GR

ORIGINAL SUBMISSION

SPHERIX

December 23, 2008

RECEIVED
DEC 30 2008
BY:----------

Office of Food Additive Safety
HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

To Whom It May Concern:

Enclosed please find three copies of "Generally Recognized As Safe (GRAS) Notification for the Use of Stevia Rebaudiana (Bertoni) Bertoni". This GRAS notification has been prepared by Spherix Incorporated for McNeil Nutritionals, LLC.

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., CEO, Spherix Incorporated, 6430 Rockledge Drive, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ckruger@spherix.com.

Should you have any questions or concerns, please contact me at the number listed above.

Sincerely,

Claire L, Kruger, Ph.D., D.A.B.T.
Chief Executive Officer

Enclosures:

Original and 2 copies of "Generally Recognized As Safe (GRAS) Notification for the Use of Stevia Rebaudiana (Bertoni) Bertoni"

Original and 2 copies of the GRAS Panel Consensus Statement for the above-referenced GRAS Notification

6430 Rockledge Drive, #503
Westmoreland Building
Bethesda, MD 20817
Tel.: (301) 897-2540
Fax: (301) 897-2567
www.spherix.com

RECEIVED
DEC 3 0 2008
BY:

Generally Recognized As Safe (GRAS) Notification for the Use of *Stevia Rebaudiana* (Bertoni) Bertoni

Prepared for:

McNeil Nutritionals, LLC
Fort Washington, Pennsylvania

Prepared by:

Spherix Incorporated
Bethesda, Maryland

August 21, 2008

Generally Recognized As Safe (GRAS) Notification for the Use of *Stevia Rebaudiana* (Bertoni) Bertoni

Prepared for:

McNeil Nutritionals, LLC
Fort Washington, Pennsylvania

Prepared by:

Spherix Incorporated
Bethesda, Maryland

August 21, 2008

CONTENTS

I. GRAS EXEMPTION CLAIM 1

A. NAME AND ADDRESS OF SPONSOR 1
B. COMMON OR USUAL NAME OF GRAS SUBSTANCE 1
C. INTENDED USE 1
D. BASIS FOR GRAS DETERMINATION 1
E. AVAILABILITY OF INFORMATION 5
F. SIGNATURE 5

II. DESCRIPTION OF SUBSTANCE 6

A. COMMON OR USUAL NAME(S) 6
B. DEFINITION 6
C. SOURCE 6
D. CHEMICAL NAMES AND SYNONYMS 6
E. C.A.S. NUMBER 7
F. CHEMICAL FORMULA 7
G. MOLECULAR WEIGHT 7
H. STRUCTURAL FORMULA 8
I. PHYSICO-CHEMICAL PROPERTIES 9
J. TERMINOLOGY 9
K. MANUFACTURING PROCESS 10
1. General description of steviol glycosides production 10
2. Manufacturing Process 1 11
3. Manufacturing Process 2 12
L. SAFETY OF RAW MATERIALS AND CHEMICALS USED IN THE PROCESS 15
M. SPECIFICATIONS 16
1. Current Regulatory Specifications 16
2. Potential impurities 18
3. McNeil's Stevia Product Specifications and Batch Analyses 19
N. STABILITY OF THE STEVIOL GLYCOSIDES 21

III. HISTORY OF USE AND INTENDED USE 23

A. HISTORY OF USE 23
B. INTENDED USE 30
1. McNeil Stevia Intake Calculation using MRCA Data: 30
2. McNeil Stevia Intake Calculation using NHANES Data: 32
3. Other Publicly Available Intake Estimates of Steviol Glycosides as Intense Sweeteners: 33

IV. SAFETY 36

A. ABSORPTION, METABOLISM, DISTRIBUTION, AND EXCRETION (ADME) OF STEVIOSIDE 37
1. Introduction: 37
2. Review of Studies: 38

B. TOXICOLOGY STUDIES 51
1. Introduction: *51*
a) Acute Toxicity 53
(1) Introduction 53
(2) Review of studies 53
b) Subchronic toxicity 54
(1) Introduction 54
(2) Review of studies 54
c) Chronic toxicity 76
(1) Introduction 76
(2) Review of studies 77
C. REPRODUCTIVE/DEVELOPMENTAL TOXICITY 86
1. Introduction *86*
2. Review of studies *86*
D. GENOTOXICITY AND MUTAGENICITY 99
1. Introduction *99*
2. Review of studies *99*
a) Bacterial Mutagenicity 99
b) Mammalian *In Vitro* and *In Vivo* Genotoxicity 102
(1) Stevioside 102
(2) Steviol 104
E. PHYSIOLOGICAL EFFECTS 111
1. Introduction *111*
2. Effect of Stevioside on Blood Glucose and Insulin levels *114*
a) Studies in Animals: 114
(1) Introduction 114
(2) Review of studies 114
(3) In Vitro Studies: 120
(4) Other related studies 121
b) Studies in Humans: 135
(1) Introduction 135
(2) Review of Studies 135
3. Effect of Stevioside on Blood Pressure *145*
a) Studies in Animals: 145
(1) Introduction 145
(2) Review of studies 145
b) Studies in Humans: 155
(1) Introduction 155
(2) Review of studies 155

V. REFERENCES 166

TABLES

Table 1. Terminology 9
Table 2. WHO Chemical and Physical Properties of a Stevia Extract Product 17
Table 3. Steviol Glycosides Specifications and Batch Analyses 18
Table 4. Concentration of Major Glycosides in *S. rebaudiana* (Bertoni) Bertoni 24
Table 5. Non-Glycosidic Components of *S. rebaudiana* (Bertoni) Bertoni 27
Table 6. Food Use Levels of Steviol Glycosides 28
Table 7. International Estimates of Stevioside and Steviol Intake 30
Table 8. MRCA Intake Calculation* 32
Table 9. Estimated Intakes of Steviol Equivalents from Use of McNeil Product in High-Intensity Table-Top Sweeteners, NHANES 2003-2004 33
Table 10. FSANZ Estimated dietary exposure to steviol glycosides* 34
Table 11. Predicted dietary exposures of rebaudioside A based on sweetener substitution[a] 35
Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside 42
Table 13. Relative feed consumption, body weight gain and feed conversion efficiency of hamsters fed steviol during the first 3 months (98 days) of the chronic toxicity/carcinogenicity study 55
Table 14. Amount of steviol ingested by hamsters during the first 3 months (98 days) of the chronic toxicity/carcinogenicity study 55
Table 15. Body weight gain of the rats in the 4-week study 56
Table 16. Feed consumption of the rats in the 4-week study during selected periods 57
Table 17. Body weight of rats in the 13-week study 60
Table 18. Selected clinical chemistry parameters of the rats in the 13-week study on day 89 61
Table 19. Experimental design and rebaudioside A consumption 62
Table 20. Body weight (g) of animals during selected intervals 63
Table 21. Serum chemistry values (mean ± SD) at week 13 in males 64
Table 22. Serum chemistry values (mean ± SD) at week 13 in females 65
Table 23. Tissue and Organs Collected for Examination 67
Table 24. Effect of steviol glycosides on blood chemistry variables in male rats at interim (week 4) and terminal (week 13) study periods 72
Table 25. Reference range for relevant blood chemistry variables in male rats (Charles River 2006) 73
Table 26. Effect of steviol glycosides on blood chemistry variables in female rats at interim (week 4) and terminal (week 13) study periods 73
Table 27. Reference range for relevant blood chemistry variables in female rats (Charles River 2006) 75
Table 28. Incidence of neoplasms in hamsters fed steviol for up to 22 months (male) and 18 months (female) 78
Table 29. Acute Animal Toxicology Studies 79
Table 30. Subchronic Animal Toxicology Studies 81
Table 31. Chronic Animal Toxicology Studies 84
Table 32. Mating performance and fertility 91
Table 33. Gestation length and gestation index 91
Table 34. Litter size and offspring survival 92
Table 35. Sperm analysis and morphology 93

Table 36. Reproductive and Developmental toxicity studies 94
Table 37. Studies of genotoxicity with purified stevia extract and its major components, stevioside and redaubioside A 106
Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin 122
Table 39. Glycosylated hemoglobin at baseline and changes from baseline to weeks 4, 8, 12, and 16[a] 137
Table 40. Fasting Glucose, Insulin, C-Peptide, Body Weight, Blood Pressure, and Lipids at Baseline and Following Treatment[a] 138
Table 41. Serum Chemistry and Hematology Parameters at Screening (Week -2) and the Changes from Screening to Week 16[a] 139
Table 42. Human Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin. 141
Table 43. Animal Studies of the Effects of Steviol Glycosides on Blood Pressure 149
Table 44. Characteristics of the steviol glycosides and placebo groups at baseline and post-treatment: group 1 (type 1 diabetes) 158
Table 45. Characteristics of the steviol glycosides and placebo groups at baseline and post-treatment: group 2 (type 2 diabetes) 159
Table 46. 24-hour Ambulatory Blood Pressure Monitor Readings at Baseline (Week 0) and the Change from Baseline to Week 4[a,b] 160
Table 47. Supine and Standing Blood Pressure and Heart Rate Measurements During Meal Tests at Week 0 and 4 161
Table 48. Changes from Baseline to Treatment of Blood Pressure Measurements in the Subgroup of Subjects with Baseline Systolic Blood Pressure Below the Sex-Specific Median[a,b] 161
Table 49. Serum Chemistry and Hematology Parameters at Screening (Week -1) and the Changes from Screening to Week 4[a] 162
Table 50. Human Studies of the Effects of Steviol Glycosides on Blood Pressure 163

FIGURES

Figure 1. Production of Stevia (Process 1) 14
Figure 2. Production of Stevia (Process 2) 15
Figure 3. Effect of 90-Day oral steviol glycosides treatment on body weight in male rats. 69
Figure 4. Effect of 90-Day oral steviol glycosides treatment on body weight in female rats. 70

I. GRAS EXEMPTION CLAIM

A. Name and Address of Sponsor

McNeil Nutritionals, LLC
601 Office Center Drive
Fort Washington, PA 19034

Contact: Richard Reo
Director, Regulatory Affairs
215-273-4953

B. Common or Usual Name of GRAS Substance

The substance that is the subject of this Generally Recognized As Safe (GRAS) determination is steviol glycosides.

C. Intended Use

The steviol glycosides product from McNeil Nutritionals (hereinafter, "McNeil Stevia") is intended to be used as a tabletop sweetener, including packet, tablet and granular powder form.

D. Basis for GRAS Determination

This GRAS determination for the use of steviol glycosides as a table- top sweetener as described in Section C of this chapter is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of steviol glycosides from the intended uses specified above, has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that steviol glycosides are safe, and GRAS, under the intended conditions of use, the safety of the intake of steviol glycosides have been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of steviol glycosides as an ingredient in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

000009

- McNeil Stevia fully complies with the established food grade Joint FAO/WHO Expert Committee on Food Additives (JECFA) specifications for steviol glycosides (JECFA 2007); this allows for seven steviol glycosides (rebaudioside A, stevioside, rebaudioside B, steviolbioside, rebaudioside C, dulcoside A and rubusoside) to be present, the sum of which accounts for a minimum of 95% of the dried substance. The rebaudioside A content of McNeil Stevia accounts for ≥ 80% of the steviol glycoside content.
- All steviol glycosides are metabolized to steviol, which is the basis for appropriately extrapolating results from studies on extracts that are comprised predominantly of stevioside to extracts containing predominantly rebaudioside for purposes of safety assessment. Based on relative molecular weights, stevioside quantities are multiplied by 0.40 and rebaudioside A quantities by 0.33 to convert to steviol equivalents for comparison of Estimated Daily Intake (EDI) to an Acceptable Daily Intake (ADI).
- Intake of McNeil Stevia has been estimated using both NHANES and MRCA data. Intake as a table top sweetener is estimated to be 17 and 35 mg steviol equivalents/day at the mean and 90th percentile of intake for ages ≥ 19 years (equivalent to 0.28 and 0.58 mg steviol equivalents/kg/day at the mean and 90th percentile of intake) (NHANES 2003-2004) and 7 and 18 mg steviol equivalents/day at the mean and 90th percentile of intake for all ages, eaters only (equivalent to 0.14 and 0.33 mg steviol equivalents/kg/day at the mean and 90th percentile of intake) (MRCA 1992-1993).
- Older, publicly available studies on steviol glycosides included crude or semi-purified extracts that were poorly defined, however, more recent, pivotal studies on well defined extracts that address comparative metabolism, acute, repeat-dose, chronic, genetic and reproductive toxicity and clinical safety have become available to document the safety of steviol glycosides at the proposed EDI. A 90-day rodent study using two sources of McNeil Stevia at up to 4% in the diet was also conducted and showed no adverse treatment related effects. The NOAEL was determined to be the highest amount tested.
- Steviol glycosides, including stevioside and rebaudioside A, are completely metabolized to steviol by the microbial flora of the caecum. Steviol thus formed is readily absorbed and rapidly glucuronidated in both humans and rats, with excretion occurring via the bile (and into feces) in rats and via the urine in humans. Based on the similarity between rats and humans in metabolism of stevioside and rebaudioside A to the primary metabolite, steviol glucuronide, it can be concluded that both human studies and rodent toxicological studies conducted with stevioside are also relevant for assessing human safety of rebaudioside A.

- Steviol glycosides have very low acute toxicity and there is no evidence of carcinogenicity, developmental, reproductive or genotoxic effects.
- Steviol glycosides extracts have been used for many years as high-intensity sweeteners in several countries; the extracts typically contain predominantly stevioside, and rebaudioside A is also common. Other steviol glycosides may be present and all have the common steviol backbone. All are metabolized (deglycosylated) to steviol in the gastrointestinal tract. Because of the similar metabolic fate of steviol glycosides, it is expected that these components would not only be toxicologically equivalent, but would be expected to produce similar physiological effects.
- Some animal and human studies of steviol glycosides have reported hypotensive and hypoglycemic effects, however, critical new published studies resolve questions about potential untoward effects on blood pressure in both normal and hypotensive subjects and on glucose homeostasis in both normoglycemic and diabetic subjects after long term repeated exposure to steviol glycosides. Studies have shown that steviol glycosides are well tolerated in humans and unlikely to have adverse effects on blood pressure or blood glucose at levels well exceeding maximum expected intakes:
    - A number of studies have examined the effect of oral steviol glycosides on normal and hypertensive humans. Results from two longterm clinical trials (1-2 years) in China studying men and women with mild to moderate essential hypertension have suggested antihypertensive effects of stevioside (purity undefined) at intakes of 750 and 1500 mg/day. However, other recent studies have not shown measurable effects of steviol glycosides on blood pressure in humans. In a separate trial of men and women with type 2 diabetes, more than half of whom were taking antihypertensive medications, consumption of as much as 1000 mg/day of rebaudioside A for 16 weeks produced no clinically important changes, or statistically significant differences from control, in blood pressure. An additional 4-week study in healthy adults with normal and low-normal blood pressure found no effect of 1000 mg/day rebaudioside A on blood pressure. Collectively, these studies support the overall conclusion of safety of ingestion of steviol glycosides and lack of untoward adverse effects on blood pressure in normal, hypertensive or hypotensive individuals.
    - Several studies have measured glucose homeostasis after ingestion of stevioside and steviol glycosides extracts; steviol glycosides were reported to reduce post-prandial glucose response in some trials. However, in a three-month study of subjects with type 2 diabetes, fasting blood glucose and glycosylated hemoglobin (HbA_{1c}) were not significantly lowered by intake of 1500 mg/d of stevioside

compared with placebo. An additional study of type 2 diabetic subjects also reported no significant changes in HbA1c, glucose, insulin and C-peptide between rebaudioside A (1,000 mg/day) and placebo groups. These data support the overall conclusion of safety of ingestion of steviol glycosides and lack of untoward adverse effects on glucose homeostasis in normal and type 2 diabetic individuals.

- Although the database is extensive, many of the key studies in humans which investigated the effects of stevioside on blood glucose and blood pressure did not report the purity of stevioside or mixtures of stevioside. This did not allow absolute assessment for these studies of whether the purity of stevioside would be representative of the expected specifications of stevioside that humans would be exposed to in the diet. In addition, as only one level was used in the pivotal human studies, it precluded the establishment of a NOAEL on which an ADI could be established. Therefore, these data were not used by Food Standards, Australia and New Zealand (FSANZ) or JECFA to establish the ADI; rather the chronic animal data was used for this purpose.
- The adequacy of the existing database provides a basis for eliminating the need for any additional safety factors in setting an ADI for steviol glycosides, such as the 2-fold uncertainty factor used by JECFA in its 2005 derivation of a temporary ADI (2 mg steviol equivalents per kg bw/d). The latter was specifically applied to account for uncertainty in potential effects on blood glucose and blood pressure. The recent well-designed clinical studies demonstrate no effects on either blood glucose or blood pressure, and collectively strengthen the clinical evidence of safety, so that an additional 2-fold safety factor for uncertainty is not warranted. Therefore, an ADI of 4 mg steviol equivalents/kg bw/d, derived by applying a 100-fold safety factor to the NOAEL of 970 mg stevioside/kg bw/d (equivalent to 383 mg/kg bw/kg steviol equivalents) in a 2-year rat study, is appropriate. This ADI covers steviol glycoside mixtures with different ratios of stevioside/rebaudioside. The ADI for steviol glycosides, set in 2008 by FSANZ, is also 4 mg steviol equivalents/kg bw/d. FSANZ did not apply an additional safety factor after concluding that potential effects on either blood glucose or blood pressure were not expected at the projected maximum human intake.
- The ADI of 4 mg/kg bw/d steviol equivalents is greater than the proposed EDI of up to 0.58 mg/kg bw/d steviol equivalents for the use of steviol glycosides as a table top sweetener.

The GRAS status of steviol glycosides (compliant with the established food grade JECFA specifications for steviol glycosides [which is inclusive of McNeil Stevia]), under the intended conditions of use proposed by McNeil Nutritionals, has been made through the deliberations of Roger Clemens, Ph.D., A. Wallace Hayes, Ph.D., John Thomas, Ph.D., and William Waddell, M.D. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of steviol glycosides and the potential human exposure to steviol glycosides resulting from its intended use as a table top sweetener, and have concluded:

> *There is no evidence in the available information on steviol glycosides that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when steviol glycosides are used at levels that might reasonably be expected from the proposed applications. McNeil Stevia is GRAS for use as a table top sweetener.*

Therefore, McNeil Stevia is safe and is GRAS at the proposed levels of ingestion. McNeil Stevia is therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

E. Availability of Information

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., CEO, Spherix Incorporated, at 6430 Rockledge Drive, Westmoreland Bldg. #503, Bethesda, MD 20817. Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ckruger@spherix.com

F. Signature

Pursuant to the criteria provided in proposed 21 CFR 170.36, McNeil Nutritionals, LLC, hereby notifies the Food and Drug Administration that the use of McNeil Stevia as a table top sweetener under the intended conditions of use is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because McNeil Nutritionals, LLC, has determined that such use is generally recognized as safe (GRAS) through scientific procedures.

12/23/08

Signature Date

II. DESCRIPTION OF SUBSTANCE

A. COMMON OR USUAL NAME(S)

Steviol glycosides

B. DEFINITION

The specified GRAS substance (McNeil Stevia) is a mixture of steviol glycosides which is obtained by extracting leaves of *Stevia rebaudiana* (Bertoni) Bertoni (*S. rebaudiana* (Bertoni) Bertoni) with hot water or a solvent such as ethanol. Stevioside and rebaudioside A are the principal steviol glycosides of the specified substance. Rebaudioside C and dulcoside A are secondary steviol glycosides. Other steviol glycosides including rubusoside, steviolbioside, and rebaudioside B may also be present.

