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Toxicology of Rebaudioside A: A Review

by

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Toxicology of Rebaudioside A: A Review

This review of safety data regarding high-purity **rebaudioside A** (rebiana), the subject of two GRAS (generally recognized as safe) notifications,[1,2] was conducted for the Center for Science in the Public Interest. Much of the recent research was published in a Food and Chemical Toxicology supplement on **rebaudioside A**.[3] All the rebiana used in the studies published in that supplement met all current specifications for **steviol glycosides** set by the Joint FAO/WHO Expert Committee on Food Additives (Carakostas et al., 2008 and JECFA, 2007). The research described in this supplement was peer reviewed and said to be conducted in compliance with Good Laboratory Practices (GLP) and Good Clinical Practices (GCP) requirements.

Background

Rebaudioside A is a **steviol glycoside** derived from the herb *Stevia Rebaudiana* (bertoni). **Rebaudioside A** and **stevioside** (Fig. 1) are the two main **steviol glycosides** found in the *S. Rebaudiana* herb and are the two predominant derivatives used in high-potency sweeteners. **Stevioside** differs from **rebaudioside A** by having one less glucose moiety. **Steviol glycosides** have been used as food and medicine in Japan and South America for many years, but **stevia** in the leaf or extracted form is permitted to be sold in the U.S. only as a dietary supplement, as defined in section 201(ff)(1) of the Federal Food, Drug, and Cosmetic Act. In 2007, JECFA specified that **steviol glycoside** sweeteners must be composed of at least 95% of the known **steviol glycosides** (JECFA, 2007). Products that consist predominantly of **rebaudioside A** are referred to as rebiana.

The temporary acceptable daily intake (ADI) for **steviol glycosides** set by JECFA is 0-2 mg/kg bw/day (based on **steviol** content) with a **steviol** equivalent of 0-6 mg/kg bw/day of **rebaudioside A** (**steviol** equivalent=[**stevioside**]*(0.4); [**rebaudioside A**]*(0.33)). JECFA has concluded that there is insufficient data to give **steviol glycosides** a permanent ADI. The FDA has not yet authorized **stevia** as a food additive nor has the FDA considered it to be generally recognized as safe (GRAS). (Data for the above background information was obtained from Carakostas et al. 2008 and correspondence by the author with the Food and Drug Administration (FDA).)

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[1] GRAS notification numbers 252 and 253. We have not obtained a copy of No. 252 and so cannot comment on any data that may have accompanied that notification. Furthermore at least one other company, Wisdom Natural Brands, may have self-affirmed its stevia product as GRAS, and not notified the FDA.

[2] See Appendix A for list of abbreviations

[3] Authors of the supplement are affiliated with manufacturers or potential users of **rebaudioside A** (see Appendix B).

JECFA's Requirements

The JECFA has made the following requests for research before it set a permanent ADI for rebiana and reduced the safety factor to 100 (currently 200):

- detailed information on specifications
- human studies conducted in normotensive and hypotensive subjects to gain information on potential hypotensive effects;
- human studies with subjects with insulin-dependent and insulin-independent diabetes to gain information on the effects on glucose homeostasis (Carakostas et al. 2008).

Below we discuss those issues and indicate several additional concerns.

Comparative Metabolism

1. Humans

Renwick et al. (2008) reviewed the literature on the metabolism of **stevioside** and **rebaudioside A** by intestinal microbiota. Investigators used this review to try to establish that toxicity studies on **stevioside** are relevant for assessing the toxicity of **rebaudioside A**. Gardana et al. (2003) provided a comprehensive study on **steviol glycoside** hydrolysis in the human gut. The study was carried out under anaerobic conditions with mixed bacterial cultures from fecal samples of healthy human volunteers. **Stevioside** was completely hydrolyzed to **steviol** after 10 hours of incubation, with steviolbioside as an intermediate. Steviolbioside formation peaked at 2-4 hours and **steviol** was first detected at 3-4 hours of incubation. **Rebaudioside A** was completely hydrolyzed to **steviol** after 24 hours of incubation, with steviolbioside as an intermediate. Steviolbioside was detected at 6-7 hours and peaked at 12-15 hours of incubation. **Steviol** was unchanged by incubation with intestinal microflora after 72 hours of incubation. Because stevioside and rebaudioside A are metabolized at different rates, toxicity assessments of **stevioside** cannot definitively be extrapolated to assess the risk of **rebaudioside A**.

Hutapea et al. (1997) reported that **stevioside** has a steviol-16,17-epoxide metabolite (Fig. 2) when incubated for 48 hours with rat intestinal microflora. Epoxides are of concern because they are highly reactive with nucleophiles, such as DNA. The creation of the steviol-16,17-expoxide metabolite that was seen in the Hutapea study could not be replicated by Gardana et al. (2003) and Koyama et al. (2003). Renwick et al. (2008) speculated that the HPLC-UV (high performance liquid chromatography-ultraviolet) instrument used to detect the epoxide metabolite in the Hutapea study was not highly specific. However, given the possibility of epoxide formation from steviol and/or its glycosides based on their structures (Fig. 1), the creation of an epoxide metabolite in the human system needs to be further investigated.

Wheeler et al. (2008) conducted human metabolism studies that reported similar metabolic and elimination pathways (Fig. 3) but not identical pharmacokinetics for **rebaudioside A** and **stevioside**. Healthy, adult, male subjects received a single oral dose of 5 mg/kg of 98.7% pure **rebaudioside A** and 4.2 mg/kg of 96.6% pure **stevioside** (each ~1.6 mg/kg of **steviol** equivalents). Plasma, urine, and fecal samples were collected during a pre-dose period and up to 72 hours post-dose. Both glycosides were hydrolyzed in the GI tract into **steviol**, which was absorbed and conjugated to a **glucuronide**. **Steviol glucuronide** was predominantly excreted in the urine and accounted for 59% and 62% of the **rebaudioside A** and **stevioside**, respectively. **Steviol** excreted in the urine only accounted for 0.04% and 0.02% of **rebaudioside A** and **stevioside**, respectively. **Steviol glucuronide** was not detectable in the feces, but **steviol** in the feces accounted for 4.8% and 5.2% of **rebaudioside A** and **stevioside**, respectively. The half-life ($t_{1/2}$) for both glycosides was approximately 14 hours (Tables 1 and 2). However, only 64.2% of **rebaudioside A** and 67.22% of **stevioside** was accounted for in the urine and feces after 72 hours of dosing. Plasma **steviol glycosides** were not measured in this study.

