeostasis or blood lipids in subjects with diabetes mellitus.

11. Discussion and conclusion

Stevia has been the food substance with "multiple personas" due to its historical status as an ethnobotanical in South America, as an approved food ingredient in Japan and as a counter-culture herbal ingredient in the US and Europe. This long history of use as therapeutic, food, herb and subject of research has both helped and hindered the development of stevia-based sweeteners in countries with strong food regulatory systems. The common perception that stevia's long history of use was sufficient to substantiate its safety regardless of the scientific gaps remaining to be resolved led to a number of false starts in successfully bringing this natural sweetener onto the market in many countries, including the US.

By definition, the process to substantiate the Generally Recognized as Safe (GRAS) status of a substance requires suppliers and users to demonstrate that an ingredient is safe for its intended use in foods, and to ensure safety studies required to reach that conclusion are publicly available. Consistent with this process, the objectives of the scientific and regulatory program reported in this Supplement were two-fold. First, complete a safety assessment process that comprehensively examined the safety of rebiana for use in food and beverages by consumers around the world. Then second, publish the results of this examination in a way that addresses the scientific gaps and resolves the confusion caused by decades of studies conducted with poorly characterized stevia products and studies conducted using intravenous administration, for example, to evaluate potential therapeutic benefits.

All of the studies reported in this Supplement were conducted under applicable current Good Laboratory Practice and Good Clinical Practice guidelines and all papers underwent multiple peer reviews before being accepted for publication. The scientific program was guided by critical reviews of the large body of scientific literature regarding stevia and steviol glycosides, global regulatory requirements for determining the safety of low-calorie sweeteners, a thoughtful analysis of the outstanding scientific questions regarding the acceptability of steviol glycosides for use as a sweetener, and the input of numerous experts from around the world. As a result, the program sought to evaluate and address any uncertainties regarding the safety of purified steviol glycosides for the broadest possible global scientific and regulatory constituency.

The due diligence of the rebiana scientific program reported in this Supplement reflects a commitment by industry partners to, at long last, fully address regulatory requirements for this naturally occurring sweetener by providing the scientific basis to conclude high purity rebaudioside A (rebiana), produced under current GMP to food-grade standards, is safe and appropriate for introduction into the global marketplace.

Conflict of interest statement

Author Brusick received financial support from Cargill for consulting services and manuscript preparation.

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