C. SOURCE

Leaves of *S. rebaudiana* (Bertoni) Bertoni of the family *Asteraceae (Compositae)*.

The plant from which steviol glycosides are extracted has an alternate leaf arrangement and an herbaceous to semi-woody growth habit, with flowers arranged in indeterminate heads. The flowers are small and white with a pale purple throat. The pollen can be highly allergenic. The plant is self-incompatible and probably insect pollinated. The seeds are small, have very little endosperm and are dispersed in the wind via a hairy pappus.

D. CHEMICAL NAMES AND SYNONYMS

The following are the chemical names of seven steviol glycosides:

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

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

Rebaudioside C: 13-[(2-O-α–L-rhamnopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid β-D-glucopyranosyl ester

Dulcoside A: 13-[(2-O-α–L-rhamnopyranosyl-β–D-glucopyranosyl)oxy] kaur-16-en-18-oic acid β-D-glucopyranosyl ester

Rubusoside: 13-β-D-glucopyranosyloxy kaur-16-en-18-oic acid β-D-glucopyranosyl ester

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

Rebaudioside B: 13-[(2-O-β–D-glucopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid

E. C.A.S. NUMBER

The following are the C.A.S. numbers of seven steviol glycosides:

Stevioside	57817-89-7
Rebaudioside A	58543-16-1
Rebaudioside C	63550-99-2
Dulcoside A	64432-06-0
Rubusoside	64849-39-4
Steviolbioside	41093-60-1
Rebaudioside B	58543-17-2

F. CHEMICAL FORMULA

The following are the chemical formulae of seven steviol glycosides

Stevioside	$C_{38}H_{60}O_{18}$
Rebaudioside A	$C_{44}H_{70}O_{23}$
Rebaudioside C	$C_{44}H_{70}O_{22}$
Dulcoside A	$C_{38}H_{60}O_{17}$
Rubusoside	$C_{32}H_{50}O_{13}$
Steviolbioside	$C_{32}H_{50}O_{13}$
Rebaudioside B	$C_{38}H_{60}O_{18}$

G. MOLECULAR WEIGHT

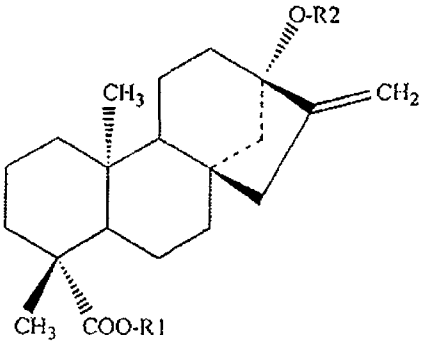
The following are the molecular weights of seven steviol glycosides

Stevioside	804
Rebaudioside A	966
Rebaudioside C	950
Dulcoside A	788

Rubusoside	642
Steviolbioside	642
Rebaudioside B	804

H. STRUCTURAL FORMULA

The structural formulas of seven named steviol glycosides are:



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

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

I. PHYSICO-CHEMICAL PROPERTIES

Description	Odorless or having a slight characteristic odor; White to light yellow powder
Solubility	Freely soluble in water and in ethanol
pH	Between 4.5 and 7.0 (1 in 100 solution)
Sweetness	As a sweetener, it is about 200-300 times sweeter than sucrose.
Stability	Two principal steviol glycosides of the specified additive, stevioside and rebaudioside A, are reasonably stable under the elevated temperatures used in food processing and do not undergo browning or caramelization when heated

J. TERMINOLOGY

The specified GRAS substance (McNeil Stevia) is a mixture of steviol glycosides which is obtained by extracting leaves of the perennial shrub, *S. rebaudiana* (Bertoni) Bertoni of the family *Asteraceae (Compositae)*. The plant from which steviol glycosides are extracted (*S. rebaudiana* (Bertoni) Bertoni) is native to Paraguay, but has since been introduced as a crop in Brazil, Korea, Mexico, United States, Indonesia, Tanzania, China and Canada. It is one of two members of the genus that produces and that has a relatively high concentration of sweet steviol glycosides (Brandle et al. 1998). The fresh leaves, dried leaves, leaf extracts, and components of steviol glycosides leaf extracts have been either consumed as food additives or dietary supplements. Because of its wide use, the plant and/or its components have been studied and are described in the literature using specific terms. Commonly used terms and their definitions are listed in Table 1.

Table 1. Terminology

Term	Refers To:
Stevia plant	The plant, *S. rebaudiana* (Bertoni) Bertoni
Steviol glycosides	Group of sweet diterpene components of stevia leaves
Stevioside	Most abundant steviol glycoside found in stevia leaves
Rebaudioside A	Second most abundant steviol glycoside found in stevia leaves
Stevia extract	Typically prepared from the leaves using various solvents. Primarily composed of steviol glycosides, but proportion may vary depending on extract method.
Steviol	The aglycone metabolite of stevioside; it is not present in the plant itself.

000017

K. Manufacturing Process

1. General description of steviol glycosides production

The specified GRAS substance (McNeil Stevia) is extracted from leaves of *S. rebaudiana* (Bertoni) Bertoni. It is a mixture of steviol glycosides with stevioside and rebaudioside A as principal steviol glycosides and with rebaudioside C and dulcoside A as secondary steviol glycosides. Of the above four major steviol glycosides, rebaudioside A has been identified as the least bitter and with the least persistent aftertaste, and thus is the most desirable component in the steviol glycosides extract. The production process can be divided into several basic stages.

Extraction of steviol glycosides. Typically, steviol glycosides are extracted from dry leaves of *S. rebaudiana* (Bertoni) Bertoni with various polar solvents including hot water or organic solvents like alcohol. In some cases, the leaves are pretreated with non-polar solvents, such as chloroform or hexane to remove essential oils, lipids, chlorophyll, and other non-polar substances. The extraction process can be operated batchwise or continuously (such as counter flow extraction). When operated batchwise, the extract is removed by draining or other known techniques. Plant debris and other impurity particles are removed from the extract with known techniques such as centrifugation, press filtration and membrane filtration. Flocculants such as calcium hydroxide and aluminum sulfate may be used to facilitate the removal of undesired accompanying substances. The recovered extract contains steviol glycosides, plant pigments and other extracting solvent (water or polar solvents like ethanol) as soluble components.

Isolation and purification of steviol glycosides. The extract is then purified and steviol glycosides are recovered by crystallization or direct spray drying. For example, steviol glycosides are recovered by absorption onto a macroporous ion exchange resin followed by elution with a water soluble organic solvent. Minerals, pigments and other charged impurities in the eluent may be further removed by passing the eluent through a mixed ion exchange resin column (cation/anion). Crude steviol glycosides can be recovered by crystallization (cooling/evaporative; continuous/batchwise) or spray drying.

Refined steviol glycosides enriched with rebaudioside A. Rebaudioside A can be enriched in the final product by recrystallization in water/ethanol, for an example. Final product can be obtained by crystallization or spray drying.

2. Manufacturing Process 1

The petitioner has two suppliers to supply steviol glycosides. Both manufacturing processes produce steviol glycosides that meet product specifications. There are small differences in the production processes. The flow-scheme of one production process (Process 1) is shown in Figure 1. All chemical reagents used in the process, the adsorption resin and the ion exchange resins are food grade.

Stevia dry leaves (Step 1): The raw materials used in the manufacture of McNeil Stevia are the crushed leaves of the perennial shrub *S. rebaudiana* (Bertoni) Bertoni of the family *Asteraceae (Compositae)*. The leaves are dried and ground.

Extraction with hot water (Step 2): The ground leaves of *S. rebaudiana* (Bertoni) Bertoni are mixed with hot water (b)(4) Subsequently, the aqueous extract is removed by draining using pressure in order to achieve the maximum amount of extract. This extraction process is repeated 2 to 3 times. The extract is allowed to cool to room temperature.

Separation with centrifugation (Step 3): Plant debris and other impurity particles are separated with centrifugation. The recovered extract contains steviol glycosides, plant pigments and other water-soluble components.

Adsorption on resin and elution with ethanol (Step 4): The water extract is passed through a macroporous ion exchange resin on which stevia is absorbed. The stevia is then eluted with ethanol.

Ethanol removal by evaporation (Step 5): Ethanol in the stevia solution (water/ethanol) is recovered by distillation.

Demineralization and decolorization with ion exchangers (Step 6): Stevia solution is demineralized by passing through a mixed bed containing both cation and anion ion exchangers. In this step, water soluble pigments in the stevia solution are also removed by absorption onto the ion exchangers. In addition, charged impurities are removed as well. For regeneration of the ion exchange resins, concentrated solutions of hydrochloric acid and sodium hydroxide are used.

Evaporation concentration under reduced pressure (Step 7): The demineralized/decolorized stevia solution is concentrated in an evaporator under reduced pressure.

Spray drying (Step 8): Crude stevia powder is obtained by spray drying.

Recrystallization in ethanol (Step 9): Stevia is refined to enrich rebaudioside A by dissolving the crude stevia in hot ethanol and recrystallization by cooling).

Separation of crystal with centrifugation (Step 10): The crystals are separated with centrifugation and washed with cold ethanol. The mother liquor is recycled to the crystallizer (Step 9).

Dissolution of crystal in water and concentration under reduced pressure (Step 11): The rebaudioside A enriched and refined stevia extract is redissolved in water and its solution is concentrated by evaporation under reduced pressure.

Micro-filtration (Step 12): Foreign particles including microbial load are removed by passing the concentrated stevia solution through a microfilter.

Spray drying (Step 13): The refined McNeil Stevia enriched with rebaudioside A is finally recovered by spray drying.

3. Manufacturing Process 2

The flow-scheme of Process 2 is shown in Figure 2. All chemical reagents used in the process and the adsorption resin are food grade.

Stevia dry leaves (Step 1): The raw materials used in the manufacture of McNeil Stevia are the crushed leaves of the perennial shrub *S. rebaudiana* (Bertoni) Bertoni of the family *Asteraceae (Compositae)*. The leaves are dried and ground.

Extraction with ethanol (Step 2): Stevia is extracted from ground leaves of *S. rebaudiana* (Bertoni) Bertoni with a mixture of ethanol and demineralized water by counter-flow extraction in stainless steel extraction equipments.

Membrane separation (Step 3): Plant debris and other impurity particles are then separated with polyamide membrane filter (b)(4) . The recovered extract contains steviol glycosides, plant pigments and other ethanol/water soluble components.

Adsorption on resin and elution with ethanol (Step 4): The extract is passed through a macroporous adsorption resin on which stevia is absorbed. Stevia is then eluted with ethanol/water mixture.

Concentration by evaporation (Step 5): Stevia solution in ethanol/water is concentrated by distillation in a stainless steel outside loop evaporator.

Crystallization to obtain crude crystal (Step 6): To enrich rebaudioside A, Stevia is crystallized by cooling the stevia solution with temperature reduction rate controlled. Crude crystals are recovered by centrifugation. Mother liquor is recycled to Step 4.

Recrystallization in ethanol (Step 7): Stevia is refined by dissolving the crude stevia in hot ethanol/water and recrystallized by cooling. Refined crystals are recovered by centrifugation. Mother liquor is recycled to Step 4

Drying (Step 8): The refined McNeil Stevia slurry enrich with rebaudioside A is redissolved in water and is finally dried in a spray dryer.

Figure 1. Production of Stevia (Process 1)

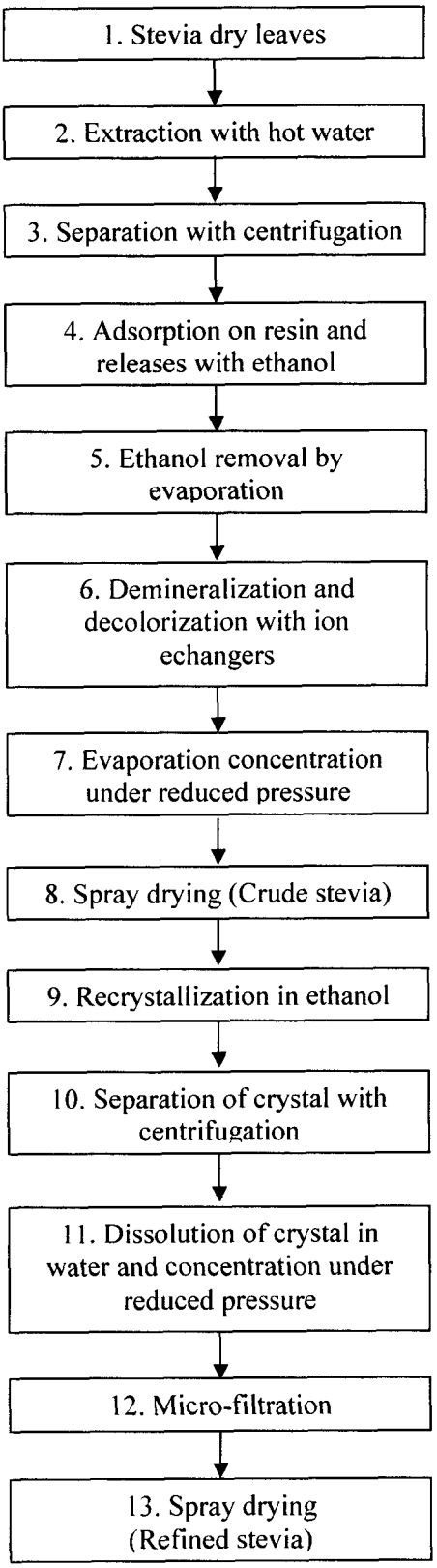
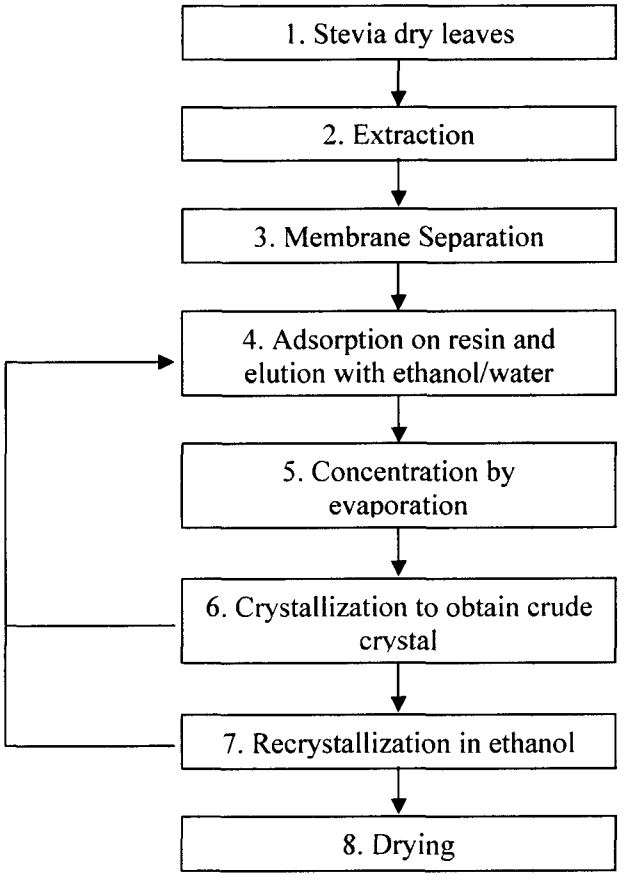


Figure 2. Production of Stevia (Process 2)



L. Safety of Raw Materials and Chemicals Used in the Process

Raw materials for the production of steviol glycosides are the crushed leaves of non-genetically modified cultivars of *S. rebaudiana* (Bertoni) Bertoni. The leaves are free of pesticides and heavy metals. Leaves are packaged and can be stored in a cool and dry environment (10-40 °C and relative humidity of 45% to 75%) for up to 2 years when naturally dried.

The processing aids used in the process include ethanol, water and ion exchange resins, hydrochloric acid and sodium hydroxide solution. Food grade ethanol is used in steviol glycosides extraction (Process 2) and refinery (in both Process 1 and 2). Macroporous adsorption resin used for the recovery of steviol glycosides is safe for food applications per 21 CFR 173.25. Anion and cation exchange resins used for demineralization and decolorization

(Process 1) are also safe for food application per 21 CFR 173.25. Hydrochloric acid and sodium hydroxide are used in the regeneration of ion exchangers (Process 1). Both chemicals are technical grade allowed for use in food processes. After regeneration, the resins are rinsed exhaustively with demineralized water. Water used in the processes meets the standards for drinking water quality.

M. SPECIFICATIONS

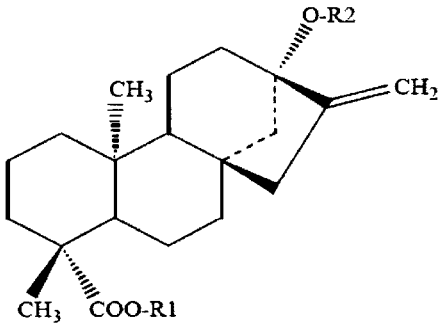
1. Current Regulatory Specifications

The composition of stevia (or stevia extract) depends on the composition of the leaves, influenced by soil and climate, and on the extraction and purification processes used. In response to the call for data on "stevioside" for the 63rd JECFA meeting (Joint meeting of the United Nations' World Health Organization [WHO] and Food and Agriculture Organization [FAO]), submissions from several countries showed that the main components of the commercially available extracts of stevia are stevioside and rebaudioside A, in various amounts ranging from about 10-70% stevioside and 20-70% rebaudioside A. The information indicated that most commercial products contained more than 90% steviol glycosides with the two main steviol glycosides comprising about 80% of the material. The 63rd Joint Expert Committee on Food Additives (JECFA) required that the summed content of stevioside and rebaudioside A was not less than 70% and established a minimum purity of 95% total steviol glycosides. Analytical data showed that most of the remaining 5% could be accounted for by saccharides other than those associated with the individual steviol glycosides. Noting that the stevia could be produced with high purity (at least 95%) and that all the steviol glycosides hydrolyze upon ingestion to steviol, on which the temporary JECFA-assigned ADI is based, the 68th JECFA decided it was unnecessary to maintain a limit for the sum of stevioside and rebaudioside A content. The Committee moved to establish specifications that cover a range of steviol glycoside compositions; the newly revised specification of stevia product requires at least 95% of the total of the seven named steviol glycosides.

The JECFA specifications for a stevia product are presented in Table 2. No specifications were given for pesticide residues.

000024
SPHERIX

Table 2. WHO Chemical and Physical Properties of a Stevia Extract Product

C.A.S. number	**Stevioside: 57817-89-7** **Rebaudioside A: 58543-16-1**
Chemical formula	**Stevioside: $C_{38}H_{60}O_{18}$** **Rebaudioside A: $C_{44}H_{70}O_{23}$**
Structural formula	

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

Formula weight	**Stevioside- 804.88** **Rebaudioside A: 967.03**
Assay	**Not less than 95% of the total of the seven named glycosides, on the dried basis**

Characteristics	
Parameter	**Value**
Color	White to yellow
Form	Crystalline Powder
Odor	Slight, characteristic
Purity	
Steviol glycosides	≥ 95%
Solubility	Freely soluble in water and ethanol
Identification	The main peak in the chromatogram obtained by following the procedure in Method of Assay corresponds to either stevioside or rebaudioside A
pH (Vol. 4)	Between 4.5 and 7.0 (1 in 100 solution)
Ash	Not more than 1%
Loss on drying	Not more than 6% (105°C, 2 hour)
Residual solvents	Not more than 200 mg/kg methanol (Method I in Vol. 4, General Methods, Organic Components, Residual Solvents)
Arsenic	Not more than 1 mg/kg
Lead	Not more than 1 mg/kg
Source: 68th JECFA (2007) and published in FAO FECFA Monographs 4 (2007).	