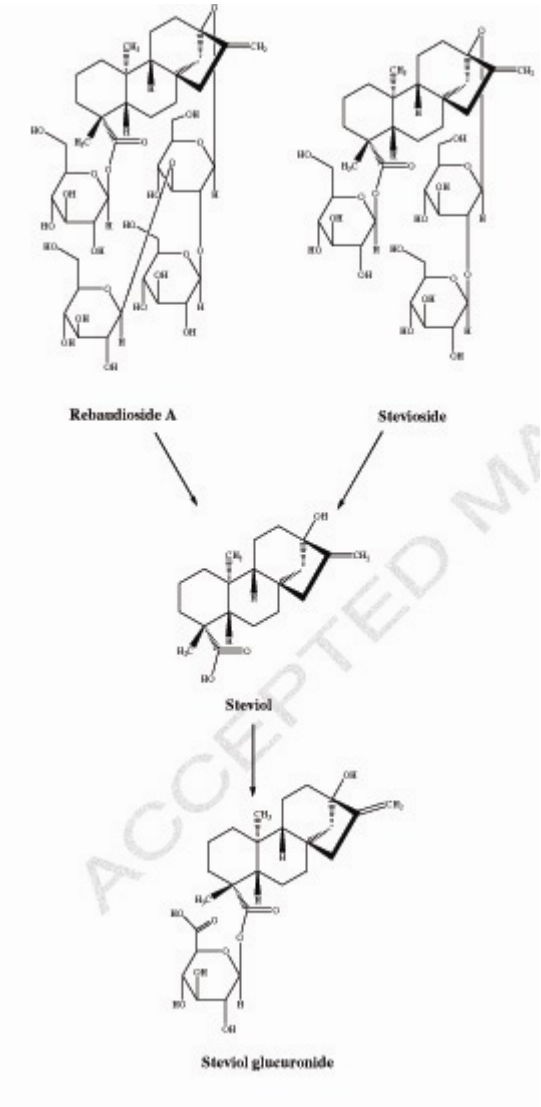

Figure 1 Components of **steviol glycoside** metabolism (Wheeler et al., 2008)

Geuns et al. (2003) reported low transport of **rebaudioside A** and **stevioside** in Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line often used to detect absorption rates of drugs, with apparent permeability coefficients of 0.11×10^{-6} and 0.16×10^{-6} cm/s, respectively. They reported that **steviol** was transported much more efficiently than the two glycosides, with an apparent permeability coefficient for absorptive transport of 38.6×10^{-6} cm/s. Though it is apparent that the majority of the **stevioside** and **rebaudioside A** are hydrolyzed into **steviol**, which is absorbed by the GI tract, it is possible that transport and/or absorption of **rebaudioside A** and **stevioside**

occurs. It is also likely that **steviol** metabolites are not being excreted within 72 hours, and that may account for the remainder of the dose that was not measured in the feces or urine. The unaccounted-for fraction of **rebaudioside A** and **stevioside** after 72 hours (~5 half-lives) of dosing needs to be further investigated. If harmful metabolites are being formed after absorption, it is important to understand their toxicokinetics in order to properly assess their potential toxicological relevance. A more complete study would measure plasma concentrations and excretion of unhydrolyzed **steviol glycosides**.

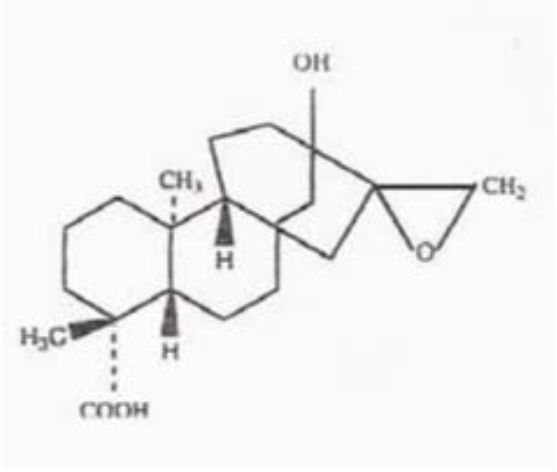

Figure 2 steviol-16,17α-epoxide (Brusick et al. 2008)

In the human metabolism study, **rebaudioside A** and **stevioside** had different pharmacokinetic results for certain parameters when **steviol** and **steviol glucuronide** were measured (Tables 1 and 2). For instance, there was a longer T_{max} and lower C_{max} of **steviol glucuronide** and **steviol** when the patients were administered **rebaudioside A** compared to **stevioside**. **Stevioside** toxicity studies may be a good way to predict the toxicity of **rebaudioside A**, but they cannot be used in place of directly testing **rebaudioside A** itself. The toxicity of **stevioside** and **rebaudioside A** should be studied individually since each will potentially be used as ingredients in human foods.

Table 1 Mean pharmacokinetic parameters for **steviol** in men (Wheeler et al., 2008)

		Treatment			
Parameter	Units	N	Rebaudioside A	N	Stevioside
C_{max}	(ng/mL)	1	227 (NA)	1	121 (NA)
T_{max}	(hr)	1	72.0 (NA)	1	6.00 (NA)
AUC_{0-t}	(ng*hr/mL)	0	NA (NA)	0	NA (NA)
AUC_{0-inf}	(ng*hr/mL)	0	NA (NA)	0	NA (NA)
$t_{1/2}$	(hr)	0	NA (NA)	0	NA (NA)
$?_z$	(1/hr)	0	NA (NA)	0	NA (NA)
$Ae_u(0-72)$	(mg)	8	0.0510 (0.0877)	8	0.0238 (0.0675)
CL_R	(L/hr)	0	NA (NA)	0	NA (NA)
$Ae_f(0-72)$	(mg)	6	5.88 (6.95)	7	6.50 (7.08)

The values given are mean ± SD. NA = Not applicable.

Table 2 Mean pharmacokinetic parameters for **steviol glucuronide** in men (Wheeler et al., 2008)

		Treatment			
Parameter	Units	N	Rebaudioside A	N	Stevioside
C_{max}	(ng/mL)	8	1588 (700)	8	2222 (1078)
T_{max}	(hr)	8	12.0 (6.02, 24.0)	8	8.00 (6.00, 12.0)
AUC_{0-t}	(ng*hr/mL)	8	33904 (15139)	8	39928 (20129)
AUC_{0-inf}	(ng*hr/mL)	4	46197 (18604)	4	53211 (23782)
$t_{1/2}$	(hr)	4	14.8 (3.32)	4	14.0 (5.61)
$?_z$	(1/hr)	4	0.0483 (0.00908)	4	0.0551 (0.0221)
$Ae_u(0-72)$	(mg)	8	106 (24.0)	8	112 (36.8)7)
CL_R	(L/hr)	8	3.73 (2.01)	8	3.36 (2.51)
$Ae_f(0-72)$	(mg)	6	0 (0)	7	0 (0)

The values given are mean ± SD. T_{max} is presented as Median (Min, Max).