2. Potential impurities

The impurities occurring in steviol glycosides are due primarily to compounds extracted from the Stevia leaves. They are typical plant materials, such as pigments and saccharides. One literature study [Pasquel et al 2000] reports identification of the following substances in the nonglycosidic fractions of extracts of stevia leaves, obtained using supercritical fluid extraction (SCFE): spathulenol; decanoic acid; 8,11,14-ecosatrienoic acid; 2-methyloctadecane; pentacosane; octacosane; stigmasterol; β-sitosterol; α- and β-amyrine; lupeol; β-amyrin acetate; and pentacyclic triterpene. These highly non-polar substances represented 56% of the total non-glycosidic extracts, 44% remaining unidentified.

The specified GRAS substance (McNeil Stevia with >95% total steviol glycosides) will contain, in addition to saccharides other than those associated with the individual steviol glycosides, residual extraction/recrystallization solvent and possibly residues of ion-exchange resins used in the manufacturing process. Because of its highly non-polar character, the level of the non-glycosidic fraction in the resulting McNeil Stevia product can be considered insignificant.

For the purpose of the safety assessment, relevant purity criteria are set for total steviol glycosides, total ash, arsenic, lead and residual solvents. JECFA did not deem it necessary to

include purity criteria for other heavy metals or microbiological parameters in the specifications of steviol glycosides.

Analytical results on preparations support a setting of maximum limits of 1 ppm for both arsenic and lead, a maximum limit for residual solvents of 200 ppm and a maximum limit for loss-on-drying of 6%.

3. McNeil's Stevia Product Specifications and Batch Analyses

The product specifications set by McNeil for its Stevia product are the same as the product specifications set by JECFA. McNeil Stevia product specifications and analytical batch data for the steviol glycoside product produced by McNeil's two suppliers that comply with these specifications are presented in Table 3.

Table 3. Steviol Glycosides Specifications and Batch Analyses

C.A.S. number	Stevioside: 57817-89-7 Rebaudioside A: 58543-16-1
Chemical formula	Stevioside: $C_{38}H_{60}O_{18}$ Rebaudioside A: $C_{44}H_{70}O_{23}$
Formula weight	Stevioside- 804.88 Rebaudioside A: 967.03
Assay	Not less than 95% of the total of the seven named glycosides, on the dried basis (Stevioside, Rebausioside A, Rebaudioside C, Dulcoside A, Rubusoside, Steviolbioside, Rebaudioside B)

Characteristics

Parameter	**Value in Specifications[1]**	**Lot 8.80.010**	**Lot 8.80.020**	**Lot 8.80.030**	**Lot 071103**	**Lot 071104**	**Lot 071105**
Color	White to yellow	White	White	White	White	White	White
Form	Crystalline Powder	Powder	Powder	Powder	Fine powder	Fine powder	Fine powder
Odor	Slight, characteristic	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Purity	Total steviol glycosides ≥ 95%	95.56%	97.44%	99.09%	95.95%	95.90%	96.25%
Solubility	Freely soluble in water and ethanol	Freely soluble in water	Freely soluble in water	Freely soluble in water	Freely soluble in water and in ethanol	Freely soluble in water and in ethanol	Freely soluble in water and in ethanol
Identification	Stevioside or rebaudioside A [2]	Complies	Complies	Complies	Complies	Complies	Complies
pH	Between 4.5 and 7.0 (1 in 100 solution)	6.0	6	6	5.6	5.5	5.5
Ash	Not more than 1%	<1%	<1%	<1%	0.03%	Complies	Complies
Loss on drying	Not more than 6% (105°C, 2 hour)	0.014%	0.59%	0%	0.86%	1.14%	1.08%
Residual solvents	Not more than 200 mg/kg methanol	Complies	Complies	Complies	Complies	Complies	Complies
Arsenic	Not more than 1 mg/kg	Not detected	Not detected	Not detected	Complies	Complies	Complies
Lead	Not more than 1 mg/kg	Not detected	Not detected	Not detected	Complies	Complies	Complies

1. Same as the JECFA Specifications (68th JECFA in 2007 and published in FAO JECFA Monographs 4 in 2007).
2. The main peak in the chromatogram obtained by following the procedure in Method of Assay (JECFA monograph, 2007) corresponds to either stevioside or rebaudioside A

000028

N. STABILITY OF THE STEVIOL GLYCOSIDES

McNeil Stevia is a mixture of steviol glycosides. Among them, stevioside and rebaudioside A are reasonably thermally stable under the elevated temperatures used in food processing and do not undergo browning or caramelization when heated. In response to a request by the 63rd JECFA for information on the hydrolytic stability of steviol glycosides in acidic foods, the Committee received results of thermal and hydrolytic stability studies for the specified material (Kuznesof, 2007): It appears that citric acid solutions (pH 2-4) of steviol glycosides (ca. 1000 mg/l; ca. 29% stevioside, 62% rebaudioside A) are highly stable for at least 180 days at 20°C. At elevated temperatures (80°C, in water, 8 hour), however, the same samples showed 4% and 8% decomposition at, respectively, pH 4.0 and 3.0. At 100°C, decomposition was expectedly higher: 10% and 40% at, respectively, pH 4.0 and pH 3.0. Also, at 100°C decomposition was 4% at pH 6.0, but increased to about 16% at pH 8.0. And in an acidic beverage (pH 3.8) held at 24°C for one year, essentially no decomposition of steviol glycosides (ca. 94% rebaudioside) was noted. Isosteviol (CAS no. 27975-19-5) was identified as a decomposition product in the tested samples.

The JECFA Committee also had available a summary of literature studies that addressed the stabilities of stevioside and rebaudioside A, which it considered relevant to its evaluation because the specified material includes products that may be 95% stevioside or 95% rebaudioside A. The studies are summarized as follows (Kuznesof, 2008):

- Refluxing stevioside (5 hour; 0.4% hydrochloric acid (pH 1.16, aq. methanol)): identifiable hydrolysis products: 49% steviol, 29% steviolbioside, 4% 13-O-β-D-glucopyranosyl-steviol, and 19% 13-O-β-D-glucopyranosyl-steviol-β-D-glucopyranosyl ester.
- Heating stevioside or rebaudioside A (citric or phosphoric acid solutions; 5.7 days): no decomposition at 60°C; at 100°C, decomposition products noted, but not identified. No steviol was observed.
- Stevioside (aqueous acid, pH 2.5 and 3.0; 80°C and 100°C): After one hour, maximum decomposition (10%) noted at pH 2.5 and 100°C. No information on the acid used.
- Stevioside and rebaudioside A (650 mg/l; 100°C): Decomposition in neutral solutions after 13 hours; 40% decomposition in acid solutions (citric acid, pH 2.6; phosphoric acid, pH 2.4) after 4 hours.
- Stevioside (130 mg/l; citric acid; room temperature; 6 months): 2.5% loss at pH 4.0; 10% loss at pH 3.0.

- Stevioside (1000 mg/l; pH 2.6 - citric acid solution; 4°C and 22°C): stable for up to 4 months. Rebaudioside A (22°C; pH 2.6): slightly less stable than stevioside; some decomposition after three months. At 37°C, both substances begin to decompose after 2 months; ca. 15% decomposed at 4 months. These data also suggest that, in citric acid solutions, stevioside is more thermally stable than Rebaudioside A.
- Rebaudioside A (1000 mg/L; phosphoric acid - pH 2.6) showed slightly greater thermal stability than stevioside (1000 mg/L; phosphoric acid - pH 2.6).
- Stevioside (500 mg/l; room temp.; solutions of 10 g/l citric and phosphoric acids (pH: 2.1 and 1.6, respectively): decomposition begins after one month; stable in 5% acetic acid (pH 2.6). In the phosphoric acid solution (4 months), decomposition reached 75%; in citric acid, decomposition was 20%. The greatest stability was observed for the acetic acid solution.

The 68th JECFA concluded that steviol glycosides are sufficiently thermally and hydrolytically stable for food use, including acidic beverages, under normal conditions of processing/storage.

It has been shown that McNeil Stevia is stable after storage at 25°C±2°C and relative humidy of 60%±10% for 11 months. There was no loss of rebaudioside A, the major steviol glycoside in the tested product. The product is also stable at 105°C for 12 hours without losing rebaudioside A.

III. HISTORY OF USE AND INTENDED USE

A. HISTORY OF USE

The fresh stevia leaves, dried leaves, leaf extracts, and components of stevia leaf extracts have been historically consumed as a sweetener. The characteristic sweetness of stevia leaves results from the presence of relatively high quantities of sweet steviol glycosides. The major (most abundant) steviol glycosides include stevioside, rebaudioside A, rebaudioside C, and dulcoside A. Stevioside is estimated to be approximately 200-300 times sweeter than sugar (Bovanova et al. 1998). Rebaudioside A appears to have the most favorable sensory attributes of the four major steviol glycosides (Brandle et al. 1998).

Total glycosides in stevia leaves are reported to range from 6.4 to 16.2% by weight (w/w) of dried leaves (Kolb et al. 2001; Tamura et al. 1984). Stevioside, the most abundant glycoside occurring in stevia leaves, is reported to occur in concentrations ranging from below the limit of detection to as high as 32.8% w/w dry leaves (Yoda et al. 2003; Nikolova-Damyanova et al. 1994). Stevia leaves analyzed in these studies were sourced from various countries including Russia (Bondarev et al. 2003), Brazil (Ferreira et al. 2006a), Japan, Korea, and Paraguay (Hashimoto et al. 1978) and were extracted and analyzed by various methods. Although reported concentrations of the major glycosides other than stevioside vary, these studies consistently report that rebaudiosides A and C and dulcoside A occur in decreasing quantities, respectively, compared to stevioside. A summary of quantitative data are presented in Table 4.

Other than the four major steviol glycosides, a number of minor glycosides have been isolated from stevia leaves. These include rebaudiosides B (Makapugay et al. 1984), D, E (Sakamoto et al. 1977; Makapugay et al. 1984), and F (Brandle et al. 1998), dulcoside B (Kobayashi et al. 1977), and rubusoside (Hirokado et al. 1980). In addition, apigenin-4'-O-glucoside, luteolin-7-O-glucoside, kaempferol-3-O-rhamnoside, and quercetin-3-O-arabinoside have been identified (Rajbhandari and Roberts 1983). These minor glycosidic components are not well characterized and few investigators, if any, report quantities in stevia plants or plant extracts.

Table 4. Concentration of Major Glycosides in *S. rebaudiana* (Bertoni) Bertoni

	Sample Description	Total Glycosides	Stevioside	Rebaudioside A	Rebaudioside C	Dulcoside A
	Plants (mean or range, % w/w dry leaves)[1]					
Ahmed and Dobberstein 1982	leaves, dried/powdered	--	ID	--	ID	ID
Bondarev et al. 2003	leaves, lyophylized/powdered[2]	--	~1.2	~0.79	1.4	--
Brandle et al. 1998 (review)	leaves, dried	--	9.1	3.8	0.6	0.3 ("A" not specified)
Geuns 2003 (review)	leaves, dried	--	4 - 20	--	--	--
Hashimoto et al. 1978	leaves, dried/crushed	--	0.8 - 2.9	0.8 - 2.3	--	--
Kobayashi et al. 1977	Crude glycoside mixture	--	--	ID	--	--
Kolb et al. 2001	leaves, dried/ground to powder	6.38-16.19	3.78 - 9.84	1.62 - 7.01	--	--
Makapugay et al. 1984	leaves, powdered/dried	--	4.6 - 8.1	1.9 - 3.7	0.85 - 2.1	0.41 - 0.66
Mauri et al. 1996	leaves, dried[3]	--	3 - 8	~ 1	--	0.2
Metivier and Viana 1979	leaves, dried	--	3.7 (short day); 7.2 (long day)	--	--	--
Mizukami et al. 1982	leaves, dried[4]	--	8.5 - 9.7	--	--	--
Nevopim et al. 1998	leaves, dried	--	0 - 6.5	--	--	--
Nikolova-Damyanova et al. 1994	ground leaves; air-dried[5]	--	32.8	22.5	--	--
Nishiyama et al. 1992	leaves, dried/powdered[6]	--	4.6 - 13.5	--	--	--
Pasquel et al. 2000	leaves, dried[7]	--	3 to 11 parts	1 part	--	--
Rajbhandari and Roberts 1983	leaves, dried	--	5	2	--	--

000032

000033

Table 4. Concentration of Major Glycosides in *S. rebaudiana* (Bertoni) Bertoni

	Sample Description	Total Glycosides	Stevioside	Rebaudioside A	Rebaudioside C	Dulcoside A
	Plants (mean or range, % w/w dry leaves)[1]					
SCF 1999 (Review)	leaves, dried	--	4 - 13	2 - 4	1 – 2	0.4 - 0.7
Shibata et al. 1991	Leaves from mature shoots; freeze-dried	--	5 - 10	--	--	--
Tamura et al. 1984	leaves of plants from stem-tip cultures, dried[8]	11.2 - 12.3	4.9 - 5.6	4.9 - 5.3	1.0 - 1.1	0.3 - 0.4
Tamura et al. 1984	Leaves of plants from seeds, dried	10.0 ± 1.5	4.4 ± 1.0	4.1 ± 1.3	1.0 ± 0.6	0.5 ± 0.8
Tamura et al. 1984	Leaves of plants from cuttings, dried	12.0 ± 0.7	5.7 ± 0.3	5.9 ± 0.4	1.0 ± 0.1	0.3 ± 0.1
WHO 2000 (review)	leaves, dried	--	5 - 10	--	--	--
Yoda et al. 2003	leaves, fresh, triturated[9]	--	0.042 - 0.085	0.034 - 0.051	--	--
	Minimum	6.38	LOD	0.08	0.06	0.2
	Maximum	16.19	32.8	22.5	2.1	0.7
	Extracts (mean or range, % w/w)					
Ferreira et al. 2006b	commercial product	--	72 ± 2	23 ± 2	--	--
Hirokado et al. 1980 (Japanese)	14 commercial products[10]	--	0.47 - 37.9	0.18 - 11.9	--	--
Liu and Li 1995	commercial product[11]	--	46.2 - 45.8	--	--	--
Mauri et al. 1996	commercial product	--	56.2	29.1	--	--
Nikolova-Damyanova et al. 1994	commercial product[12]	--	58.1 - 59.1	24.7 - 25.0	--	--
WHO 2005 (review)	commercial products	≥ 95	--	--	--	--

Table 4. Concentration of Major Glycosides in *S. rebaudiana* (Bertoni) Bertoni

	Sample Description	Total Glycosides	Stevioside	Rebaudioside A	Rebaudioside C	Dulcoside A
	Plants (mean or range, % w/w dry leaves)[1]					
Yoda et al. 2003	leaf extracts	--	7.1 - 23.9	4.5 - 18.2	--	--
	Minimum	--	0.47	0.18	--	--
	Maximum	--	59.1	29.1	--	--

1 Values are % w/w dry leaves, unless otherwise noted. For extracts, percent (%) indicates % w/w of the commercial product or extract.
2 Values estimated from graph; mg/g units converted to %.
3 Values are as stated in the introduction of this paper (no primary reference provided).
4 Values represent the range of means for enzyme assay, TLC densitometry, and GLC methods (results were similar).
5 Values are based on the sum of three extractions.
6 Values represent the range of means determined by several methods.
7 Reported rebaudioside A yield varied depending upon treatment.
8 Values represent the range of means for two different plant strains (results were similar).
9 Units appear to be % w/w fresh leaves.
10 Steviolbioside was also detected.
11 Values represent range of means for two different commercial products and two different analysis methods (results were similar).
12 Values represent the range of means for HPLC and TLC methods (results were similar).

Abbreviations:
CE: Capillary electrophoresis
GLC: Gas liquid chromatography
HPLC: High-performance liquid chromatography
LOD: Limit of detection
ID: Identified but not quantified
TLC: Thin-layer chromatography

In addition to major and minor steviol glycosides, a number of other components have been isolated from stevia leaves. These include oils, hydrocarbons, sterols, enzymes, macronutrients, and other constituents (Table 5). The majority of these components have been identified by one or two investigators; very little information is available specific to characterization or quantity.

Table 5. Non-Glycosidic Components of *S. rebaudiana* (Bertoni) Bertoni

Component Identified	Reference
Minor Components	
centauredin (5,7,3'-trihydroxy 3,6,4'-trimethoxy flavone)	Rajbhandari and Roberts 1983
Quercitin	Rajbhandari and Roberts 1983
Enzymes	
glucosyltransferase I	Shibata et al. 1995
glucosyltransferase II B	Shibata et al. 1995
Oils and Minor Organic Constituents	
total sesquiterpines	Cioni et al. 2006
Khusinol	Yoda et al. 2003
Nerolidol	Yoda et al. 2003
Spathulenol	Cioni et al. 2006
Monoterpines	Cioni et al. 2006
labdane diterpines	Brandle et al. 1998
sterebins A-H	McGarvey et al. 2003; Oshima et al. 1988
sterebins I-N	McGarvey et al. 2003
Austroinulin	Yoda et al. 2003
6-O-acetylaustroinulin	Yoda et al. 2003
7-O-acetylaustroinulin	Yoda et al. 2003
Jhanol	Yoda et al. 2003
manoyl oxide	Yoda et al. 2003
3-keto-manoyl oxide	Yoda et al. 2003
Triterpenes	Yoda et al. 2003
alpha-amyrine	Yoda et al. 2003
beta-amyrine	Yoda et al. 2003
epi-alpha-cadinol	Yoda et al. 2003
beta-carophyllene	Cioni et al. 2006
caryophyllene oxide	Cioni et al. 2006
Octadecanol	Yoda et al. 2003
beta-pinene	Cioni et al. 2006
beta-sitosterol	Cioni et al. 2006
n-tetracosane	Yoda et al. 2003
n-pentacosane	Yoda et al. 2003
n-octacosane	Yoda et al. 2003
giberrellin A20	Alves and Ruddat 1979
Total essential oils	Cioni et al. 2006
Macronutrients and Aggregates	
Protein	SCF 1999

Table 5. Non-Glycosidic Components of *S. rebaudiana* (Bertoni) Bertoni	
Component Identified	**Reference**
Lipids	SCF 1999
Total carbohydrate	SCF 1999
Water-soluble substances	SCF 1999

Steviol glycosides are currently used in several countries including China, Japan, South Korea, Israel, Mexico, Paraguay, Brazil, Argentina, Germany, Belgium, Italy, the UK and Switzerland to sweeten food products and in the United States as a dietary supplement (Geuns et al. 2006; USAID 2004). Recently, Rebaudioside A purified from *S. rebaudiana* (Bertoni) Bertoni has been determined to be GRAS by Cargill, Inc. (GRN 253) for use as a "General-purpose sweetener in foods generally, excluding meat and poultry products, at levels determined by current good manufacturing practices" and by Whole Earth Sweetener Company LLC (GRN 252) for use as a "Sweetener in selected beverages (including sweetened teas, diet soft drinks, energy drinks and flavored waters) and cereals (oatmeal, cold cereal, and cereal bars) at levels ranging from 150 to 500 mg/kg; and as a tabletop sweetener at 30,000 mg/kg).

Some international food uses of steviol glycosides reported to JECFA are presented in Table 6 (WHO 2006). Steviol glycosides are also used to sweeten teas in several countries throughout the world; glycoside concentrations in these teas are lower than typical concentrations in foods to which steviol glycosides have been added (WHO 2006).

Table 6. Food Use Levels of Steviol Glycosides	
Food Type	**Maximum Use Level Reported (mg/100g food)**
Beverages	50
Desserts	50
Yogurt	50
Cold confectionery	50
Sauces	100
Pickles	100
Delicacies	100
Sweetcorn	20
Bread	16
Biscuits	30
Source: Modified from WHO 2006	

000036

Available estimates reported by JECFA of steviol glycoside intake are reported as steviol or stevioside and are either based on current market uses of steviol glycosides or assume replacement of all dietary sugars (Table 7). Based on the total demand for steviol glycosides, Japan estimated that the per capita intake of steviol glycosides (as steviol, assuming 40% conversion from steviol glycosides) was 0.04 mg/kg bw/d (WHO 2006). No other estimates of current per capita intake were provided to JECFA. The European Commission's Scientific Committee on Food (SCF) provides a per capita estimate of 2.4 g dry stevia powder (400 mg stevioside or 6.7 mg stevioside/kg bw/d based on 60 kg) for individuals living in Europe (SCF, 1999) while a recent study in Korea estimated a stevioside intake from various processed foods of 0.008 mg/kg bw/d (Chung et al. 2005).

Using the Global Environment Monitoring System – Food Contamination and Monitoring Assessment Programme (GEMS/Food), the JECFA estimated intake of stevioside as steviol. To generate these estimates, it was assumed that all dietary sweeteners would be replaced with steviol glycosides at the minimum reported relative sweetness of glycoside to sucrose (200:1). Body weights were assumed to be 50 kg (Japan) or 60 kg (all other countries). Using this method, intake of stevioside ranged from 0.1 (Japan, per capita) to 12.5 (United States, maximum potential consumption) mg/kg bw/d and estimated steviol glycoside intake (as steviol) ranged from 0.8 (Africa) to 5.0 (United States) mg/kg bw/d (Table 7). For estimates considering replacement of all sugar in the diet, true values were stated to likely be only 20-30% of these estimates, given that replacement of all sweeteners with steviol glycosides are unlikely (WHO 2006). Considering this over estimation, a truer estimate of mean per capita intake in the U.S. considering replacement of all dietary sugar would be approximately 2.5 to 3.8 mg/kg bw/d stevioside. Table 7 provides a summary of available intake estimate data.

Table 7. International Estimates of Stevioside and Steviol Intake

Region or Country	Estimated Intake of Stevioside[1]	Estimated Intake of Steviol
	(mg/kg bw/d)	
Middle East[2]	6.0 – 8.0	2.4 – 3.2
Far East[2]	3.5 – 4.0	1.4 – 1.6
Africa[2]	2.0 – 3.3	0.8 – 1.3
Latin America[2]	8.3 – 8.8	3.3 – 3.5
Europe[2]	8.3 – 8.8	3.3 – 3.5
Europe (per capita)[3]	6.7	2.7
Japan (per capita)[4]	0.1	0.04
Japan (maximum consumption)[5]	7.5	3.0
Korea[6]	0.008	<.001
United States (maximum consumption)[5]	12.5	5.0

Source: Modified from WHO 2006, unless otherwise noted.
[1] Converted from steviol intake data based on a 40% conversion rate.
[2] Estimated using the WHO Global Environment Monitoring System – Food Contamination and Monitoring Assessment Programme. Estimates assumed that all dietary sugars would be replaced by steviol glycosides at a ratio of 200:1 and body weight of 60 kg.
[3] Source: SCF 1999; based on estimated per capita intake of 400 mg stevioside, body weight of 60 kg, and 40% conversion to steviol.
[4] Data provided to JECFA.
[5] Estimates assumed that all dietary sugars would be replaced by steviol glycosides at a ratio of 200:1. Estimates for Japan and the U.S. are based on consumer body weights of 50 and 60 kg, respectively.
[6] Chung et al. 2005; estimated intake from various processed foods.

B. Intended Use

McNeil Stevia is intended to be used as a table top sweetener including packet, tablet and granular powder form. Two estimates of intake were prepared, using data from Market Research Corporation of America (MRCA) in addition to food consumption data reported in the United States Department of Health and Human Service's 2003-2004 National Health and Nutrition Examination Survey (NHANES). These estimates are compared to data from other publicly available sources giving estimates of high intensity sweetener intake.

1. McNeil Stevia Intake Calculation using MRCA Data:

An estimate of McNeil Stevia intake for its use as a sweetening ingredient is based intake estimates prepared for McNeil by the Market Research Corporation of America (MRCA) (Table 8). The methodology involved calculation of intake based on optimal use levels for the foods and beverages contained in specified categories of use. These optimal use levels were provided by McNeil. MRCA applied these use levels to its menu census database, which involves 2000 American families, who record the frequency with which foods/beverages in these categories were actually consumed by all household members over a 14-day period. Food consumption from each of the designated categories for each participant was totaled for the 14-days and

divided by 14 to yield an average daily consumption level. Finally, the frequency and use level information was linked to a USDA database which lists the quantity of a particular food/beverage commonly consumed per eating occasion. To ensure that the intake estimate accounted for maximum potential ingestion, MRCA assumed that the sweetener would replace, all added sweeteners, i.e., both nutritive and artificial, in all foods and beverages in the twenty-four categories of use.

MRCA prepared intake calculations for the "Total Population" of survey participants and also for a subgroup designated "Eaters Only", i.e., those individuals from the total sample who during the survey period actually consumed a food/beverage from one or more of the selected use categories. The data are presented as frequency distributions (percentiles) of intake for various age groups and for the total population (i.e., "All ages"), and are also broken down by food category. This permits evaluation of the contribution that each category makes to the overall intake estimate.

Customarily, the "All ages", 90th percentile of intake for "Eaters Only" for all food categories is viewed by health authorities as representing the maximum potential exposure to an ingredient (FDA, 2006). This lifetime exposure estimate would describe individuals who consume large quantities of foods and beverages containing the particular ingredient, every day, over an entire lifetime. While health authorities use this approach to defining maximum exposure potential, it must be recognized that it is overly liberal. Simply put, it would not be possible for an individual to be a high level consumer of all foods and beverages from all twenty-four categories of use, every day, over an entire lifetime. That is, individuals who would choose to consume large quantities (i.e., at the 90th percentile) of a sweetened carbonated soft drink, would not also be drinking excessive quantities of other sweetened beverages, e.g., milk, coffee, tea, juices, alcoholic beverages on the same day. Moreover, it is also unlikely that an individual would consume excessive amounts of a food or beverage on a daily basis over an entire lifetime. That is why projected "mean" intakes are seen to be more realistic estimates of intake, although they too routinely overestimate actual consumption.

McNeil used the following assumptions for the estimation:

- McNeil Stevia is 200-300 times sweeter than sugar.
- The McNeil Stevia amount in a usual packet is equivalent in sweetness to two tsps of sugar.
- McNeil Stevia-containing packets would replace all high intensity sweeteners in all tabletop sweetener applications.

000039

Intake was estimated on a "14-Day average", "all ages", "eaters only", projected intake at the "mean" and "90th percentile". It is a conservatively derived number, reflecting use as a full replacement of all tabletop high intensity sweeteners, not just sweetener packets.

Table 8. MRCA Intake Calculation*

All Ages, Eaters Only, McNeil Stevia Intake (mg/day)				All Ages, Eaters Only, McNeil Stevia Intake (mg/kg bw/d)			
Stevia Extract		Steviol Equivalents[a]		Stevia Extract		Steviol Equivalents[a]	
Mean	90th percentile	Mean	90th percentile	Mean	90th percentile	Mean	90th percentile
20	50	7	18	0.40	0.92	0.14	0.33

[a]McNeil Stevia extract product is approximately 36% steviol equivalents by weight
*Calculated using MRCA's 1992-1993 Menu Census Survey and USDA's 1987-1988 database.

2. McNeil Stevia Intake Calculation using NHANES Data:

Estimates of potential intake of McNeil Stevia resulting from the intended use as a table top sweetener were calculated using food consumption data reported in the United States Department of Health and Human Service's 2003-2004 National Health and Nutrition Examination Survey (NHANES). The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States (NCHS 2006).

As part of the examination, trained dietary interviewers collect detailed information on all foods and beverages consumed by respondents in the previous 24 h time period (midnight to midnight). A second dietary recall was administered by telephone 3 to 10 days after the first dietary interview, but not on the same day of the week as the first interview. A total of 9043 respondents provided complete dietary intakes for the Day 1 recall, and 8354 of the individuals provided a complete Day 2 recall.

The data files used to process the NHANES 2003-2004 dietary recalls include 6940 food codes, each identified by a unique number and descriptive name. The database was reviewed, and all food codes corresponding to one of the intended use category of table top sweetener were identified.

Using the list of food codes and the NHANES 2003-2004 dietary recall data files from individuals with 2 complete days of dietary recall, an estimated mean and 90th percentile 2-day average intake of the individual product categories and also all categories combined was

determined. Two-day average intakes represent the total number of servings consumed during the two days of recall divided by two (i.e., $(Intake_{Day\ 1} + Intake_{Day\ 2})/2$).

Intakes were calculated for subpopulations; the estimates were generated using survey sample weights to adjust for differences in representation of subpopulations; results therefore are representative of the U.S. population.

Estimates of intakes are shown in Table 9.

Table 9. Estimated Intakes of Steviol Equivalents from Use of McNeil Product in High-Intensity Table-Top Sweeteners, NHANES 2003-2004

		2-day average intakes					
		high-intensity sweetener teaspoon equivalents/d [a]		mg stevia extract/d [b]		mg steviol equivalents/d [c]	
Age Group	% Users	Mean	90th Percentile	Mean	90th Percentile	Mean	90th Percentile
Under 1 yr	0.3	0.3	0.3	4	4	2	2
1-5 yr	0.3	0.6	1.0	8	14	3	5
6-11 yr	1.2	0.8	1.0	11	14	4	5
12-18 yr	1.3	2.3	6.0	32	84	12	30
≥19 yr	14.3	3.3	7.0	46	98	17	35

a Units of intake correspond to the equivalent sweetness provided by a teaspoon of sugar; most individual packets of high-intensity sweeteners are equal in sweetness to two teaspoons of sugar. These forms include packets, tablets and granular powders.
b Values based on 14 mg stevia extract per teaspoon equivalent of sugar (weight of one teaspoon of granulated sugar is 4.2 g; assume that stevia extract is 300 times sweeter than sugar on a weight basis).
c McNeil Stevia extract product is approximately 36% steviol equivalents by weight.

3. Other Publicly Available Intake Estimates of Steviol Glycosides as Intense Sweeteners:

A dietary exposure assessment was undertaken by FSANZ (2008) to estimate dietary exposure to steviol glycosides. Food consumption data from the 1995 Australian and 1997 New Zealand National Nutrition Surveys were used for the exposure assessments. The population groups assessed were the Australian population (2 years and above), the New Zealand population (15 years and above) and children (2-6 years for Australia only). (Table 10)

The Applicant provided FSANZ with information on proposed levels of use for steviol glycosides for specific food groups and the expected proportion of products in each food group

that would be using steviol glycosides instead of sugar or another intense sweeteners after 20 years. Based on this information, dietary exposure assessments were conducted for a "30% sugar replacement scenario".

At its 63rd meeting, JECFA also estimated the intake of steviol glycosides would likely be 20-30% of total sugar replacement in foods (WHO 2004). Based on this assumption, dietary exposure assessments were also conducted for a "30% market share scenario" of the sugar replacement dietary estimate.

Table 10. FSANZ Estimated dietary exposure to steviol glycosides*

Population group	Mean consumers mg/day		90th percentile consumers mg/day	
	Sugar Replacement	30% Market Share Sugar Replacement	Sugar Replacement	30% Market Share Sugar Replacement
2 years and above (Australia)	57	37	107	63
2-6 years (Australia)	40	24	74	41
15 years and above (New Zealand)	40	29	74	49

*Consumption assuming expected proportion of products in each food group that would be using steviol glycosides instead of sugar or another intense sweeteners

For both scenarios, the major contributors to steviol glycosides dietary exposure for Australians aged 2 years and above and for new Zealanders aged 15 years and above were predicted to be formulated beverages, tabletop sweeteners and carabonated soft drinks. For Australian children aged 2-6 years, the major contributors were fruit and vegetable juices and products, formulated beverages, carbonated soft drinks, breakfast cereals, mueslins and muesli bars, and flavoured milks and yoghurts for both scenarios. Beverages were predicted to be major contributors to steviol glycosides exposures, because they are consumed in large volumes.

A sweetener substitution method was used to predict dietary exposures for the sweetener rebaudioside A (Renwick 2008) (Table 11). The analysis used data available from countries with different patterns of sweetener approvals and also for population groups with high potential intakes, such as children and diabetic subjects. These data were used to predict the potential intakes of a novel intense sweetener, such as rebaudioside A, by adjusting the reported intakes of different sweeteners in mg/kg bw by their relative sweetness intensities. This approach assumes that the new sweetener would follow the same pattern and extent of use as the existing sweeteners.

Table 11. Predicted dietary exposures of rebaudioside A based on sweetener substitution[a]

Population Group	Intakes of intense sweeteners expressed as sucrose equivalents in mg/kg bw/d		Predicted intakes of rebaudioside A in mg/kg bw/d		Predicted intakes of rebaudioside A expressed in steviol equivalents (mg/kg bw/d)[b]	
	Average consumer	High consumer	Average consumer	High consumer	Average consumer	High consumer
Non-diabetic adults	255	675	1.3	3.4	0.4	1.1
Diabetic adults	280	897	1.4	4.5	0.4	1.4
Non-diabetic children	425	990	2.1	5.0	0.6	1.7
Diabetic children	672	908	3.4	4.5	1.1	1.7

[a] Adapted from Renwick 2008
[b]Calculation based on the molecular weight of rebaudioside A (967) being about three times that of steviol (318).

Based on this method, estimated intakes of rebaudioside used as a general purpose sweetener, which would be a more extensive use than just for table top sweetener, are between 0.4 and 1.7 mg/kg bw/d. This includes populations such as diabetic children, for whom intake would be predicted to be highest.

000043

IV. SAFETY

The steviol glycosides in highest concentrations in plant extracts, namely stevioside and rebaudioside A, are two of approximately ten other steviol glycosides. In most toxicology studies, the administered amount was expressed as mg/kg bw of stevioside, and was used to assess the toxicity of steviol glycoside extracts. Studies on metabolism of steviol glycosides demonstrate that they are all hydrolyzed upon ingestion to steviol (Gardana et al. 2003); this supports the use of the steviol equivalents as the standard measure of quantity to assess safety of the extract.

Steviol glycosides including stevioside and rebaudioside A are completely metabolized to steviol by the microbial flora of the caecum. Steviol thus formed is readily absorbed and rapidly glucuronidated in both humans and rats, with excretion occurring via the bile (and into feces) in rats and via the urine in humans.

Steviol glycosides have very low acute toxicity and there is no evidence of carcinogenicity, developmental toxicity, reproductive toxicity. Weight of evidence also suggests that it does not exhibit anygenotoxicity. Studies in animals and humans have been conducted to investigate possible antihypertensive and antiglycemic effects of steviol glycosides. These studies have shown that steviol glycosides are well tolerated at levels far in excess of anticipated intakes and unlikely to have adverse effects on blood pressure, blood glucose or other parameters in normal, hypotensive or diabetic subjects (`Curry and Roberts, 2008, Maki et al. 2008a; Maki et al. 2008b).

The adequacy of the existing database provides a basis for eliminating the need for any additional safety factors in setting an ADI for steviol glycosides, such as the 2-fold uncertainty factor used by JECFA in its 2005 derivation of a temporary ADI (2 mg/kg bw/d steviol). The latter was specifically applied to account for uncertainty in potential effects on blood glucose and blood pressure. The recent well-designed clinical studies demonstrate no effects on either blood glucose or blood pressure, and collectively strengthen the clinical evidence of safety, so that the additional 2-fold safety factor for uncertainty is not warranted. Therefore, an ADI of 4 mg/kg bw/d, derived by applying a 100-fold safety factor to the NOAEL of 970 mg/kg bw/d (equivalent to 383 mg/kg bw/kg steviol) in a 2-year rat study, is appropriate. This ADI covers steviol glycoside mixtures with different ratios of stevioside/rebaudioside. The ADI for steviol glycosides, set in 2008 by Food Standards, Australia and New Zealand (FSANZ), is also 4 mg/kg bw/d. FSANZ did not apply an additional safety factor after concluding that potential effects on

either blood glucose or blood pressure were effects unexpected at the projected maximum human intake (FSANZ, 2008).

A. Absorption, Metabolism, Distribution, and Excretion (ADME) of Stevioside

1. Introduction:

Studies on the absorption, distribution, metabolism, and excretion of extracts from steviol glycosides have focused on the stevioside component; information is also available for rebaudioside A and the other steviol glycosides. Results from studies evaluating the absorption and fate of stevioside from several animal species as well as humans indicate that it is appropriate to extrapolate results from toxicity studies in rats and hamsters to humans.

The absorption of stevioside after oral administration is extremely low and none of the digestive enzymes of the gastrointestinal (GI) tract of different animals or humans are able to degrade stevioside into steviol, the aglycone of stevioside. However, stevioside is metabolized to steviol by the bacterial flora of the cecum in rats and hamsters and bacteria isolated from the human colon also metabolize stevioside to steviol *in vitro*. In the same manner, rebaudioside A and the other steviol glycosides (rebaudioside C, dulcoside A, rubusoside, steviolbioside and rebaudioside B) are degraded ultimately to steviol under anaerobic conditions by the gut microflora (Gardana et al. 2003).

Steviol glucuronide is the final product from steviol glycoside metabolism in humans, rat and hamsters. In humans, steviol, not excreted in the feces, is absorbed from the intestines, and transported to the liver in the portal blood. In the liver, the steviol glucuronide is formed, which is released into the blood and filtered out by the kidneys into the urine. Rebaudioside A is excreted as steviol glucuronide in the urine in humans, with lesser amounts of steviol in the feces following oral administration. Similarily, in rats, steviol glycosides are transported to the liver as steviol where they are glucuronidated. The difference in route of excretion of the glucuronide in rats (primarily via the bile) is reported to be due to the lower molecular weight threshold for biliary excretion in rats compared to humans (Wheeler, 2008)

Based on the similarity between rats and humans in generating the primary metabolite, steviol glucuronide, through the classical phase II detoxification mechanism, following administration of either rebaudioside A or stevioside, it can be concluded that both human and rodent toxicology studies conducted with stevioside are also relevant for assessing human safety of rebaudioside A.

2. Review of Studies:

Studies conducted to elucidate the absorption and ultimate fate of stevioside, the major sweet component isolated from the leaf of the plant *S. rebaudiana* (Bertoni) Bertoni, are summarized in Table 12.

The absorption of stevioside after oral administration has been shown to be extremely low (Koyama et al. 2003a, Geuns et al. 2003b) and none of the digestive enzymes from the GI tract of different animals or humans are able to degrade stevioside into steviol, the aglycone of stevioside (Hutapea et al. 1997; Koyama et al. 2003b). In studies with rats and hamsters, stevioside is metabolized to steviol by the bacterial flora of the cecum (Nakayama et al. 1986; Koyama et al. 2003a). Bacteria isolated from the human colon are able to transform stevioside into steviol *in vitro* (Hutapea et al. 1997; Koyama et al. 2003b; Gardana et al. 2003). In pigs, oral administration of stevioside results in complete degradation into steviol, the only compound that is detected in the feces (Geuns et al. 2003b).