2. Rats

Roberts et al. (2008) investigated the metabolism of **stevioside**, **rebaudioside A**, and **steviol** in Sprague-Dawley rats in order to determine the toxicokinetic and metabolic similarities between **stevioside** and **rebaudioside A**. The three compounds were radiolabeled with ^{14}C in the $=CH_2$ group of the **steviol** moiety (Fig. 1). The rats were given a single oral dose of 5 mg/kg bw **rebaudioside A**, 4.2 mg/kg bw **stevioside**, and 1.6 mg/kg bw **steviol** (molar equivalents). Even though the investigators concluded that the pharmacokinetics of **stevioside** and **rebaudioside A** in rats are similar, while that of **steviol** is different, it appears that most of the pharmacokinetic parameters are quite different for all three compounds in rats of the same sex (Table 3). The main radioactive component in plasma was always **steviol** after rats were dosed with ^{14}C-**stevioside**, ^{14}C-**rebaudioside A**, and ^{14}C-**steviol**. **Steviol glucuronide** and two unidentified metabolites were also found in the plasma in lower concentrations than **steviol**. The absorption through the gut from **rebaudioside A** treatment was 71% for males, 82% for females; from **stevioside** treatment was 78% for males and 81% for females; from **steviol** treatment was 97% for males and 99% for females. **Steviol** was excreted predominantly in the feces and was the primary metabolized component of the parent glycoside. Unlike the human studies, limited urinary elimination was reported. **Steviol glucuronide** was the primary form of the metabolized glycoside found in the bile of cannulated rats. **Steviol glucuronide** from the bile proceeds to the GI tract where it is deconjugated back to **steviol**. **Steviol** is then either re-circulated back to the liver or is excreted in the feces (Fig. 3). The investigators propose that because of the pharmacokinetic similarities

between **stevioside** and **rebaudioside A**, information from **stevioside** safety studies can be used to extrapolate safety data on **rebaudioside A**. However, the pharmacokinetic parameters in rats are different enough that toxicity data from **stevioside** may not be reliably extrapolated to **rebaudioside A**. Independent toxicity studies on **rebaudioside A** would be needed to make any conclusive statements about its safety.

The difference in excretion pathways between humans and rats is explained by the different molecular weight thresholds for human and rat biliary excretion of organic anions such as **steviol glucuronide** (Renwick, 2008b). **Steviol** and **steviol glucuronide** are subject to enterohepatic re-circulation in the rat (Roberts and Renwick, 2008). Roberts et al. (2008) proposed that the rat is an ideal model for studying **steviol glycoside** toxicity in humans due to their similarities in **steviol glycoside** metabolism. However, due to the differences in **steviol glycoside** metabolism between humans and rats (Fig. 3) the rat does *not* appear to be an ideal model for studying **steviol glycoside** toxicity. **Steviol glycosides** are hydrolyzed to their aglycone by similar microflora in the guts of the two species, but **steviol** and its **glucuronide** conjugate undergo enterohepatic circulation in the rat. The rat's primary path of elimination is through the feces, while in humans **steviol** is conjugated to **glucuronide** and predominantly eliminated in the urine. While studies in rats are certainly useful, rat studies may lead to inappropriate conclusions because of the differences in metabolism between rats and humans.

Table 3 Pharmacokinetic parameters in **rats** following administration of single oral doses of ^{14}C **rebaudioside A**, ^{14}C-**stevioside**, and ^{14}C-**steviol** (Roberts et al., 2008)

	Administered Substance					
Parameters For Total Radioactivity	Rebaudioside A		Stevioside		Steviol	
	Male	Female	Male	Female	Male	Female
C_{max} (ng equiv./g)	90	177	101	279	114	264
T_{max} (h)	2	8	4	8	0.25	0.25
AUC_{72} (ng equiv.h/g)	645	3329	1617	4287	1251	1604
$AUC^{?}$ (ng equiv.h/g)	630	3349	1607[a]	4359	1926[a]	1926[a]
k (h^{1})	0.1462	0.0721	0.0795[a]	0.0460	0.0437[a]	0.0427[a]
$T_{1/2}$ (h)	5	10	9[a]	15	16[a]	16

[a] Not all of the criteria for reliability were met (see the methods section)

C_{max} – maximum observed plasma concentration

T_{max} – time of maximum observed plasma concentration

AUC_{72} – AUC calculated from zero to 72h after administration

$AUC_{?}$ – AUC extrapolated to infinity using the terminal slope

Dietary Intake Assessment

To project **rebaudioside A** intakes, Renwick et al. (2008) used a substitution method that takes into account actual intake data of high consumption artificial sweeteners (expressed as sucrose equivalents). Renwick et al. (2008) determined that this method was conservative enough for purposes of toxicity assessments. They found that the highest predicted intake of **rebaudioside A** would be in children and diabetics, but predicted that dietary exposure would always be less than 6 mg/kg bw/day. The estimates were calculated from published intake data of existing artificial sweeteners which had varying age ranges for children. In order to be conservative, Renwick et al. used data from the age group of each study that showed the highest intake. The predicted daily intake of **rebaudioside A** in average consumers was 1.3, 2.1 and 3.4 mg/kg bw in the general population, children, and children with diabetes, respectively. The predicted daily intake of **rebaudioside A** in high consumers was 3.4, 5.6, and 4.5 mg/kg bw in the general population, children, and children with diabetes, respectively.

Hemodynamic Effects

According to a 4-week study by Maki et al. (2008a), 1,000 mg/day **rebaudioside A** did not significantly alter resting, seated systolic blood pressure, diastolic blood pressure, mean arterial pressure, heart rate, or 24-hour ambulatory blood pressure responses in patients with low-normal to normal blood pressure compared with a placebo. 1,000 mg/day is 7-10 times the predicted average daily intake and 2-4 times the daily intake for high-intake consumers. A secondary analysis noted small changes in diastolic blood pressure and mean arterial pressure. The investigators assert that those findings are clinically insignificant.

Glucose Homeostasis

According to Maki et al. (2008b), **rebaudioside A** does not affect glucose homeostasis or resting blood pressure in patients with type 2 diabetes mellitus. The patients in this study were dosed for 16 weeks with 1,000 mg/day **rebaudioside A**. The investigators found no hypoglycemia in the **rebaudioside A** group compared to the placebo. However, there was a small but significant increase in alanine transaminase (ALT) levels in the **rebaudioside A** group (1.7 U/L) and a decrease in the placebo group (-1.5 U/L). The investigators suggest that the elevation in ALT levels was likely due to random variation and claim it has no clinical significance since mean levels of ALT stayed within normal range. No explanation of "clinically significant" or "normal range" was provided by the investigators. Further investigation would be necessary to determine one of the many possibly causes of the elevated ALT levels.