In one study, bacteria from the human colon also formed steviol epoxide *in vitro*; however, this metabolite was metabolized to steviol (Hutapea et al. 1997). The epoxide may not be formed *in vivo*, however, due to the anaerobic conditions of the human colon (Geuns 2003). In contrast to the previously cited studies, Hutapea et al. (1999) found several other metabolites in the blood, feces and urine of hamsters 3, and 24 hours, respectively, after ingestion of stevioside. The chromatogram of urine showed the presence of steviol-16,17 alpha-epoxide, stevioside, 15 alpha-hydroxysteviol, steviolbioside and isosteviol. All of these compounds were also detected in feces, along with steviol. The chromatogram of plasma shows the presence of steviol-16, 17 alpha-epoxide, stevioside, 15 alpha-hydroxysteviol and steviolbioside. It was not possible, however, to quantify the amount of each of these metabolites. In addition, since the extracted fractions were treated with H-5 beta-glucuronidase/sulfates at 55°C for 3 h, some of the metabolites might have been produced from this treatment. Work by Hutapea et al. (1997) using *in vitro* digestion of stevioside by intestinal microflora showed the production of 90% of the theoretical amount of steviol from digestion of stevioside. Koyama et al. (2003b) also reported that throughout the incubation period of stevioside with human intestinal microflora, there was no peak except for steviol on both the full scan ion and monitor ion chromatograms of steviol obtained by negative ion LC/ESI/MS analysis. No degradation of steviol at high or low concentrations was observed over the 24-h incubation period. Steviol was confirmed in this study to be the final product from stevioside metabolism. The investigators suggested that the epoxide noted by Hutapea et al (1997) might have been the result of conditions that were not

sufficiently anaerobic allowing oxidation to occur. Secondly, the analysis using HPLC-UVis not highly specific and so another peak may have been erroneously attributed to steviol-16,17-epoxide. In the same manner as stevioside, rebaudioside A is degraded, ultimately to steviol, by rat intestinal microbial cells under anaerobic conditions (Koyama et al. 2003a; Gardana et al. 2003).

Ishii-Iwamoto and Bracht (1995) evaluated steviol chromatographically and observed that under *in vitro* conditions stevioside does not permeate the cell membrane of hepatocytes. The results were consistent with their earlier report (Ishii et al. 1987) and the findings of Kemmelmeier et al. (1984) who demonstrated similar results in human erythrocytes. They concluded that the reported metabolic transformation of intravenously injected [^{131}I] iodine-stevioside is either a specific characteristic of this derivative or depends on factors that are absent in the isolated perfused rat liver.

Steviol, not excreted into the feces, is absorbed from the intestines in both animals and humans (Nakayama et al. 1986; Koyama 2003b). The uptake of steviol by the GI tract was demonstrated in experiments with everted gastrointestinal sacs (Koyama et al. 2003b) and Caco-2 cell monolayers (Geuns et al. 2003b). In humans, stevioside is excreted mainly as a steviol glucuronide in the urine. The excretion route suggests that after degradation of stevioside to steviol by bacteria in the colon, part of the steviol is absorbed by the colon and transported to the liver by portal blood (Cardoso 1996; Nakayama et al. 1986; Geuns et al. 2007). In the liver, the steviol glucuronide is formed, which is released into the blood and filtered out by the kidneys into the urine in humans. The high levels of steviol glucuronide in the urine suggest that there is no accumulation of steviol derivatives in the human body.

Roberts and Renwick (2008) examined the toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol in rats to determine whether toxicological studies conducted previously with stevioside would be applicable to the structurally-related glycoside, rebaudioside A. The results of their study indicated that rebaudioside A, stevioside, and steviol are extensively absorbed as steviol after oral administration, and are metabolized and excreted rapidly with the majority of the radioactivity eliminated in the feces via the bile. This study is in agreement with the results reported by Gardana et al (2003), Geuns et al. (2003b, 2007) and Koyama et al. (2003b) who showed that both rebaudioside A and stevioside are metabolized to steviol by gut microflora. The results from the toxicokinetic and metabolism studies of both rebaudioside A and stevioside indicate a close similarity in handling of all steviol glycosides in the Sprague-Dawley rat, which corroborate the metabolic results from previously reported studies administering stevioside in the Wister rat (Nakayama et al. 1986). In the Roberts and Renwick study, single, oral quantities of the radiolabelled compounds were readily and

extensively absorbed with plasma-concentration time profiles following similar patterns for both stevioside and rebaudioside A. Elimination of radioactivity from plasma was essentially complete within 72 hours. All plasma samples had similar metabolic profiles; the predominant radioactive components were steviol, with lower amounts of steviol glucuronide(s) and even lower level of one or two other metabolites. Rebaudioside A, stevioside, and steviol were metabolized and excreted rapidly; with 60.3% radioactivity eliminated in the feces within 48 h. Urinary excretion accounted for less than 2% of the administered amount for all compounds in both intact and bile duct-cannulated rats, and the majority of the absorbed amount was excreted via the bile. When these compounds were administered into the intact and bile duct-cannulated rats, radioactivity in the feces was present primarily as steviol. The predominant radioactive compound detected in the bile of all cannulated rats was steviol glucuronide(s), indicating deconjugation in the lower intestine. The authors concluded that the toxicokinetic and metabolism of rebaudioside A and stevioside are handled in an almost identical manner.

Geuns et al. (2007) demonstrated the metabolism of stevioside in healthy human subjects. They analyzed stevioside, free steviol and steviol metabolites in blood, feces and urine after 3 days of stevioside administration (250 mg capsules; 3 times per day). No free stevioside was detected in feces, indicating complete metabolism by gut microflora to steviol. A small percent of the steviol generated was excreted in the feces. The remainder and majority is readily absorbed. Steviol glucuronide was the only metabolite detected in either blood or urine. No free steviol was detected, indicating rapid glucuronidation of all absorbed steviol. Theoretically, about 300 mg steviol can be generated from 750 mg stevioside by gut microflora metabolism. Geuns et al compared this amount of steviol to the amount of steviol detected in feces, blood and urine collected after 24 hours (total stevioside intake at 24 hours was 750 mg). About 23 mg of free steviol was directly excreted in the feces. About 277 mg was absorbed, based on about 102 mg steviol glucuronide recovered in the blood and about 102 mg steviol glucuronide excreted into the urine. Calculated total recovery was 75.5%, a percent recovery noted as similar to the recovery expected from this analytical methodology. The data suggested no accumulation of steviol derivatives in the human body.

Wheeler et al. (2008) recently reported the pharmacokinetics of rebaudioside A and stevioside after a single oral administration in healthy men. This randomized, double-blind, cross-over study assessed the comparative pharmacokinetics of steviol and steviol glucuronide following a single oral administration of rebaudioside A and stevioside in healthy adult male subjects. Steviol glucuronide appeared in the plasma of all subjects after administration of rebaudioside A or stevioside, with median T_{max} values of 12.0 and 8.0 hours post-administration, respectively. Steviol glucuronide was eliminated from the plasma, with similar $t_{1/2}$ values of

000048
SPHERIX

approximately 14 hours for both compounds. Administration of rebaudioside A resulted in a significantly (approximately 22%) lower steviol glucuronide geometric mean C_{max} value than administration of stevioside.

Rebaudioside A has one additional glucose moiety that must be removed prior to absorption as steviol in the colon, and therefore, it is not unexpected that the formation of steviol from stevioside would be more rapid than that of rebaudioside A. These observations support the earlier *in vitro* and *in vivo* findings of Koyama et. al., (2003a; 2003b), which demonstrated hydrolysis of stevioside and rebaudioside A to the aglycone steviol within 10 and 24 hours, respectively following incubation with human microflora. Studies with steviol -17-[^{14}C] also demonstrated almost total absorption of the aglycone from the rat lower bowel following intracecal administration (Wingard et al. 1980).

In addition, the geometric mean AUC_{0-t} value for steviol glucuronide after administration of rebaudioside A was approximately 10% lower than administration of stevioside. Steviol glucuronide was excreted primarily in the urine of the subjects during the 72-h collection period accounting for 59% and 62% of the rebaudioside A and stevioside quantities, respectively. No steviol glucuronide was detected in the feces. The study demonstrated that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans with steviol glucuronide excreted primarily in the urine and steviol in the feces. These data are consistent with those from metabolism studies in rats where administration of radiolabelled rebaudioside A and stevioside resulted in metabolism to steviol, followed by extensive glucuronidation to steviol glucuronide. However, in contrast to rats, rebaudioside A and stevioside are excreted mainly as steviol glucuronide in the urine, with lesser amounts of steviol in the feces following oral administration. The difference in excretion of steviol glucuronide primarily via the bile in rats is associated with the lower molecular weight threshold for biliary excretion in rats compared to humans (Renwick and Tarka, 2008; Wheeler et al. 2008). It has been demonstrated that the toxicokinetic and metabolic profile of rebaudioside and stevioside is handled in an almost identical manner in rats (Roberts and Renwick, 2008).

Based on the similarity between rats and humans in generating the the primary metabolite, steviol glucuronide, through the classical phase II detoxification mechanism, following administration of either rebaudioside A or stevioside, it can be concluded that both human and rodent toxicological studies conducted with stevioside are relevant for assessing human safety of rebaudioside A.

Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside

Reference	Test Material(s)	Methods	Results and Discussion
Cardoso et al. 1996	^{131}I-Stevioside (radiochemical purity given as 97%; purity of stevioside not given)	Distribution and metabolism of ^{131}I-Stevioside in rats was studied administering intravenously; rats terminated at 1,3,5,10,30,45,60,120,240, and 1440 minutes after injection; blood and tissue samples taken. Urine and feces collected in metabolic cages for 24 h.	Plasma radioactivity decreased rapidly and reached 5.6% of the injected quantity 60 minutes after administration, showing a fast distribution in the body. The highest concentration of radioactivity was observed in the liver and small intestine after 10 and 120 minutes, respectively. At 2 h after injection, the radioactivity eliminated in the bile was 52% of the original quantity. Excretions of radioactivity in the urine and feces were done at 24 hours; the radioactivity excreted into the feces and urine was 35.30 and 34.70% of the administered quantity, respectively. Results of RP-HPLC analysis of the bile showed that stevioside was degraded *in vivo* (accounted for 37.3% of radioactivity) and steviol appeared as a major metabolite (47.3%); 15.4% was due to an unidentified metabolite with a retention time of 8.0 minutes. In the urine, analysis did not show the presence of steviol, however, stevioside and an unknown metabolite with the same retention time as that observed in the bile was noted.
Gardana et al. 2003	Steviol extracts containing either 85% (w/w) stevioside or 90% (w/w) rebaudioside A	Experiments were carried out under strict anaerobic conditions in batch cultures inoculated with mixed fecal bacteria from volunteers. Hydrolysis was monitored by HPLC. Isolated bacterial strains from fecal materials incubated in selective broths were added to stevioside and rebaudioside A.	Stevioside and rebaudioside A were completely hydrolyzed to their aglycone steviol in 10 and 24 hours, respectively. The human intestinal microflora was not able to degrade steviol.

000050

000051

Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside

Reference	Test Material(s)	Methods	Results and Discussion
Geuns et al. 2003b	Stevioside (purity 96%) Steviolbioside (3%) and rebaudioside A (0.5%) were the main impurities.	Absorption and metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and Steviol using the Caco-2 system were studied.	Stevioside administered orally was completely converted into steviol by the bacteria of the colon. Steviol was the only compound that could be detected in feces. This is in agreement with published results where steviol is found as the only possible metabolite produced by the intestinal microflora under anaerobic conditions. No stevioside or steviol was detected in the blood. These results suggest that steviol, which is only sparingly soluble, is not taken up from the colon of pigs. Only a minor fraction of stevioside and rebaudioside A was transported through the Caco-2 cell layer; results suggested carrier-mediated transport. The authors explained the hydrophilic nature of stevioside and rebaudioside A may be responsibe for the poor transport through the Caco-2 monolayers and everted gastrointestinal sacs, as well as the poor absorption in an *in vivo* situation. The authors speculated that the discrepancy between relatively high absorptive transport of steviol and the lack of steviol in the blood may be explained by the fact that in the Caco-2 study, steviol is applied as a solution facilitating the uptake, whereas in the colon, steviol probably is adsorbed to compounds present in the colon, the contents of which are concentrated as water is withdrawn.

Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside

Reference	Test Material(s)	Methods	Results and Discussion
Geuns et al. 2007	Stevioside (97% purity; impurities are 2.8% steviolbioside and a trace of rebaudioside A, 0.3%)	Stevioside was given three times daily to 10 healthy subjects. On the third day, six blood samples were taken before breakfast and before the first stevioside was administered, and after breakfast and the first stevioside was administered. Subjects collected a 24 h urine sample. A 24 h feces collection was done on the fourth day of the experiment.	Fasting plasma glucose and insulin concentrations were not significantly different before and after test article consumption. No significant differences were detected in electrolytes excreted in the 24 h urine and no indications of tissue damage (alkaline phosphates, alanine aminotransferase, glutamic pyruvate transaminase and creatine kinase). No breakdown of stevioside occurred after 6 h of incubation in stomach juice. No stevioside or free steviol detected in blood samples at any time. No stevioside or free steviol were detected in urine samples, however, after enzymatic hydrolysis of the conjugated steviol, steviol was detected. No stevioside or steviol conjugates were detected in feces samples, however, free steviol was detected. Of the daily quantity of 750 mg stevioside, authors estimated 300 mg free steviol could be produced by the bacteria in the colon. Total recovery of steviol calculated as the sum of the amounts found in blood, urine and feces varied between 36.5% and 101.4% with an average recovery of 75.5%, which is similar to the methodological recovery in spiked samples.
Hutapea et al. 1997	Stevioside extract (purity not given)	Stevioside was incubated with salivary alpha-amylase, pancreatic alpha-amylase, saliva, pepsin, gastric secretion, pancreatin and intestinal brush border membrane enzymes of mice, rats, hamsters, as well as with intestinal microflora of mice, rats, hamsters and humans.	None of the enzymes digested stevioside except the microflora of the rat and hamster cecal contents, which hydrolyzed it to steviol, and the microflora of mouse cecal content and human feces, which hydrolyzed it to both steviol and steviol-16,17 alpha-epoxide. Steviol-16,17 alpha-epoxide was then completely converted back into steviol. Authors suggest that steviol might be the only metabolite produced by the intestinal microflora from various animals and humans.
Hutapea et al. 1999	Stevioside extract (purity not given)	A method of analysis using HPLC was developed for the separation and quantitation of the metabolites of stevioside. The applicability of this method was demonstrated in the detection and separation of stevioside and its metabolites found in blood, feces, and urine of hamsters after ingestion of stevioside.	The chromatogram of urine shows the presence of steviol-16,17 alpha-epoxide, stevioside, 15 alpha-hydroxysteviol, steviolbioside and isosteviol. All of these compounds were also detected in feces, including steviol. The chromatogram of plasma shows the presence of steviol-16,17 alpha-epoxide, stevioside, 15 alpha-hydroxysteviol and steviolbioside.

000052

000053

Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside

Reference	Test Material(s)	Methods	Results and Discussion
Ishii-Iwamoto and Bracht 1995	Stevioside (crystallized to 99% purity)	Stevioside was perfused into rat livers to evaluate hepatic metabolism; concentrations of 0.2 and 0.5 mM were recirculated during 2 h.	Samples were taken periodically and analyzed for total sugars and free glucose. The latter is released from endogenous sources, especially glycogen. The concentration of stevioside was calculated from the difference between total sugars and free glucose. Concentration of stevioside remained constant during the whole perfusion time. Possible formation of hydrolysis products was investigated chromatographically with negative results. Metabolic transformation of stevioside to steviol did not occur in the perfused liver.
Koyama et al. 2003a	Stevia mixture (main components: rebaudioside A, stevioside, rebaudioside C, dulcoside A) Steviol	Absorption in rats (*in vivo* with a single oral administration and ex vivo using rat everted sac method) and hepatic metabolism in rats and humans using incubation with liver microsomes of both stevia mixture and steviol was investigated.	Ex vivo using rat everted sac method, no absorption of stevia mixture was observed, but significant absorption of steviol was noted. *In vivo*, after single oral administration of steviol, a peak steviol concentration in plasma was observed 15 minutes after administration demonstrating rapid absorption. After stevia mixture, steviol concentration in plasma increased steadily over 8 h, suggesting degradation preceding absorption as steviol. Incubation with liver microsomes from rats and humans produced monohydroxy and dihydroxy metabolites of steviol. The intrinsic clearance of steviol in human liver microsomes was 4-tikmes lower than that found in rat liver microsomes. The purpose of the study was to evaluate the evidence for the validity of extrapolating toxicity data for stevia related compounds from rats to humans. From previous work the authors indicated that absorption of steviol from the human intestine can be predicted to occur in an analogous manner to absorption from rat intestine. Current data suggests that metabolism of steviol is similar between rats and humans.

Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside

Reference	Test Material(s)	Methods	Results and Discussion
Koyama et al. 2003b	Stevia mixture (main components: rebaudioside A, stevioside, rebaudioside C, dulcoside A) Enzymatically modified stevia (main components: alpha-glucosylrebaudioside A, alpha glucosylstevioside, alpha-glucosylrebaudioside C, alpha glucosyldulcoside A) Rebaudioside A Stevioside	Human intestinal metabolism of test materials examined by incubating stevia mixture and other stevia components with pooled human fecal homogenates under anaerobic conditions.	Stevia mixture, enzymatically modified stevia, stevioside and rebaudioside A were completed eliminated within 24 hours, whereas no degradation of steviol occurred during the incubation period. Stevia mixture, stevioside and rebaudioside A appeared to be hydrolyzed to steviol by human intestinal microflora; enzymatically modified stevia appeared to be metabolized via stevia components and finally, to steviol. Steviol was determined to be the final product from stevia and its related compounds including enzymatically modified stevia in human intestinal microflora. Authors suggest that there are no apparent species differences in intestinal metabolism of stevia mixture between rats and humans.

000054

000035

Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside

Reference	Test Material(s)	Methods	Results and Discussion
Nakayama et al. 1986	^{3}H Stevioside (radiochemical purity given as 95%; purity of stevioside not given)	ADME of ^{3}H Stevioside administered orally at a level of 125 mg/kg to rats was studied.	Level of radioactivity in the blood reached a maximum at 8 hours, with a biological half-life of 24 hours. Radioactivity remaining the body at 48 hours was 30.7% of the quantity. At 120 hours, the percentages of radioactivity excreted into the feces and expired air were 68.4% and 23.9% respectively. Excretion of radioactivity in the urine was 2.3%. Radioactivity excreted into the bile at 72 hours was 40.9% of the original quantity. From results of biliary and fecal excretion it was concluded that entero-heaptic circulation occurs in the body. Thin-layer radiochromatograms of metabolites in the GI contents were evaluated. Stevioside was detected as a major component of the stomach contents at 1 h and minor metabolites were also detected. In the small intestinal contents, stevioside and metabolites accounted for 33 and 67% of total radioactivity, respectively. The highest peak obtained in solvent system I was at the position of glucose. Stevioside, steviolbioside and steviol accounted for 7.6, 8.0 and 7.5%, respectively, of the radioactivity in the small intestinal contents at 4 hours, while they accounted for 39.4%, 16.7% and 5.1% , respectively, in the cecal contents at the same time. At 24 hours, stevioside was not detectable in the cecal contents, but steviol and unidentified metabolites accounted for 15.6 and 68.0% of the radioactivity, respectively. Steviol was found to be a major metabolite in feces. Steviol was found to be the major metabolite in feces. Ingested stevioside is metabolized by bacterial flora in the cecum to steviolbioside, steviol and glucose, and they are absorbed as they are or after further decomposition. Absorbed glucose is metabolized and excreted in expired air while most of the steviol is conjugated in the liver and excreted into the intestinal tract through the bile.

Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside

Reference	Test Material(s)	Methods	Results and Discussion
Roberts and Renwick, 2008	Rebaudioside A (>97% purity), stevioside (>97% purity) and steviol (>97% purity)	Radiolabelled rebaudioside, stevioside and steviol administered via gavage to study toxicokinetic and metabolism in rats. In pharmacokinetics study, 27 rats per sex per test compound received a single oral quantity of the test substance and blood samples were collected for analyzing steviol and steviol glucuronide(s) by HPLC A single quantity of each test substance was administered to 5 intact rats per sex and 5 bile duct cannulated rats per sex. Urine and feces was collected after administration at specific time intervals for analysis. For metabolic experiments, proportional pools of feces extracts were prepared from intact animals administered with radiolabelled rebaudioside A., stevioside or steviol and from cannulated animals administered with radiolabelled rebaudioside A or stevioside. Likewise, proportionate pools of bile samples collected during the 48-h period were prepared from selected cannulated animals administered with radiolabelled rebaudioside A, stevioside or steviol.	Rebaudioside A, stevioside, and steviol are extensively absorbed after oral administration. Toxicokinetic and metabolism studies of both rebaudioside A and stevioside indicate a close similarity in handling of all steviol glycosides in the Sprague-Dawley rat. Single, oral quantities of the radiolabelled compounds were readily and extensively absorbed with plasma-concentration time profiles following similar patterns for stevioside and rebaudioside A. Elimination of radioactivity from plasma was essentially complete within 72 hours. All plasma samples had similar metabolic profiles; the predominant radioactive components as steviol, with lower amounts of steviol glucuronide(s) and low level of one or two other metabolites. Rebaudioside A, stevioside, and steviol were metabolized and excreted rapidly; with the majority of the radioactivity eliminated in the feces within 48 h. Urinary excretion accounted for less than 2% of the administered amounts for all compounds in both intact and bile duct-cannulated rats, and the majority of the absorbed quantity was excreted via the bile. When these compounds were administered into the intact and bile duct-cannulated rats, radioactivity in the feces was present primarily as steviol. The predominant radioactive compound detected in the bile of all cannulated rats was steviol glucuronide(s), indicating deconjugation in the lower intestine. The authors concluded that the toxicokinetic and metabolism data on rebaudioside A and stevioside are handled in an almost identical manner.

000056

Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside

Reference	Test Material(s)	Methods	Results and Discussion
Wingard et al. 1980	Stevioside and Rebaudioside A (purity not given)	Test articles were incubated anaerobically with microbial whole-cell suspensions from rat cecum and aerobically with whole-cell suspensions from rat cecum and aerobic incubation with sonic cell-free extracts prepared from rat cecal contents. Intestinal absorption of steviol-17-[^{14}C] released in the lower bowel was assessed after oral and intracecal administration.	Test articles were degraded to steviol *in vitro*. Steviol was readily absorbed from the lower bowel of the rat after oral or intracecal administration. The radioactivity was excreted in the urine of bile duct ligated animals (approximately 94% of amount administered) and in the bile of bile duct cannulated and intact animals (approximately 105% of amount administered, biliary excretion; approximately 96% of amount administered, fecal excretion).

000057

Table 12. Absorption, Metabolism, Distribution and Excretion of Stevioside

Reference	Test Material(s)	Methods	Results and Discussion
Wheeler et al. 2008	Stevioside (>96.6% purity) and rebaudioside A (>98.7% purity)	Subjects received a single oral administration of 5 mg/kg rebaudioside A and 4.2 mg/kg stevioside, providing exposure of approximately 1.6 mg/kg of steviol equivalents to study pharmacokinetics.	Steviol glucuronide appeared in the plasma of all subjects after administration of rebaudioside A or stevioside, with median T_{max} values of 12.0 and 8.0 hours post-administration, respectively. Steviol glucuronide was eliminated from the plasma, with similar $t_{1/2}$ values of approximately 14 hours for both compounds. Administration of rebaudioside A resulted in a significantly (approximately 22%) lower steviol glucuronide geometric mean C_{max} value than administration of stevioside. In addition, the geometric mean AUC_{0-t} value for steviol glucuronide after administration of rebaudioside A was approximately 10% lower than administration of stevioside. Steviol glucuronide was excreted primarily in the urine of the subjects during the 72-h collection period. No steviol glucuronide was detected in the feces. The study demonstrated that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans with steviol glucuronide excreted primarily in the urine and steviol in the feces. Authors concluded that the data obtained in this study is consistent with those from metabolism studies in rats where administration of radiolabelled rebaudioside A and stevioside resulted in metabolism to steviol, followed by extensive glucuronidation to steviol glucuronide. In addition, based on the similarity in human metabolism to the primary metabolite steviol glucuronide following administration of rebaudioside A or stevioside through the classical phase II detoxification mechanism, it was concluded that previous human studies and rodent toxicological studies conducted with stevioside are relevant for assessing human safety of rebaudioside A.

000058

B. Toxicology Studies

1. Introduction:

Steviol glycosides are not acutely toxic in oral toxicity studies (Medon, 1982). In subchronic studies of up to 13 weeks duration, no adverse test-article related toxicity has been reported (Akashi and Yokoyma, 1975 (as cited by Yamada 1985); Taskulkao et al. 2001; Curry and Roberts, 2008). The predominant finding in subchronic oral toxicity studies has been a reduction in body weight in rats given very high levels of steviol glycosides. Curry and Roberts (2008) reported that Wistar rats given rebaudioside A as 5% of their diet for 90-days had reduced feed 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 concentration 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 4,000 mg/kg bw/d, the highest amount tested. A subchronic study of McNeil Stevia (steviol glycoside extract containing rebaudioside A and stevioside, as the primary and secondary steviol glycosides, respectively), from two sources prepared by two different manufacturers, evaluated potential toxicity in male and female Sprague Dawley (Crl) rats following repeated exposures for 90 days in the diet (McNeil, 2008 unpublished data) also reported similar results.

There are three studies in the literature that investigated chronic toxicity of stevioside over a two-year period in rats. Yamada et al. (1985) and Xili et al. (1992) reported NOAELs of 550 mg/kg bw/d and 600 mg/kg bw/d, respectively, the highest amounts tested in each study. Toyoda et al. (1997) reported body weight reductions related to the amount of stevioside administered that were considered toxicologically significant in males (2000 mg/kg bw/d) and females (2,400 mg/kg bw/d) who were given high quantities, along with a significantly reduced survival rate in males given high quantities. The authors considered the NOAEL in this study to be 970 mg/kg bw/d based on the reduction of body weight gain and effects on survival observed in males. The body weight gain reduction is considered toxicologically non-significant. These effects are similar to those seen for other high intensity sweeteners or other food additives with the potential for taste aversion (Curry and Roberts, 2008). Toyoda et al. (1997) 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 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.

According to JECFA (2005) neither stevioside nor rebaudioside A are genotoxic. Most recently, an additional Comet assay of stevioside was reported. (Nunes et al. 2007). Stevioside at a level of 4 mg/ml was given orally to Wister rats and was reported to induce lesions in peripheral blood, liver, brain, and spleen cells. The data reported by Nunes et al. (2007) is inconsistent and conflicting since both control and treated animals exhibit a high degree of DNA damage. In addition, since the Comet assay is only a screening assay for DNA damage, it is not indicative of heritable genetic effects, but only useful as an aid to detect the toxic effect of a compound to the genetic material (Geuns 2007). Two earlier Comet assays did not find any DNA damage (Sekihashi et al. 2002; Sasaki et al. 2002) with steviol glycosides exposure. The organs reported to show lesions in the Nunes study do not show any adverse histopathology in subchronic or chronic studies conducted earlier with any of the derivatives of the steviol glycosides (Toyoda et al. 1997; Yamada et al. 1985; Xili et al. 1992; Curry et al. 2008; McNeil 2008)

Steviol, the aglycone bacterial metabolite of stevioside, in the presence of a rat liver S9 metabolic activation system, produced positive effects in both Salmonella strain TM 677 and in an in vitro chromosome damage test in Chinese hamster lung cells. In the absence of S9 activation or in several *in vivo* tests for chromosome damage, steviol was negative. The conditions under which these positive results were seen in vitro have limited relevance to *in vivo* exposures where all the tests for genotoxicity were negative. This conclusion is corroborated by the negative results in chronic carcinogenicity assays conducted in rats and hamsters (Toyoda, 1997; Xili et al. 1992; Yamada et al. 1985).

Studies with purified extracts containing stevioside or steviol glycosides have not reported adverse reproductive or developmental effects (Mori et al., 1981; Yodyingyuad and Bunyawong, 1991; Usami et al., 1995); two early studies conducted with crude extract of leaves of *S. rebaudiana* (Bertoni) Bertoni reported adverse effects on fertility (Mazzei-Planas and Kuc, 1968; Melis, 1999). The relevance of these latter studies to the safety assessment of purified rebaudioside A is limited because of the lack of characterization of the crude steviol glycosides extracts and use of protocols that do not meet regulatory guidelines for reproductive safety studies. The Joint Expert Committee on Food Additives (JECFA) reviewed the reproductive studies published during that time in 1999 and 2004 and concluded that steviol glycosides meeting the JECFA monograph specifications did not pose a reproductive hazard to humans based on the weight of the evidence from the safety data (JECFA 1999, 2005). Recently Curry et al. (2008) presented the first report evaluating the reproductive and developmental toxicity of well-characterized and purified rebaudioside A extract. The results corroborate the results of other studies in hamsters and rats which administered well defined, high-purity steviol

glycosides extracts. Both Yodyingyuad and Bunyawong (1991) and Mori et al. (1981) used stevioside (90% and 96% purity, respectively) and reported no significant effects on hamster or rat reproduction, respectively. Roberts and Renwick (2008) have demonstrated through comparative metabolism studies that animal toxicity studies conducted with both rebaudioside A and stevioside can be used for human safety evaluation. Therefore, this study along with the two previously reported reproductive toxicity studies with purified stevioside clearly support a conclusion that consumption of purified steviol glycosides does not present a reproductive or developmental hazard.

a) Acute Toxicity

(1) Introduction

One acute toxicity study of stevioside and its metabolite steviol was retrieved from the public literature (Toskulkao et al. 1997). In addition, in the introduction to several of the papers dealing with subchronic and chronic toxicity that were retrieved and reviewed, information regarding the acute toxicity was cited.

(2) Review of studies

The acute toxicity of stevioside and steviol was investigated in three animal species including rat, mouse and hamster. Stevioside at a level as high as 15 g/kg bw was not lethal to either mice, rats or hamsters. Hamsters were found to be more susceptible to steviol than rats or mice. LD_{50} values of steviol in hamsters were 5.20 and 6.10 g/kg bw for males and females, respectively. In rats and mice, LD50 values of steviol were higher than 15 g/kg bw in both sexes.

The oral LD_{50} of a steviol glycosides extract containing 53.1% stevioside in mice was reported to be more than 4.2 g/kg by Akashi and Yokoyama (1975) as cited by Yamada et al. (1985).

Medon et al. (1982, abstract) reported an LD_{50} greater than 2.0 g/kg for the crude extract of *S. rebaudiana* (Bertoni) Bertoni following gastric intubation to male Swiss-Webster mice.

The oral LD_{50} of stevioside of 93.5% purity was more than 15 g/kg for mice of both sexes (Akashi and Yokoyama, 1975), cited by Toyoda, et al, 1997; however, citing the same paper, Yamada et al. (1985) give the oral LD_{50} as more than 1.5 g/kg. An oral LD_{50} of 8.2 g/kg of purified (% purity not stated) stevioside for both rats and mice (sex and strain not specified) was reported by Mitsuhashi et al. (1976) as cited by Alvarez (2006). Medon et al (1982, abstract) reported an LD_{50} greater than 2.0 g/kg following gastric intubation to male Swiss-Webster mice.

Medon et al (1982) have reported in an abstract that the LD_{50}s for steviolbioside, dulcoside A and rebaudiosides A are all greater than 2.0 g/kg following gastric intubation to male Swiss-Webster mice.

b) Subchronic toxicity

(1) Introduction

A number of subchronic toxicity studies were performed that utilized purified steviol glycosides in rats and hamsters. The endpoints evaluated in these studies included clinical signs, feed intake, clinical chemistry and hematology, gross and histopathology, organ and body weight and neurobehavioral screening. In 13-week studies, NOAELS were established at the highest amounts tested, up to 5% in the diet of steviol glycosides.

(2) Review of studies

In rats (sex not stated) given a steviol glycosides extract (53.1% stevioside) in their diet at levels of 0.28, 1.40 and 7.00% for 3 months, the highest amount (7.00%) caused a slight decrease in body weight gain in the males (Akashi et al. 1975 as cited by Yamada et al. 1985). No other adverse effects were mentioned.

No toxicological findings from dietary administration of a 95.6% pure stevioside preparation at levels of 0.31, 0.62, 1.25, 2.5, and 5 % for 13 weeks were reported in male or female F344 rats (10 male and 10 female per group) (Aze et al. 1991, abstract only, article is in Japanese).

Toskulkao et al. (2001) evaluated the toxicity of steviol (~90% purity) in hamsters of both sexes. In the first part of their study, they evaluated the levels of steviol in order to determine the level for a subsequent carcinogenicity study in hamsters. The chronic study evaluated the carcinogenic potential of steviol at 0, 100 and 500 mg/kg in the diet for 22 months in both male and female hamsters (described under the chronic toxicity section). In the dose-ranging study, the animals were given 0, 100 and 500 mg/kg/day in the diet for 13 weeks. The animals were weighed once a week, and their condition was checked daily. Steviol at a level of 100 or 500 mg/kg diet had no significant effect on body weight gain or feed conversion efficiency (Table 13), and showed no observable signs of toxicity or abnormal behavior. This level of addition resulted in consumption of 38.7 mg/kg/day and 199.8 mg/kg/day steviol in males and 37.4 and 183.1 mg/kg/day steviol in females, respectively (Table 14). No observable signs of toxicity or abnormal behavior were reported. No adverse effects on feed consumption,

body wight gain or feed conversion efficency were reported (Table 15). Based on these results, the maximum tolerable amount of steviol given in the diet was estimated to be higher than 500 mg/kg diet for both sexes in hamsters.

Relative feed consumption, body weight gain, food conversion efficiency and amount of steviol ingested by hamsters fed steviol during the first 3 months (98 days) of the chronic toxicity/carcinogenicity study is shown in tables 13 and 14, respectively.

Table 13. Relative feed consumption, body weight gain and feed conversion efficiency of hamsters fed steviol during the first 3 months (98 days) of the chronic toxicity/carcinogenicity study

Sex	Steviol (mg/kg diet)	Body weight gain (g)	Relative feed consumption (g)	Feed conversion efficiency (%)
Male	0 (control)	89.9±5.3	613.1±30.8	14.7±0.9
	100	98.2±6.7	628.1±24.7	15.6±1.1
	500	87.5±4.8	580.5±19.5	15.1±0.8
Female	0 (control)	129.4±5.0	681.7±18.5	18.9±0.7
	100	122.8±5.0	686.4±12.7	17.9±0.7
	500	120.2±4.3	683.6±14.6	17.6±0.6

Values are means ±SEM of 9 hamsters/group.
From: Toskulkao et al. 2001

Table 14. Amount of steviol ingested by hamsters during the first 3 months (98 days) of the chronic toxicity/carcinogenicity study

Sex	Steviol (mg/kg diet)	Relative total feed consumption (g)	Relative steviol consumption (g)	Mean body weight (g)	Steviol consumption (mg/kg/day)
Male	0 (control)	5517	0	139.2	0
	100	5652	565	149.0	38.7
	500	5224	2612	133.4	199.8
Female	0 (control)	6135	0	175.5	0
	100	6177	617	168.5	37.4
	500	6152	3076	171.5	183.1

Values are means ±SEM of 9 hamsters/group.
From: Toskulkao et al. 2001

000063

Curry and Roberts (2008) conducted a comprehensive study evaluating the subchronic toxicity of rebaudioside A in Wister rats. The first part of the study was a 4-week palatability study, Wister rats were administered rebaudioside A at dietary concentrations of 0, 25,000, 50,000, 75,000 and 100,000 ppm. During the first week of treatment, males receiving 25,000, 50,000, 75,000, or 100,000 ppm rebaudioside A achieved mean dosages of 3,455, 6,981, 10,180, and 14,167 mg/kg bw/d, respectively, and during Week 4, they achieved mean dosages of 2,610, 5,505, 8,248, and 11,672 mg/kg bw/d, respectively. Females receiving 25,000, 50,000, 75,000, or 100,000 ppm rebaudioside A achieved mean dosages of 3,724, 7,125, 10,789, and 14,119 mg/kg bw/d, respectively, during the first week of the study, and mean dosages of 2,963, 6,217, 9,189, and 13,126 mg/kg bw/d, respectively, during Week 4.

There were no deaths and no adverse clinical signs in relation to treatment. The mean body weight gains of males and females receiving levels of 50,000 ppm or above were significantly lower than those of the controls during the first 4 days of treatment. After 4 weeks, a statistically significant difference in body weight gain was observed only in females at the 100,00 ppm level. (Table 15).

Table 15. Body weight gain of the rats in the 4-week study

Sex	Quantity (ppm)	Day 0–4	Day 4–7	Day 7–11	Day 11–14	Day 0–28
Male	0[a]	22 ± 1.3[b]	16 ± 1.8	21 ± 2.9	14 ± 2.7	119 ± 13.0
	25,000	20 ± 2.8	18 ± 2.1	24 ± 3.8	15 ± 3.4	120 ± 14.9
	50,000	14 ± 5.2**	16 ± 3.5	20 ± 5.6	12 ± 2.8	104 ± 21.0
	75,000	17 ± 4.4**	16 ± 2.4	19 ± 3.2	14 ± 2.3	108 ± 13.7
	100,000	12 ± 3.3**	18 ± 3.7	22 ± 3.9	14 ± 4.2	108 ± 14.1
Female	0	14 ± 2.6	8 ± 1.7	12 ± 2.0	6 ± 3.9	65 ± 11.5
	25,000	10 ± 4.8	9 ± 4.0	11 ± 1.9	6 ± 2.4	58 ± 12.6
	50,000	9 ± 2.7*	8 ± 1.8	9 ± 3.3	5 ± 2.3	52 ± 10.6
	75,000	9 ± 4.9*	9 ± 1.7	11 ± 3.6	6 ± 1.8	60 ± 10.9
	100,000	5 ± 5.4**	7 ± 3.3	10 ± 3.4	5 ± 2.6	48 ± 11.3**

Compared to the control *$p < 0.05$; **$p < 0.01$.
[a] n = 10/sex/group.
[b] Average weight gain in grams ± standard deviation
From: Curry and Roberts, 2008

There was a significant reduction in feed consumption in males in the 50,000, 75,000, and 100,000 ppm groups and in females of the 75,000 and 100,000 ppm groups compared to the controls on Day 1 of treatment. On Day 6, males and females in the two highest level groups (75,000 and 100,000 ppm) showed a significant increase in feed consumption compared to

controls. On Day 7, males in the three highest level groups had a significant increase in feed consumption compared to controls. All of the treated males had higher feed consumption compared to controls on Days 10 and 12. Average feed consumption of the rats from Day 1 to 28 in males was 22, 24, 23, 23 and 24 g/animal and 19, 18, 18, 19 and 19 g/animal in the 0, 25,000, 50,000, 75,000 and 100,000 ppm levels respectively. Feed consumption in rats in the 4-week study is shown in Table 16.

Table 16. Feed consumption of the rats in the 4-week study during selected periods

Day	Males				
	0 ppm[a]	25,000 ppm	50,000 ppm	75,000 ppm	100,000 ppm
−3	19 ± 2.0[b]	19 ± 2.9	18 ± 3.4	20 ± 8.0	19 ± 2.3
1	20 ± 2.2	18 ± 2.3	16 ± 3.4*	17 ± 2.9*	13 ± 3.3**
2	22 ± 10.8	23 ± 2.6	20 ± 4.9	18 ± 2.7	19 ± 7.0
3	21 ± 1.6	21 ± 2.7	21 ± 3.8	20 ± 3.2	21 ± 2.5
4	21 ± 1.2	22 ± 1.6	20 ± 2.3	21 ± 2.4	26 ± 8.1
10	22 ± 1.9	25 ± 1.9*	23 ± 2.5*	25 ± 1.8**	24 ± 1.4**
12	22 ± 2.4	25 ± 2.9*	23 ± 2.2*	26 ± 2.3**	25 ± 2.2**
14	20 ± 2.9	25 ± 3.4	23 ± 5.0	24 ± 2.4	26 ± 4.1**
15 to 18	24 ± 1.7	26 ± 1.2	25 ± 3.0	25 ± 1.8	25 ± 2.8
19 to 21	23 ± 1.8	25 ± 1.7	24 ± 4.1	24 ± 2.9	26 ± 2.9*
22 to 25	24 ± 1.7	26 ± 2.0	25 ± 3.3	26 ± 1.9	27 ± 3.7**
26 to 21	18 ± 1.5	21 ± 1.7*	19 ± 1.7	18 ± 2.0	20 ± 2.3
1 to 28	22	24	23	23	24*
Day	**Females**				
	0 ppm[a]	**25,000 ppm**	**50,000 ppm**	**75,000 ppm**	**100,000 ppm**
−3	18 ± 3.6	18 ± 2.7	18 ± 2.8	20 ± 8.7	16 ± 2.5
1	17 ± 3.1	16 ± 2.4	15 ± 2.6	12 ± 3.3**	11 ± 5.1**
2	18 ± 2.6	22 ± 7.5	18 ± 4.7	18 ± 4.6	19 ± 8.2
3	19 ± 2.9	18 ± 2.9	15 ± 1.9	17 ± 5.3	16 ± 4.9
4	18 ± 1.5	16 ± 3.0	18 ± 2.1	17 ± 2.2	18 ± 3.7
10	17 ± 2.5	18 ± 2.1	17 ± 2.2	19 ± 2.0	19 ± 2.6
12	19 ± 1.8	18 ± 2.7	18 ± 2.3	19 ± 2.4	19 ± 2.8
14	17 ± 2.8	17 ± 2.8	18 ± 2.7	18 ± 1.4	19 ± 2.4
15 to 18	19 ± 1.6	19 ± 1.5	19 ± 1.2	20 ± 1.4	20 ± 2.2
19 to 21	19 ± 2.2	19 ± 1.7	16 ± 5.5	19 ± 1.9	20 ± 2.6
22 to 25	20 ± 1.0	20 ± 1.5	20 ± 1.7	20 ± 1.2	21 ± 2.2
26 to 21	18 ± 1.9	17 ± 1.6	17 ± 0.9	15 ± 1.7*	16 ± 1.9*
1 to 28	19	18	18	19	19

Compared to the control *p < 0.05; **p < 0.01.
[a] n = 9–10/sex/group.
[b] Average food consumption (g/animal) ± standard deviation.

From: Curry and Roberts, 2008

Other increases and decreases were observed in males and females throughout the study period; but these changes did not follow an amount or temporal pattern. Over the entire experimental period, the only significant difference in feed consumption observed was an increase by the males administered high levels compared to the controls. No apparent effects on feed conversion efficiency were observed; however, relatively low conversion efficiency in both sexes treated at 50,000, 75,000, and 100,000 ppm was apparent for the first 2 weeks of the study.

A few statistically significant differences in hematology results were observed between control and treated rats, but all were considered biologically irrelevant because they were small and did not occur in a pattern related to amount administered. The results of the clinical chemistry analyses revealed small but statistically significant increases in plasma creatinine in all groups of treated males and females in the 75,000 and 100,000 ppm level, which were accompanied by significant increases in urine specific gravity in the top two levels in males and all treated groups of females. Urine volume was also significantly reduced in the 75,000 and 100,000 group males. There was a significant decrease in total bile acid levels in males in the 75,000 ppm and 100,000 ppm groups. All treated female rats were observed to have significantly lower bile acid levels compared to the control rats, but these decreases did not follow a pattern related to amount administered. In addition, when one atypically high value among the control males and two atypically high values among the control females were excluded, there was no clear effect of treatment of biological significance. Organ weight analyses revealed that for males in the two highest level groups, heart weights were significantly lower after they were adjusted for body weights compared to control males. Males administered high levels also had significantly lower testes weights compared to the controls. Females in the three highest level groups had significantly lower relative adrenal gland weights compared to controls, but these decreases did not follow a pattern related to amount administered. There were no macroscopic findings considered to be related to treatment at necropsy and further, microscopic examination of the testes and epididymides did not reveal any treatment-related findings (*i.e.*, no histopathological correlates for the organ weight findings). No effects were observed on the seminiferous tubules. Other organs were not examined microscopically in this study.

Based on the results of the 4-week palatability study, levels for the 13-week study were established at 0, 12,500, 25,000, and 50,000 ppm using FDA Redbook guidance. The highest concentration of test agent in the diet in longer-term studies is generally set at 5% (50,000 ppm) so as to avoid nutritional imbalance in treated animals.

000066

In the 13 week study, during the first week of treatment, males receiving 12,500, 25,000, or 50,000 ppm rebaudioside A achieved mean dosages of 1,506, 3,040, and 5,828 mg/kg bw/d,

respectively, and during Week 13, they achieved mean dosages of 698, 1,473, and 3,147 mg/kg bw/d, respectively. Females receiving 12,500, 25,000, or 50,000 ppm rebaudioside A achieved mean dosages of 1,410, 2,841, and 5,512 mg/kg bw/d, respectively, during the first week of the study and mean dosages of 980, 1,914, and 3,704 mg/kg bw/d, respectively, during Week 13. There were no treatment-related deaths, and appearance, behavior, and sensory reactivity of the animals were reported as unaffected by treatment. Ophthalmological analyses revealed no differences between animals who were given high levels and the controls.

During the first 4 days of treatment, body weight gains were significantly lower in males and females receiving 25,000 or 50,000 ppm rebaudioside A. Following the first 4 days of treatment to study termination, body weight gains were significantly lower in all male test groups compared to the controls. Overall, body weight gains were significantly lower in treated males. Also, in females treated at 25,000 and 50,000 ppm, the overall body weight gains were slightly, but significantly lower than the controls (Table 17). During the first 3 days of the study, food intake was significantly lower in treated-males and in mid-and females administered high levels compared to the control groups. Rebaudioside A is known to resist digestion and absorption in the gut, but is degraded in the colon to the aglycone steviol prior to absorption (Koyama et al. 2003). Therefore, rebaudioside A is assumed to provide little to no nutritional value. To account for this, the caloric density of the rebaudioside A-containing diets were considered lower by 1.25%, 2.5%, and 5.0% in the low- through high level groups, respectively, in comparison to the control diet. When corrected for caloric density, overall food consumtion was found to be 94.8%, 97.5%, and 95.0% of the controls in the low- through high-level males, respectively, and 104.7%, 97.5%, and 95.0% of the controls in low- through high–level females, respectively. Over the course of the entire study, statistically significant decreases in food conversion efficiency values were limited to high-level males.

Clinical chemistry (Table 18), organ weight, and functional observational battery test results did not exhibit any significant changes. Inconsistent reductions in serum bile acids and cholesterol were observed, which were attributed to physiological changes in bile acid metabolism due to excretion of high levels of rebaudioside A via the liver. All other hepatic function test results and liver histopathology were within normal limits. In addition, macroscopic and microscopic examinations of all organs, including testis and kidneys, were unremarkable with respect to treatment-related findings. The NOAEL in the 13-week toxicity study was considered to be 50,000 ppm or approximately 4161 and 4645 mg/kg bw/d in male and female rats, respectively.

Table 17. Body weight of rats in the 13-week study

Day	Males			
	0 ppm[a]	12,500 ppm	25,000 ppm	50,000 ppm
−7	112 ± 5.9[b]	110 ± 7.6	109 ± 5.6	108 ± 7.5
1	152 ± 8.2	146 ± 9.3	146 ± 9.3	146 ± 9.1
4	170 ± 9.2	163 ± 10.6	160 ± 10.8	157 ± 10.7
8	199 ± 10.9	186 ± 13.6	183 ± 12.4	180 ± 11.9
11	211 ± 9.5	198 ± 10.7	196 ± 10.3	193 ± 8.9
22	267 ± 15.7	246 ± 17.8	248 ± 14.3	241 ± 14.5
29	293 ± 19.4	273 ± 21.0	272 ± 17.5	261 ± 18.3
43	335 ± 25.1	310 ± 24.5	308 ± 22.2	291 ± 21.3
57	362 ± 25.9	335 ± 25.8	339 ± 24.9	315 ± 23.3
71	385 ± 29.5	361 ± 27.2	356 ± 26.1	333 ± 29.0
92	407 ± 31.0	380 ± 28.0	378 ± 27.3	352 ± 29.6
Gain from 1 to 4	18 ± 1.9	17 ± 3.5	15 ± 4.2**	11 ± 3.3**
Gain from 4 to 92	236 ± 26.4	217 ± 26.9*	218 ± 21.9*	194 ± 25.3**
Gain from 1 to 92	255 ± 27.3	234 ± 27.7*	233 ± 23.3*	205 ± 26.2**
Day	**Females**			
	0 ppm[a]	**12,500 ppm**	**25,000 ppm**	**50,000 ppm**
−7	97 ± 6.3	100 ± 5.9	100 ± 6.2	98 ± 4.7
1	117 ± 6.8	123 ± 5.9	123 ± 9.9	120 ± 6.2
4	126 ± 7.3	132 ± 7.2	129 ± 10.5	123 ± 6.1
8	138 ± 8.5	143 ± 8.5	141 ± 12.6	134 ± 7.0
11	142 ± 7.0	150 ± 6.8	148 ± 11.9	139 ± 7.1
22	166 ± 10.3	173 ± 9.7	168 ± 15.0	163 ± 10.9
29	176 ± 10.9	186 ± 11.8	179 ± 16.8	174 ± 11.7
43	194 ± 13.9	202 ± 13.3	195 ± 17.3	189 ± 11.1
57	206 ± 14.6	213 ± 15.2	206 ± 18.6	201 ± 12.7
71	218 ± 16.4	222 ± 14.9	216 ± 19.1	209 ± 13.2
92	228 ± 16.2	232 ± 14.7	224 ± 20.1	217 ± 12.0
Gain from 1 to 4	8 ± 2.5	10 ± 2.7	7 ± 2.3*	3 ± 3.8**
Gain from 4 to 92	102 ± 14.4	99 ± 12.3	95 ± 13.0	95 ± 10.9
Gain from 1 to 92	111 ± 13.3	109 ± 12.3	101 ± 13.3*	98 ± 11.9**

Compared to the control *$p < 0.05$; **$p < 0.01$.
[a] n = 19–20/sex/group.
[b] Average weight in grams ± standard deviation.
From: Curry and Roberts, 2008

000068

000069

Table 18. Selected clinical chemistry parameters of the rats in the 13-week study on day 89

Parameter	Males				Females			
PPM	0[a]	12,500	25,000	50,000	0	12,500	25,000	50,000
ALP (u/L)	69 ± 15.4[b]	76 ± 14.2	77 ± 9.1	74 ± 12.6	31 ± 8.2	32 ± 7.8	35 ± 7.8	38 ± 11.4
ALT (u/L)	29 ± 4.5	27 ± 4.1	29 ± 3.0	26 ± 4.0	29 ± 14.9	24 ± 4.0	21 ± 1.5*	22 ± 3.5*
GLDH (u/L)	11 ± 7.7	10 ± 4.1	10 ± 3.4	10 ± 3.1	18 ± 37.3	7 ± 2.6	7 ± 3.4	9 ± 4.4
Bili (μmol/L)	3 ± 0.9	2 ± 0.4*	2 ± 0.5**	2 ± 0.6**	2 ± 0.7	2 ± 0.7	2 ± 0.6	2 ± 0.5**
BIAC (μmol/L)	39.8 ± 17.20	20.0 ± 8.64**	17.6 ± 6.28**	10.3 ± 8.10**	42.3 ± 34.70	18.6 ± 11.38	22.0 ± 11.17	13.5 ± 6.65
Urea (mmol/L)	6.36 ± 0.709	7.79 ± 0.721*	7.30 ± 1.286*	7.48 ± 1.023*	8.01 ± 1.172	8.65 ± 0.826	9.12 ± 1.424*	9.41 ± 0.854**
Creat (μmol/L)	40 ± 4.2	46 ± 3.7	42 ± 3.0	49 ± 9.0**	42 ± 4.5	55 ± 5.9**	51 ± 5.8**	55 ± 4.0**
Gluc (mmol/L)	6.99 ± 0.685	5.87 ± 0.661**	5.95 ± 0.679**	5.41 ± 0.776**	5.54 ± 0.701	5.97 ± 0.988	5.72 ± 0.883	5.22 ± 0.557
Chol (mmol/L)	1.59 ± 0.303	1.51 ± 0.190	1.69 ± 0.276	1.69 ± 0.247	2.01 ± 0.537	1.65 ± 0.274	1.48 ± 0.423**	1.49 ± 0.410**
Trig (mmol/L)	0.96 ± 0.381	0.53 ± 0.156**	0.63 ± 0.157**	0.63 ± 0.277**	0.43 ± 0.109	0.42 ± 0.156	0.37 ± 0.110	0.39 ± 0.095
Total Prot (g/L)	66 ± 2.5	64 ± 1.8	66 ± 3.5	65 ± 2.7	69 ± 3.0	71 ± 6.8	70 ± 5.8	70 ± 3.9
Alb g/L	36 ± 1.5	37 ± 1.1	37 ± 1.0	37 ± 1.2	40 ± 1.9	41 ± 3.6	40 ± 3.3	41 ± 2.3
Glob	30 ± 2.6	28 ± 1.0	29 ± 2.7	28 ± 2.0	29 ± 1.2	30 ± 3.5	30 ± 2.8	29 ± 1.9

ALP = alkaline phosphatase; ALT = alanine aminotransferase; GLDH = glutamate dehydrogenase; Bili = total bilirubin; BIAC = total bile acids; Creat = creatinine; Gluc = glucose; Chol = total cholesterol; Trig = triglycerides; Total Prot = total protein; Alb = albumin; Glob = globulin.
Compared to the control *$p < 0.05$; **$p < 0.01$.
[a] n = 9–10/sex/group.
[b] Average ± standard deviation.
From: Curry and Roberts, 2008

Nikiforov and Eapen (2008) evaluated the safety of rebaudioside A when administered as a dietary admix at target exposure levels of 500, 1000 and 2000 mg/kg/day to Sprague-Dawley rats for 90 days. Experimental design and mean average rebaudioside A consumption is depicted in Table 19.

Table 19. Experimental design and rebaudioside A consumption

Group No	Test article	No. of animals		Target dosage level[a]	Mean calculated test article consumption (mg/kg/day)	
		Males	Females		Males	Females
1	Basal Diet	20	20	0	0	0
2	Rebaudioside A	20	20	500	517	511
3	Rebaudioside A	20	20	1000	1035	1019
4	Rebaudioside A	20	20	2000	2055	2050

[a]Target dosage level is expressed as mg of test article per kg body weight of the animals.
From: Nikiforov and Eapen, 2008

The results of the study showed low mean body weight gains in the 2000 mg/kg/d group males generally throughout the study. These lower mean body weight gains resulted in statistically significant lower cumulative body weight gains and a statistically significant mean body weight difference that was 9.1% lower compared to the control group at the end of the administration period (study week 13). The lower mean body weights were not considered to be adverse due to the small magnitude of difference from the control group value. Because of the proportion of basal diet that was replaced with the test article containing little caloric value, the lower mean body weight gains may have been the result of the animals not consuming an equivalent number of calories as the concentration of the test article in the diet increased over the course of the study. Body weights of animals during selected intervals are shown in Table 20.

Table 20. Body weight (g) of animals during selected intervals

	Target Dietary Levels (mg/kg/day)			
Males				
	0	**500**	**1000**	**2000**
Week –1	151 ± 9.9	152 ± 7.9	151 ± 10.6	151 ± 8.8
Week 0	211 ± 12.4	211 ± 11.9	210 ± 11.2	211 ± 11.5
Week 1	273 ± 17.0	274 ± 14.0	268 ± 15.6	264 ± 17.3
Week 2	319 ± 23.2	320 ± 16.3	317 ± 19.5	311 ± 26.5
Week 4	407 ± 33.3	405 ± 24.5	396 ± 29.9	385 ± 32.7
Week 8	507 ± 52.6	492 ± 35.5	484 ± 47.8	470 ± 51.4
Week 13	563 ± 63.8	536 ± 44.6	527 ± 56.0	512 ± 55.7*
Females				
	0	**500**	**1000**	**2000**
Week –1	117 ± 10.1	115 ± 10.3	114 ± 10.5	114 ± 10.4
Week 0	148 ± 12.5	149 ± 11.9	149 ± 10.8	149 ± 11.6
Week 1	173 ± 14.3	174 ± 16.0	172 ± 15.9	171 ± 13.3
Week 2	193 ± 14.4	196 ± 18.0	193 ± 17.3	193 ± 16.5
Week 4	228 ± 19.3	235 ± 26.7	229 ± 22.6	228 ± 22.1
Week 8	266 ± 24.2	271 ± 30.2	262 ± 28.1	266 ± 23.2
Week 13	281 ± 25.0	284 ± 33.2	280 ± 31.5	276 ± 23.4

*Significantly different from the control group at .05 using Dunnett's test.
**Significantly different from the control group at .01 using Dunnett's test.
From: Nikiforov and Eapen, 2008

There were no treatment- related effects on the general condition and behavior of animals as determined by clinical observations, a functional observational battery, and locomotor activity assessments. Evaluations of the clinical pathology parameters revealed no toxicologically relevant, treatment-related effects on the biochemical and clinical variables. Serum chemistry values at week 13 are depicted in Tables 21 and 22 below). In addition, micro and macro-scopic findings revealed no treatment-related effects on any organ evaluated. Results of this study demonstrated the NOAEL under conditions of the assay was above the highest level of 2000 mg/kg/day.