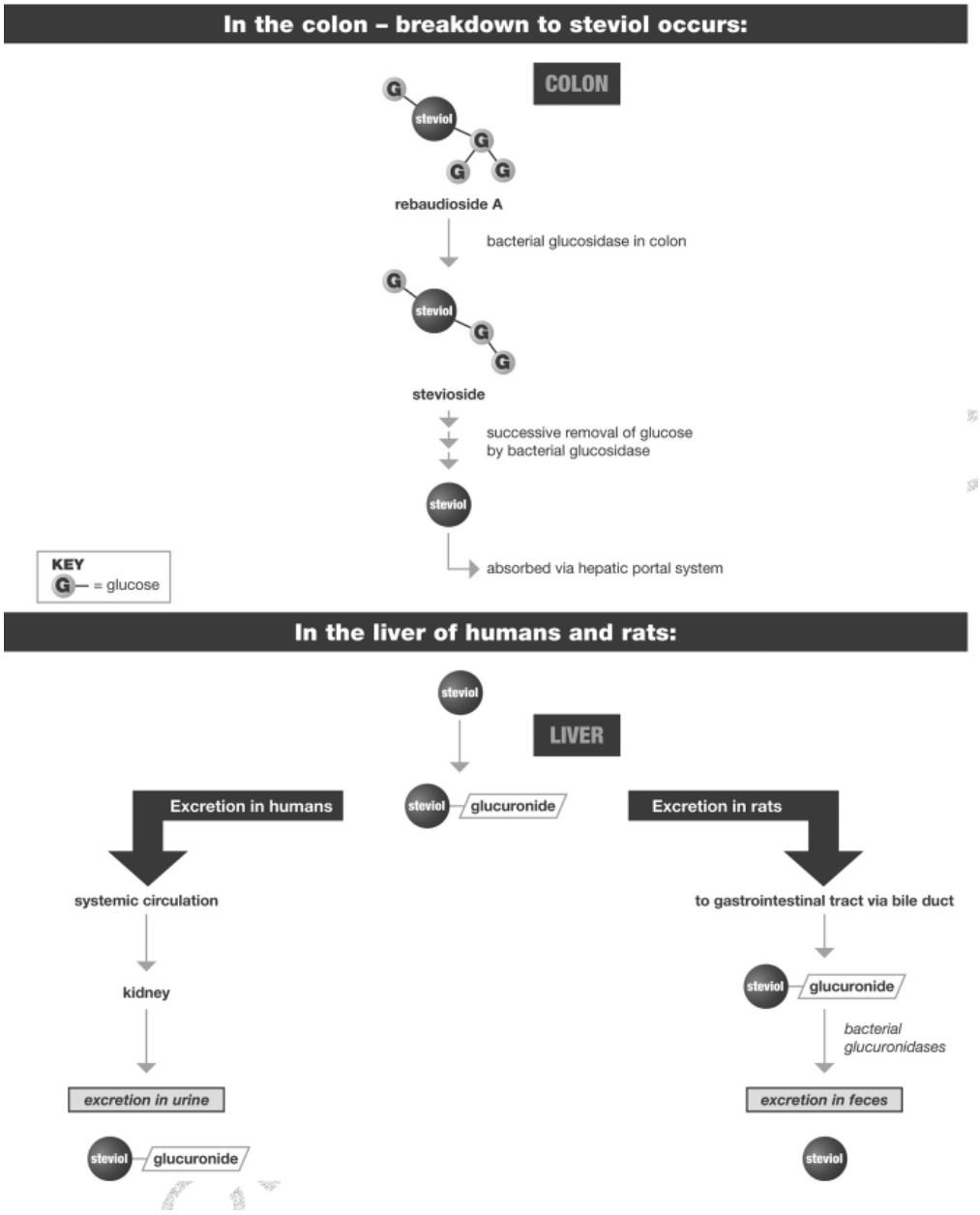

Figure 3
Summary of **Rebaudioside A** metabolism in the rat and human (Carakostas et al., 2008)

Genotoxic Effects

According to the literature review by Brusick et al. (2008) on the genotoxicity of **steviol** and stevioside, two of 16 studies showed genotoxic activity for **stevioside** and four of 15 studies (Brusick et al. did not include Pezzuto et al., 1985, and TM677 results by Matsui et al., 1996a) showed genotoxic activity for **steviol** (see Tables 4 and 5, respectively). **Rebaudioside A** was not found to cause mutations, chromosome damage, or DNA strand breakage in several *in vitro* and *in vivo* studies (Pezzuto et al, 1985; Nakajima 2000a; Nakajima 2000b; Sekihashi et al, 2002). Examples of (mostly positive) genotoxicity studies using **stevioside** include:

- **Stevioside** showed positive results in *Salmonella typhimurium* (*S. typhimurium)* strain TA98 at 50 mg/plate for 99% pure **stevioside** (Suttajit et al., 1993). The results showed a 4-fold increase in revertants without S9 extract and a 2-fold increase with S9. That study used **stevioside** pre-incubated with and without β-glucosidase. The treated and untreated samples showed roughly the same mutagenic results. Those results demonstrate that at 50 mg/plate, **stevioside** (without β-glucosidase or S9), **steviol** (**stevioside** + β-glucosidase), **stevioside** metabolite(s) (**stevioside** +S9), and **steviol** metabolite(s) (**stevioside** + β-glucosidase + S9) are all mutagenic in TA98.
- Klongpanichpak et al. (1997) did not find **stevioside** to be mutagenic in TA98 at a concentration of 50 mg/plate. However, they used S9 extract from rats, mice, hamsters, and guinea pigs; Suttajit et al. (1993) showed the strongest results without S9 extract.
- Suttajit et al. (1993) found that **stevioside** did not cause chromosomal aberrations in human lymphocytes incubated with 1, 5, and 10 mg/ml **stevioside** for 24 hours.
- Using the comet assay, Nunes et al. (2007) reported DNA breakage in blood, spleen, liver, and brain cells in Wistar rats exposed to 400 mg/kg of **stevioside** in drinking water. The strongest effects of **stevioside** were found in the liver cells.
- Matsui et al. (1996a) found that **stevioside** at doses up to 5 mg/plate was not mutagenic in *S. typhimurium* strains TA97, TA98, TA100, TA102, and TA104 with or without S9 or in *S. typhimurium* strains TA1535 and TA1537 and *E. coli* WP2 *uvrA/*pKM101 with S9. **Stevioside** was also not mutagenic in *S. typhimurium* strain TM677 with or without S9 at 10 mg/ml. **Stevioside** also gave negative results in the *umu test* with or without S9 and was negative in the spore and streak *rec-assays* with or without S9 at 10 mg/disk.

Metabolically-activated **steviol** was found to cause dose-related positive responses in several mutagenicity tests. These results indicate that a **steviol** derivative is likely responsible for its mutagenic activity, but the metabolite has not been identified (Brusick et al., 2008). The mutagenicity of **steviol** metabolites needs to be further investigated.

- Matsui et al. (1989) showed positive results for **steviol** in a plasmid mutagenesis study.
- Matsui et al. (1996a) found that **steviol** is mutagenic in *S. typhimurium* strain TM677, caused chromosome aberrations in cultured Chinese hamster lung (CHL) cells, and mutagenic in CHL cells in the presence of S9. In the same study, **steviol** produced a weak positive response with or without S9 in the *umu test.*
- Matsui et al. (1996a) found that **steviol** at doses up to 5 mg/plate was not mutagenic in *S. typhimurium* strains TA97, TA98, TA100, TA102, and TA104 with or without S9 or in *S. typhimurium* strains TA1535 and TA1537 and *E. coli* WP2 *uvrA/*pKM101 with S9. In the same study, **steviol** was also negative for spore and streak *rec-assays* and did not induce micronuclei in bone marrow erythrocytes of mice.
- A forward mutation assay using *S. typhimurium* strain TM677 found mutagenicity using 100 μg/ml **steviol** when assayed with S9 extract (Pezzuto et al., 1985).

- Suttajit et al. (1993) showed that **steviol** was not mutagenic in TA98 or TA100 at doses of 1-20 mg/plate. This study also showed that **steviol** does not cause chromosome aberrations at 0.1 and 0.2 mg/ml.

Table 4 Summary of genetic toxicity tests for **stevioside** (studies showing genotoxicity are highlighted with a box; either LED or HNED is indicated in third column)

Test	Response	LED/HNED*	Conditions	Comment	Citation
Reverse mutation in S. typhimurium, E. coli, B subtilis	Negative in all strains	Data not available	Tests conducted both with and without S9	Company sponsored testing program	Tama Biochemical C., Ltd. Safety of Stevia (Tama Report 1-20, 1981) cited in Medon et al., 1982
SCEs in human fetal cells, in vitro	Negative	Data not available	Test conditions not available	(see above)	(see above)
Chromosome aberrations in cultured rat cells, in vitro	Negative	Data not available	Test conditions not available	(see above)	(see above)
Forward mutation in S. typhimurium TM677	Negative	Data not available	Tested both with and without S9 induced by Arochlor 1254	SOT Abstract for 1982 meeting presentation	Medon et al., 1982, SOT Abstract publications
Reverse mutation in Ames strains TA98 and TA100	TA100 reported negative. TA98 reported positive, without S9	TA100 = 50 mg/plate (HNED) TA98 = 50 mg/plate (LED)	Pre-incubation method used S9 produced from rats induced with combination of Phenobarbital and 5,6-benzoflavone	Stevioside was 99% pure TA98 showed a 4-fold increase; however, a 1% impurity would be 500 ug/plate at the high concentration.	Suttajit et al., 1993
Chromosome aberrations in human lymphocytes, in vitro	Negative	10 mg/ ml	Study conducted with and without S9 from rats induced by phenobarbital and 5,6 benzoflavone	No data were provided for this study in the publication	(see above)
Reverse mutation in Ames strains plus E.coli WP_2 uvrA/pKM101	Negative in all strains tested	5 mg/plate for all strains and treatment conditions	Pre-incubation method used, Standard Ames strains plus TA102 and TA104	Stevioside purity was 83%, No toxicity was seen in the test at the highest concentration tested	Matsui et al., 1996a
Umu test	Negative	5 mg/ml	Performed according to the methods of Oda et al., 1985	S9 used was from rats treated with a combination of Phenobarbital and 5,6 benzoflavone	(see above)
Rec-assay	Negative	10 mg/paper disk	Performed	Used S9 from	(see above)

			according to the methods of Hirano et al., 1982	rats treated with Arochlor 1254	
Chromosome aberrations in CHL cells, in vitro	Negative	12 mg/ml	Treatments were for 6, 24 and 48 hours without S9 and for 6 hours with S9, maximum concentrations set at >50% toxicity	Used S9 from rats treated with Arochlor 1254	(see above)
Reverse mutation in Ames strains TA98 and TA100	Negative results in both strains for all treatment conditions	50 mg/plate	Pre-incubation method used, all S9s induced by a combination of Phenobarbital and 5,6-benzoflavone	Compared S9s from rat, mouse, hamster and guinea pig.	Klongpanichpak, et al., 1997.
Mouse lymphoma forward mutation assay	Negative	5000 µg/ml	Micro-titer method used, 3 hr exposures with and without S9 plus 24 hr treatment without S9	No toxicity observed at the maximum concentration under either treatment condition	Oh et al., 1999
Mouse micronucleus assay, in vivo	Negative	250 mg/kg	Single dose with 24 harvests of bone marrow and hepatocytes	ICR mice treated at only one dose, no toxicity reported	(see above)
Comet assay, in vivo	Negative results in all tissues examined	2000 mg/kg oral administration to ddY mice	Tissues examined for DNA damage at 3 and 24 hours post exposure	Organs included glandular stomach, colon, liver, kidney, bladder, lung, brain and bone marrow	Sasaki et al., 2002
Comet assay, in vivo	Negative results in all tissues examined	2000 mg/kg administration to BD F_1	Tissues examined for DNA damage at 3 and 24 hours post exposure	Organs included stomach, colon and liver	Sekihashi et al., 2002
Comet assay, in vivo	Positive in all tissues examined	4 mg/ml in drinking water	Blood cells examined weekly, spleen, liver and brain tissues examined at exposure termination	Wistar rats given stevia extract for 45 days in their drinking water. No DNA effects were seen before week five.	Nunes et al., 2007

- LED = Lowest concentration tested that shows a clearly positive response according to the criteria of the specific test.
- HNED = Highest concentration tested for a study with negative results

Table 5
Summary of genetic toxicity tests for **steviol** (studies showing genotoxicity are highlighted with a box; either LED or HNED is indicated in third column)

Test	Response	LED/HNED*	Conditions	Comment	Citation
Reverse mutation in Ames strains TA98 and TA100	Negative	20 mg/plate	Pre-incubation modification using S9 from rats induced by a combination of Phenobarbital and 5,6-benzoflavone	Steviol was prepared by periodae oxidation of stevioside followed by acid hydrolysis and recrystallization	Suttajit et al., 1993
Chromosome aberrations, in vitro	Negative	200 µg/ml	Studies conducted in human lymphocyte cultures with and without S9	No actual data provided to support author's conclusions	(see above)
Reverse mutation in Ames strains plus E. coli WP_2 uvrA/pKM101	Negative	5000 µg/plate	Pre-incubation modification using S9 from rats induced by Kanechlor KC 400	Negative in all standard strains plus strains TA102 and TA104	Matsui et al., 1996a
Umu test	Positive	2500 µg/plate	Performed according to methods of Oda et al., 1985 with S9 from rats induced by a combination of Phenobarbital and 5,6-benzoflavone	Approximate 2-fold increase at the high concentration considered a weak positive	(see above)
Rec-assay	Negative	10 mg/paper disk	Performed according to methods of Hirano et al., 1982	Used S9 from rats induced by PCBs	(see above)
Chromosome aberrations, in vitro	Positive	1000 µg/ml	Studies conducted in CHL cells, Cells sampled at 6, 24 and 48 hours without S9 and at 6 hours with S9	Used S9 from rats induced by PCBs Positive response only with S9	(see above)
Gene mutation in mammalian cells, in vitro	Positive	400 µg/ml	Studies conducted in CHL cells and assessed by resistance to diphtheria toxin	Used S9 from rats induced by PCBs Positive response only with S9 at highly toxic treatments (3% survival)	(see above)
Mouse micronucleus assay, in vivo	Negative	500 mg/kg	MS/Ae mouse strain used, compound administered i.p. with 24 and 48 hour harvests	Toxicity seen at 1000 mg/kg	(see above)
Reverse mutation in Ames strains TA98 and TA100	Negative	2000 µg/plate	Pre-incubation method using S9 from animals induced by a combination of Phenobarbital and 5,6-benzoflavone	Authors compared S9s from rat, mouse, hamster and guinea pig All tests negative	Klongpanichhpak et al., 1997
Gene mutation in mammalian cells, in vitro	Negative	341 µg/ml	Study conducted in mouse lymphoma cells L5178Y at the TK gene (with and without S9)	Toxicity did not exceed a RTG of 40%	Oh et al., 1999

Mouse micronucleus assay, in vivo	Negative	200 mg/kg	Single oral dose with only a 24 hour harvest of liver hepatocytes	No harvest at 48 hours	(see above)
Micronucleus assay, in vivo	Negative	4 gm/kg for hamsters and 8 gm/kg for rats and mice	Study conducted using single oral dose in mice, rats, hamsters (both sexes), bone marrow cells harvested at 24, 30, 48 and 72 hours post exposure	Toxicity seen in all species at high dose with females appearing to be more sensitive	Temcharoen et al., 2000
Comet assay, in vivo	Negative	2 gm/kg	Mice were exposed by a single oral dose and tissues collected at 3 and 24 hours post exposure	Stomach, colon, liver, kidney, and testis tissues evaluated for DNA damage	Sekihashi et al., 2002
Comet assay, in vitro	Negative	500 µg/ml	Studies conducted in TK6 and WTK1 cell cultures both with and without S9		(see above)
Plasmid mutagenesis	Positive	Not reported	Induction of xgprt mutants in plasmid pSV2-gpt in the presence of S9	Mutants analyzed and shown to be small deletions which was offered as an explanation why steviol was not mutagenic in the Ames strains	Matsui et al., 1989

- LED = Lowest concentration tested that shows a clearly positive response according the criteria of the specific test.
- HNED = Highest concentration tested for a study with negative results

Subchronic Toxicity

Curry et al. (2008a) performed subchronic tests of **rebaudioside A** in a 13-week study on Han-Wistar rats. After a 4-week palatability study, investigators dosed rats with 12,500, 25,000, and 50,000 ppm **rebaudioside A** (dosing equivalents provided in Table 6). In the 13-week study, mean body weight gain was significantly less in the first four days for males and females in the 25,000 ppm and 50,000 ppm treatment groups compared to controls. Males in all treatment groups had significantly less mean body weight gain than control groups for the length of the study. Females showed similar results but only in the 25,000 ppm and 50,000 ppm treatment groups. Investigators concluded that the reduced weight gain was not an adverse effect due to the following considerations: the effect of **rebaudioside A** on food conversion efficiency was minimal; **rebaudioside A** affects food consumption and body weight gain due to palatability issues; reduced food consumption was consistently associated with treatment groups that demonstrated reduced weight gain; and toxicity was not observed over the dose-range in the 13-week study (Flamm et al., 2003). Also, based on WHO guidance from 1987 (WHO, 1987), the body-weight-gain reductions observed in both studies would not be considered an adverse effect.

Large, but inconsistent, reductions in bile acids occurred in both studies across all treatment groups. However, liver enzyme activities (as measured in serum) and hepatic histopathology were within normal limits and did not differ significantly from controls. Mean plasma urea and creatinine concentration in several treatment groups in both studies were slightly increased from controls, but levels always remained within reference limits. Because of low urine volume, high urine specific gravity, and no change in other urinalysis parameters, investigators concluded that these results were probably not a sign of renal failure but of dehydration, possibly from the osmotic effects of high doses of **rebaudioside A**. Macroscopic and microscopic evaluation of the kidneys showed no alterations.

Absolute epidydimal weights of high-dose males and the absolute weights of the ovaries of high-dose females were significantly lower than controls. Spermatogenesis was unaffected by treatment and testicular atrophy was not detected. Microscopic histopathology did not detect any other effects on the testes.

The NOAEL for **rebaudioside A** in Han-Wistar rats for the 13-week study was determined to be 50,000 ppm for the 13-week study (4,161 and 4,645 mg/kg bw/day in males and females, respectively). This is ~2,000-fold greater than the ADI of 2.0 mg/kg bw/day of **steviol glycosides** established by JECFA and ~1,000-fold greater than the predicted human exposure.

Table 6 Average achieved **rebaudioside A** dose in treated rats (13-week study; mg/kg bw/day)

	Males: Week 1	Males: Week 13	Females: Week 1	Females: Week 13
12,500 ppm	1,506	698	1,410	980
25,000 ppm	3,040	1,473	2,841	1,914
50,000 ppm	5,828	3,147	5,512	3,704

Carcinogenicity

Studies in rats have failed to produce any evidence of carcinogenicity of **stevioside**, though rebaudioside A, the subject of the GRAS notification, itself has not been tested (Carakostas et al., 2008). Three studies support non-carcinogenicity of that substance:

- Fischer 344 rats administered 5% **stevioside** in their diet in a relatively brief 36-week study showed no increased development of pre-neoplastic or neoplastic lesions in the urinary bladders with and without an initiating dose of the bladder carcinogen N-nitrosobutyl-N-(4-hydroxybutyl) amine (Hagiwara et al., 1984).
- Xili et al. (1992) found no neoplastic or pre-neoplastic lesions in Wistar rats in a 24-month chronic toxicity and carcinogenicity study with 85% pure **stevioside** (600 mg/kg bw/day).

- A 24-month carcinogenicity study by Toyoda et al. (1997) did not find any increase in non-neoplastic or neoplastic lesions in Fischer 344 rats exposed to 2.5% and 5% of 95.6% pure **stevioside** in the diet. JECFA used the 970 mg/kg bw/day dose (2.5% dose in male rats) used in the Toyoda study to set the temporary ADI of 2 mg/kg bw/day (Carakostas et al., 2008).

It is important to note that FDA normally asks for tests in *two* rodent species, usually rats and mice, in a compound with such a high predicted exposure level. Also, all three of the aforementioned studies were done with **stevioside**, not **rebaudioside A**. It is possible that differences in metabolism and toxicokinetics would result in different risks of carcinogenicity using the two **steviol glycosides**.

Reproductive Toxicity

Older studies reported anti-fertility effects, as well as decreases in the weights of the testes, seminal vesicle, and cauda epididymides and a reduction in spermatozoa concentration, in rats administered crude **stevia** extracts (Mazzei-Plana and Kuc 1968; Olivereira Filho et al., 1989, and Melis, 1999). However, other studies with purified **stevioside** (not **rebaudioside A**, the subject of the GRAS notification) failed to produce these reproductive effects (Mori et al., 1981; Yodyingyuad and Bunyawong, 1991; Usami et al., 1995).

In a recent study, Curry et al. (2008b) found no treatment-related effects of **rebaudioside A** on mating performance, fertility, gestation lengths, and estrous cycle in the F_0 and F_1 generation of rats in a two-generation study. After a preliminary short-term study to determine appropriate dosage levels, those investigators dosed F_0 and F_1 generation rats with 0, 7,500, 12,500, and 25,000 ppm **rebaudioside A** via the diet (dosing equivalents provided in Table 7). Female rats in the 12,500 ppm and 25,000 ppm groups and male rats in the 25,000 ppm group of the F_1 generation and male and female rats in the 25,000 ppm group of the F_2 generation showed significant decreases in body weight gains compared to controls. The investigators considered those effects to be toxicologically insignificant due to the lack of adverse effects on those animals' survival, condition of their offspring, their pre-weaning reflex development, weight gain after 25 days, and timing of sexual maturation. The investigators presume that the weight-gain effects were due to the nature of a diet supplemented with high levels of intense sweeteners that normally leads to reduced consumption and nutritional content (WHO, 1987). Investigators found higher adjusted mean liver weights in females in the 12,500 ppm and 25,000 ppm F_0 and F_1 generations. There were no changes in the relative weights of the thymus glands in treated groups compared to the control group, but there was a reduction in relative and absolute weights of the spleens in several animals of the F_1 and F_2 treatment groups. However, the investigators did not find any clinically significant alterations in blood count or the histopathology of other immune system organs. There was no change on the ability of the females of the F_0 and F_1 generations to litter and rear their offspring to weaning. There was also no effect on litter size, sex ratio, and pre- and post-natal survival of offspring.

Curry et al. (2008b) concluded that **steviol glycosides** do not pose a reproductive or developmental hazard. They found the NOAEL for **rebaudioside A** for Han-Wistar rats to be 25,000 ppm for reproductive effects in the F_0 generation and for survival, growth, and general condition of F_1 and F_2 offspring.

Table 7 Average achieved **rebaudioside A** dose in treated rats (mg/kg bw/day)

	F_0 Males	F_0 Pre-paired Females	F_0 Gestating Females	F_0 Lactating Females	F_1 Males	F_1 Pre-paired Females	F_1 Gestating Females	F_1 Lactating Females
7,500 ppm	586	699	648-713	715-1,379	734	798	562-625	976-1,406
12,500 ppm	975	1,115	1,119-1,169	1,204-2,388	1,254	1,364	911-1,058	1,752-2,394
25,000 ppm	2,048	2,273	2,263-2,381	2,602-5,019	2,567	2,768	2,036-2,212	3,289-4,893

Summary and Discussion

Investigators demonstrated that **rebaudioside A** has no adverse hemodynamic effects in people with normal to low-normal blood pressure dosed with 1,000 mg/day for 4 weeks. Investigators also found no clinically significant effects of **rebaudioside A** treatment on patients with type 2 diabetes mellitus. The only concerning finding of the glucose homeostasis study was an increase in ALT levels. This finding is not of great concern since it did not lead to any adverse effects, but further investigation would be necessary to determine the cause of the increase and the long-term effects of **rebaudioside A** on ALT levels.

In both the rat and human metabolism studies, investigators demonstrate that **rebaudioside A** and **stevioside** have similar metabolic pathways within each species. However, **rebaudioside A**'s extra glucose moiety causes differences in the two compounds' pharmacokinetic parameters (Tables 1, 2, and 3). Because of those differences, toxicity data for **stevioside** cannot be assumed to be an appropriate basis for assessing the safety of **rebaudioside A**. Separate toxicity studies on **rebaudioside A** itself are necessary to make definitive conclusions about its safety.

Investigators whose studies are published in the Food and Chemical Toxicology supplement concluded from the metabolism studies that the rat is an ideal model for **steviol glycoside** human toxicity studies. Both species hydrolyze the glycosides into **steviol** by the gut microflora, but after absorption the metabolic pathways differ (Fig. 3). Since **steviol glycoside** metabolism in rats and humans is not identical, the rat may not be an ideal model for evaluating human toxicity.

Hutapea et al. (1997) reported a steviol-16,17-epoxide **stevioside** metabolite. Given the structures of **stevioside** and **rebaudioside A**, an epoxide is a likely metabolite. The

possibility of a **steviol glycoside** forming an epoxide metabolite needs to be investigated carefully, because epoxides may react with DNA and cause mutations.

Genotoxicity studies published in the Food and Chemical Toxicology supplement, as well as other studies, raise significant concerns. Suttajit et al. (1993) reported positive results for reverse mutations in the *S. typhimurium* strain TA98 with and without S9 extract at a 50 mg/plate dose of **stevioside**. Certain studies in that supplement cited the negative mutagenicity results by Klongpanichpak et al. (1997) to try to discredit the work of Suttajit, but Klongpanichpak used S9 extract while the mutagenic results from Suttajit were highest without S9. The ability of **stevioside** and **rebaudioside A** to cause reverse mutations as indicated by TA98 needs to be further investigated, because such mutations suggest the possibility of carcinogenesis. **Stevioside** also caused DNA breakage in blood, spleen, liver, and brain cells in rats (Nunes et al., 2007). The mutagenicity of this compound requires further, careful investigation.

Steviol was found positive in an *umu* test, mutagenic in a forward-mutation assay, and caused chromosome aberrations and gene mutations in mammalian cells (Matsui et al., 1996a) and plasmid mutagenesis (Matsui et al., 1989). Pezzuto et al. (1985) found that **steviol** is both toxic and mutagenic in the TM677 assay using S9 extract. Matsui's studies were all conducted with S9. These results indicate that **steviol** has a mutagenic metabolite that has yet to be identified. These finding are very important because **rebaudioside A** is hydrolyzed into **steviol** before it is absorbed by the GI tract. Before **rebaudioside A** can be generally regarded as safe, the mutagenic **steviol** intermediate needs to be identified and further studied. Overall, because of the warning flags raised by several studies, it is critical that further genotoxicity testing be conducted to clarify the potential risks.

Carcinogenicity studies have not found **stevioside** to be carcinogenic in rats (Hagiwara et al., 1984, Toyoda et al., 1997, Xili et al., 1992), but further studies on **rebaudioside A**, including a study on mice, are needed for several reasons:

- The rat is an imperfect model for evaluating **steviol glycoside** toxicity and carcinogenicity risks in humans because of the differences in metabolism in the two species.
- Several genotoxicity studies that found that **stevioside** and **steviol** cause mutations, chromosomal damage, and DNA breakage indicate the need for greater reassurance of noncarcinogenicity.
- The differences in pharmacokinetics between **rebaudioside A and stevioside** indicate the need to test **rebaudioside A** itself in two rodent species.
- Based on a maximum estimated intake level of **steviol glycosides** of 1.7 mg/kg bw/day (**steviol** equivalent), **steviol glycosides** should be considered a concern level III chemical, for which the FDA recommends carcinogenicity studies in *two* rodent species (usually mice and rats) (FDA Redbook, 2000).

The value of testing chemicals in two species is indicated by the fact that bioassays of chemicals with a variety of structures that *did not* find carcinogenicity in rats *did* find

carcinogenicity in mice (see appendix C). In sum, a lifetime carcinogenicity study in mice of **rebaudioside A** must be conducted before that substance (or other **steviol glycosides**) can be accepted as a GRAS ingredient that likely would be consumed by tens of millions of people.

In conclusion, the FDA should ensure that the genetic toxicity studies that produced either positive or conflicting results be repeated. Studies that look at potential DNA adducts related to the potential reactive metabolites (C-13 carbonium ion or the epoxide) of **steviol** would be a strong addition to the genotoxicity data. Finally, the FDA should require carcinogenicity[4] and toxicology studies in rats and in mice before accepting **rebaudioside A** as a GRAS substance or approving it as a food additive. Ideally, all those studies would be conducted by an independent party, such as the National Toxicology Program of the National Institute of Environmental Health Sciences.

[4] Ideally, the studies would include an in utero phase and follow the animals for almost their entire lives, instead of prematurely ending the study after 104 weeks. Huff J, Jacobson MF, Davis DL. 2008. The limits of 2-year bioassay exposure regimens for identifying chemical carcinogens. *Environ Health Perspect*: doi:10.1289/ehp.10716. [Online 30 June 2008] http://ehp.niehs.nih.gov/docs/2008/10716/abstract.html

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Appendix A: List of Abbreviations

ADI=Acceptable Daily Intake
Ae_u=amount excreted in urine
Ae_f=amount excreted in feces
ALT=Alanine Transaminase
AUC=Area Under the Curve
Bw=body weight
CL_R=clearance rate
cm=centimeter
C_{max}=maximum concentration
g=gram
GRAS=Generally Recognized as Safe
HPLC-UV=high performance liquid chromatography-ultraviolet
hr=hours
JECFA= Joint FAO/WHO Expert Committee on Food Additives
k=terminal rate constant
kg=kilogram
L=liter
LC-DAD-MS=liquid chromatography-diode array detector-mass spectrometry
LD_{50}=lethal dose, 50%
LED=Lowest concentration tested that shows a clearly positive response according to the criteria of the specific test
HNED=Highest concentration tested for a study with negative results
mg=milligram
min=minute
ml=milliliter
n=number
NOAEL=No Observed Adverse Effect Level
NOEL=No Observed Effect Level
ng=nanogram
NTP=National Toxicology Program
ppm=parts per million
s=second
$t_{1/2}$=half life
T_{max}=time to maximum

Appendix B: Conflicts of Interest

Authors Bisognano, Brown, Brusick, Renwick, Roberts, and Tarka received financial support from Cargill for consulting services. Authors Brown, Brusick, Renwick, Roberts, Tarka, and Wheeler received financial support form Cargill for manuscript preparation. Authors Boileau, Curry, and Fosdick are employed by Cargill, Inc. Authors Carakostas, Clos, DuBois, Prakash, and Wilkensare are employed by The Coca-Cola Company.

Appendix C: Results of Cancer Bioassays Conducted in Mice and Rats
Source: National Toxicology Program

Chemical	Male Rats	Female Rats	Male Mice	Female Mice	Reference
Primidone	EE	NE	CE	CE	NTP, 2000
1,3-Butadiene	NE	NE	CE	CE	NTP, 1984
Diphenylhydantoin (Phenytoin)	EE	NE	NE	CE	NTP, 1992
N-Methylolacrylamide	NE	NE	CE	CE	NTP, 1988
Chloroethane (ethyl chloride)	EE	EE	NA	CE	NTP, 1989
Bis (2-chloro-1-methylethyl)ether, technical grade	NE	NE	CE	CE	NCI, 1979, technical report series (trs) no. 191
5-Chloro-o-toluidine	NE	NE	CE	CE	NCI, 1979, trs no. 187
4-Chloro-o-toluidine hydrochloride	Neg	Neg	Pos	Pos	NCI, 1979, trs no. 165)
DDE	Neg	Neg	Pos	Pos	NCI, 1978, trs no. 131
Toxaphene	EE	EE	CE	CE	NCI, 1979, trs no. 37
1,1,2,2-Tetrachloroethane	EE	EE	CE	CE	NCI, 1978, trs no. 27
Aldrin	EE	EE	CE	NE	NCI, 1978, trs no. 21
Heptachlor	EE	EE	CE	CE	NCI, 1977, trs no.9
Chlordane	EE	EE	CE	CE	NCI, 1977, trs no. 8
Propylene glycol mono-t-butyl ether	EE	NE	CE	CE	NTP, 2004

EE – equivocal evidence
NE – no evidence
CE – clear evidence
NA – not available