000071

Table 21. Serum chemistry values (mean ± SD) at week 13 in males

	Target Dietary Levels (mg/kg/day) of Rebaudioside A																	
	Alb (g/dl)	TP (g/dl)	Glob. (g/dl)	A/G Ratio	Bili. (mg/dl)	Urea (mg/dl)	Creat. mg/dl)	ALP (U/L)	ALAT (U/L)	ASAT (U/L)	Glucose (mg/dl)	Chol. (mg/dl)	Ca (mg/dl)	Cl (mEq/L)	P (mg/L)	K (mEq/L)	Na (mEq/L)	TG (mg/dL)
Males	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10
0	4.3 ± 0.20	7.1± 0.33	2.8 ± 0.22	1.52 ± 0.139	0.2 ± 0.03	14.9 ± 1.39	0.3 ± 0.06	98 ± 9.9	44 ± 9.4	107 ± 31.2	113 ± 16.4	76 ± 13.6	10.7 ± 0.29	101 ± 0.8	7.2 ± 0.41	5.12 ± 0.82	143 ± 1.1	94 ± 27.5
500	4.3 ± 0.21	7.0 ± 0.36	2.7 ± 0.22	1.59 ± 0.104	0.2 ± 0.05	14.7 ± 1.75	0.4 ± 0.05	82 ± 14.0	38 ± 8.2	84 ± 10.6	112 ± 9.7	65 ± 10.2	10.6 ± 0.38	103 ± 1.7	6.9 ± 0.52	4.93 ± 0.562	144 ± 0.9	58 ± 18.9**
1000	4.3 ± 0.28	7.1 ± 0.37	2.8 ± 0.17	1.55 ± 0.110	0.2 ± 0.06	14.5 ± 2.15	0.4 ± 0.05	82 ± 14.4	45 ± 5.5	93 ± 22.0	114 ± 12.5	73 ± 19	10.7 ± 0.36	102 ± 1.8	7 ± 0.71	5.12 ± 0.486	145 ± 1.2**	77 ± 27.8
2000	4.2 ± 0.14	6.9 ± 0.20	2.7 ± 0.24	1.56 ± 0.161	0.2 ± 0.03	14.1 ± 2.14	0.3 ± 0.06	94 ± 20.2	40 ± 8.4	100 ± 24.3	105 ± 11.1	62 ± 15	10.4 ± 0.23	103 ± 1.7	7 ± 0.45	5.19 ± 0.572	145 ± 1.2**	61 ± 18**

Note. Alb – albumin; TP = total protein; Glob. = globulin; A/G ratio = albumin/globulin; Bili. = total bilirubin; Creat. = creatinine; ALP = alkaline phosphatase; ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; *N* = number of rats/sex/group.
Note Chol. = total cholesterol; TG = triglycerides; *N* = number of rats/sex/group.
**Significantly different from the control group at 0.01 using Dunnett's test.
From: Nikiforov and Eapen, 2008

000072

Table 22. Serum chemistry values (mean ± SD) at week 13 in females

	Target Dietary Levels (mg/kg/day) of Rebaudioside A																	
	Alb (g/dl)	TP (g/dl)	Glob. (g/dl)	A/G Ratio	Bili. (mg/dl)	Urea (mg/dl)	Creat. mg/dl)	ALP (U/L)	ALAT (U/L)	ASAT (U/L)	Glucose (mg/dl)	Chol. (mg/dl)	Ca (mg/dl)	Cl (mEq/L)	P (mg/L)	K (mEq/L)	Na (mEq/L)	TG (mg/dL)
Females	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10	*N* = 10
0	4.8 ± 0.52	7.3 ± 0.42	2.5 ± 0.26	1.98 ± 0.382	0.2 ± 0.05	17.4 ± 2.1	0.4 ± 0.06	57 ± 20.0	41 ± 20.2	103 ± 44.7	109 ± 14.3	79 ± 14.5	10.6 ± 0.52	103 ± 2.5	5.8 ± 0.80	4.51 ± 0.567	144 ± 1.8	51 ± 13.7
500	4.9 ± 0.58	7.4 ± 0.59	2.5 ± 0.28	2.00 ± 0.342	0.2 ± 0.03	18.7 ± 1.94	0.4 ± 0.06	53 ± 19.9	47 ± 28.2	112 ± 65.4	107 ± 10.1	80 ± 24.4	10.9 ± 0.49	103 ± 1.3	6.3 ± 1.20	5.00 ± 0.923	145 ± 1.7	59 ± 17.1
1000	4.8 ± 0.26	7.3 ± 0.26	2.5 ± 0.17	1.93 ± 0.194	0.2 ± 0.03	17.6 ± 2.74	0.4 ± 0.06	58 ± 14.3	36 ± 9.2	100 ± 21.1	104 ± 8.6	65 ± 9.8	10.6 ± 0.18	103 ± 2.0	6.0 ± 0.65	4.76 ± 0.680	144 ± 2.1	53 ± 11.5
2000	4.8 ± 0.41	7.3 ± 0.46	2.5 ± 0.19	1.94 ± 0.236	0.2 ± 0.07	16.6 ± 4.09	0.4 ± 0.10	52 ± 6.5	35 ± 6.3	89 ± 11.6	109 ± 14.4	66 ± 7.9	10.7 ± 0.41	103 ± 1.2	6.3 ± 0.93	4.9 ± 0.646	144 ± 1.9	44 ± 4.7

Note. Alb – albumin; TP = total protein; Glob. = globulin; A/G ratio = albumin/globulin; Bili. = total bilirubin; Creat. = creatinine; ALP = alkaline phosphatase; ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; *N* = number of rats/sex/group.
Note Chol. = total cholesterol; TG = triglycerides; *N* = number of rats/sex/group.
**Significantly different from the control group at 0.01 using Dunnett's test.
From: Nikiforov and Eapen, 2008

A subchronic study of McNeil Stevia (steviol glycoside extract containing rebaudioside A and stevioside, as the primary and secondary steviol glycosides, respectively), evaluated potential toxicity in male and female Sprague Dawley (Crl) rats following repeated exposures for 90 days in the diet (McNeil, 2008). McNeil Stevia product supplied by two manufacturers (Supplier 1 McNeil Stevia product and Supplier 2 McNeil Stevia product) was incorporated in the feed at concentrations of 2.5% or 4% by weight and given continuously in feed for 7 days/week for 13 weeks (terminal kill). Control rats were given a standard rodent diet without inclusion of steviol glycosides. Interim evaluations of toxicity were performed at 29 days.

Fasted blood samples were collected from all animals via the abdominal vena cava at the interim and final necropsy. Urinalysis samples were collected in metabolism cages for approximately 7 hours the day before necropsy.

Necropsies were conducted after 4 weeks (interim phase) and 13 weeks (termination) of treatment. At necropsy, or when found moribund, all animals were anesthetized with isoflurane and exsanguinated via the vena cava for blood sample collection. Tissues and organs listed in Table 23 were collected for gross and histopathological evaluation from all necropsied animals and these were fixed in 10% neutral buffered formalin solution. Organ weights were collected for brain, heart, liver, kidneys, and ovaries with oviducts, testes, adrenals, thymus, spleen, and epididymides.

Table 23. Tissue and Organs Collected for Examination	
Adrenal glands	Ovaries with oviducts
Aorta	Pancreas
Bone (sternum, femur including joint)	Pituitary gland
Bone marrow (femur)	Prostate gland
Brain (at least three levels)	Rectum
Cecum	Salivary glands
Colon	Sciatic nerve
Duodenum	Seminal vesicles (incl. coagulating gland)
Epididymides (fixed in Bouin's solution)	Skeletal muscle
Esophagus	Skin
Eyes w/optic nerve and Harderian gland (fixed in Davidson's solution)	Spinal cord - cervical, midthoracic, lumbar
Heart	Spleen
Ileum, with Peyer's patches	Stomach
Jejunum with Peyer's patches	Testes (fixed in Bouin's solution)
Kidneys	Thymus
Larynx	Thyroid (incl. parathyroid glands, if possible)
Lacrimal gland, exorbital	Tongue
Liver	Trachea
Lung, filled w/formalin at necropsy	Urinary bladder, filled w/formalin at necropsy
Lymph nodes - mesenteric, mandibular	Uterus
Mammary gland area	Vagina
Nasal turbinates	Gross lesions

Test article intake of McNeil Stevia from Supplier 1 at 2.5% in the diet ranged from 1165-2176 mg/kg (males) and 1159-2117 mg/kg (females); intake at 4% in the diet ranged from 2017-3298 mg/kg (males) and 2431-3265 mg/kg (females). Test article intake of McNeil Stevia from Supplier 2 at 2.5% in the diet ranged from 1181-2050 mg/kg (males) and 1549-2117 mg/kg (females); intake at 4% in the diet ranged from 1988-3222 mg/kg (males) and 2587-3313 mg/kg (females).

The results of the study demonstrated no adverse clinical observations considered related to steviol glycosides treatment. Differences between treated and control groups in general gross observations were limited to occasional findings of red stains on the cage liner-paper, dark or light colored feces, or increased moisture in feces. None of these observations was considered toxicologically significant, and coloration difference of feces relative to controls was relatively

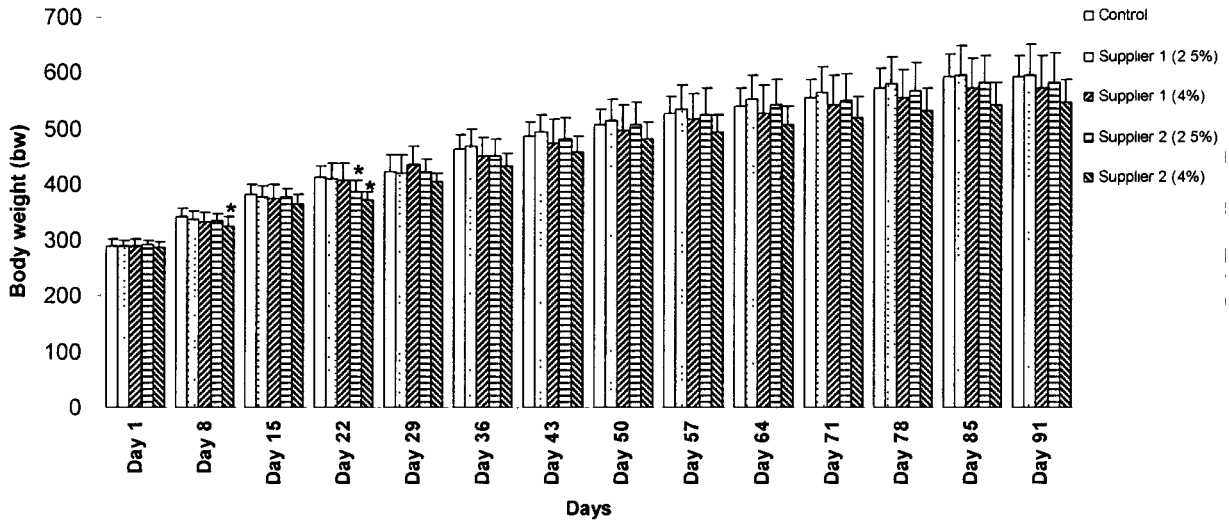
000075

constant during the study and not remarkably different with increasing concentration of steviol glycosides from McNeil Stevia Product from Supplier 1 or Supplier 2 in the diet.

Body weights of the animals were recorded weekly during acclimatization and treatment, and immediately before necropsy. Body weight gain was calculated based on body weight data. There were no treatment-related changes in mean body weights in male and female rats as compared to control rats. There were only sporadic, statistically significant indications of differences from the controls in the early weeks of study. Following week 4 and up to study termination, body weights in all treatment groups were similar and showed no statistical differences from control values (Figure 3 and 4). Although not significant, there was a trend of lower mean body weights in both sexes relative to controls. No toxicologic significance was ascribed to this finding. The difference in caloric density of the diets was related to the slight (non-statistically significant) difference in body weights between treatment and control groups. During the course of treatment, male rats receiving 2.5% and 4% McNeil Stevia from Supplier 1 or Supplier 2 experienced similar body weight gains compared to weight gains of the male rats in the control group. There was a statistically significant decrease in the body weight gain of females from both the 2.5 and 4% treatment groups which was minor and considered toxicologically insignificant. This was attributed to the caloric density of the diet.

There were no treatment-related changes in mean feed consumption in male or female rats relative to control.

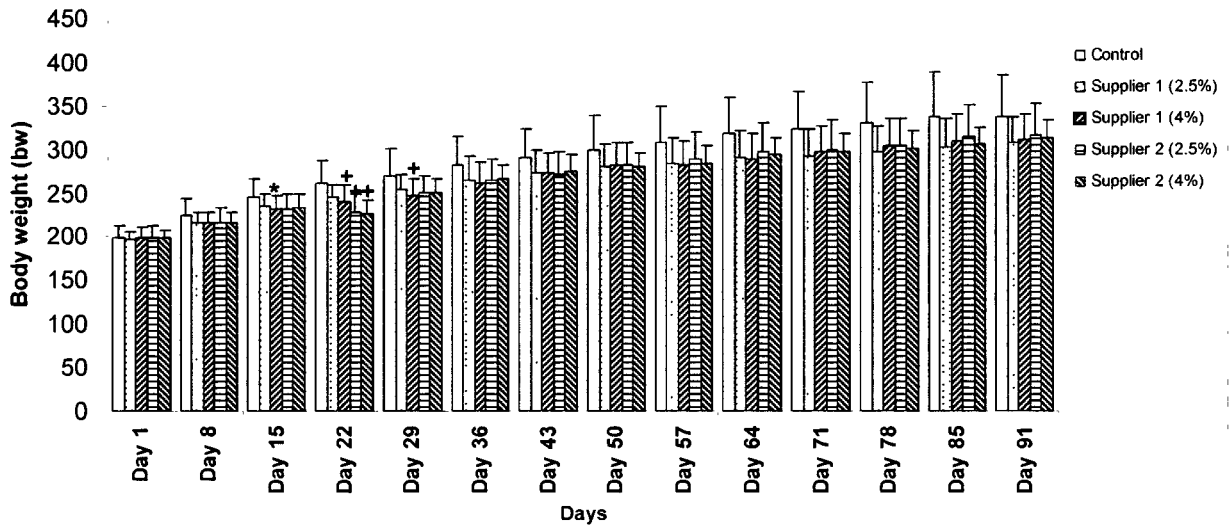
Figure 3. Effect of 90-Day oral steviol glycosides treatment on body weight in male rats.



N=10 animals/group; */+: Dunnett Test based on pooled variance significance at 5% (*) and 1% (+).

000077

Figure 4. Effect of 90-Day oral steviol glycosides treatment on body weight in female rats.



N=10 animals/group; */+: Dunnett Test based on pooled variance significance at 5% (*) and 1% (+).

No toxicologically significant differences in hematology or coagulation values were seen at the interim or final termination in comparisons of male or female rats given McNeil Stevia from Supplier 1 versus controls. A statistically significant increase in WBC values was seen at the 28 day interim interval in both males and females given 4% McNeil Stevia from Supplier 2 (increases of 1.6 to 1.7 X above control levels) and in males fed 2.5% McNeil Stevia and examined at the Day 29 interim termination (1.5 X control). Differential absolute counts showed that the differences in WBC were attributable to increases in lymphocytes in each case. These results were transient and had no apparent relationship to ingestion of steviol glycosides, as WBC values were within normal control ranges for all groups of rats in blood samples taken at the final termination following 90 days of ingestion of both steviol glycoside products. Coagulation was not statistically different in males or females given dietary McNeil Stevia from Supplier 1 or 2 at the 90 day termination.

Random indications of statistical differences of urinalysis parameters from control values in males or females at the interim termination on Day 29 were typically not corroborated by the findings at the terminal kill. The only statistical differences from control values after 90 days of ingestion of the steviol glycoside products included a decrease in urine volume and an increase in ketones in the group of males receiving 4% of the Supplier 2 product. It is not expected that

these changes were biologically significant; urine volumes were highly variable and measurement of ketones in the urine was done by a dipstick method, which is qualitative in nature.

Clinical chemistry values after consumption of McNeil Stevia from Supplier 1 and Supplier 2 for 28 and 90 days showed only random indications of statistical differences from controls (differences reported were frequently within references ranges from relevant historical controls obtained from the suppliers) (Charles River 2006) and were seen in both control and treated groups of rats (Tables 24, 25, 26 and 27). Statistical indications of increases in each parameter were slight, lacked indications of relation to dosage and none of the parameters was of sufficient magnitude to indicate a significant toxicological effect. Indications of significant increases in ASAT, ALAT, CK and ALT enzyme levels in females given 2.5% and 4% dietary levels of steviol glycosides from Supplier 2 at 28 days were not seen at the 90-day termination schedule. Similarly, significant decreases in glucose in all groups of females seen at 28 days were not significantly different from controls at 90 days. Indications of significant decreases of ASAT and ALAT at 90 days in males given either steviol glycoside product were caused by abnormally high values in control rats (well above reference ranges) and the groups given the steviol glycoside products had values that were within the acceptable reference range. A significant, 2-fold decrease in triglycerides in females given the 4% level of Supplier 2 steviol glycosides at 90 days was a singular result not seen at any other amount/source/sex comparison. All other statistical indications of differences of treated and control values were slight and were considered to lack clinical relevance generally due to the randomness of the findings and lack of corroboration from findings in the related groups of treated rats.

Table 24. Effect of steviol glycosides on blood chemistry variables in male rats at interim (week 4) and terminal (week 13) study periods

Group	Week 4					Group	Week 13				
	Control	Supplier 1 (2.5%)	Supplier 1 (4.0 %)	Supplier 2 (2.5%)	Supplier 2 (4.0 %)		Control	Supplier 1 (2.5%)	Supplier 1 (4 0 %)	Supplier 2 (2.5%)	Supplier 2 (4.0 %)
	Group 1	Group 2	Group 3	Group 4	Group 5		Group 1	Group 2	Group 3s	Group 4	Group 5
Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation
Glucose (mmol/L)	9.05	7.66**	7.85**	6.28**	6.78**	Glucose (mmol/L)	10.36	9 08	9.07	9.56	10.37
	0.68	1.22	0.61	0.45	0 55		1.09	1 09	1.31	1.44	1.35
Urea (mmol/L)	5.3	4.77	4.76	5.17	5 37	Urea (mmol/L)	5.56	4.67**	4.92**	4.65**	4.15**
	0.69	0.57	0.61	0.42	0.61		0.5	0.34	0.44	0.47	0.35
Creat (µmol /L)	21.8	20.1	22.1	30**	31 1**	Creat (µmol /L)	30 3	23.1**	27	24.1**	24.1**
	2.7	2.3	1.8	3.4	3		3.8	2	5.1	3 8	3
Bili-T (µmol/L)	1.47	1.29	1.87	2.64**	3.54**	Bili-T (µmol/L)	1.95	2 05	2.79	2.37	2.08
	1.02	1.13	1.13	1	1.3		1.12	1 4	2.44	0.52	0.9
Cholest (mmol/L)	1 51	1.38	1 38	1.47	1 45	Cholest (mmol/L)	1 63	1.9	1.69	1.68	1.75
	0.34	0.37	0.4	0.36	0.19		0.52	0.49	0.51	0 48	0 46
Trigly (mmol/L)	0.65	0.41	0 35*	0.53	0 53	Trigly (mmol/L)	0.82	1 02	0.86	0 67	0 71
	0.3	0.18	0.16	0.17	0.21		0.34	0.47	0.61	0.24	0.3
ASAT (U/L)	76.3	69.4	76.1	85.4	78.6	ASAT (U/L)	160.9	70*	74*	92.8	71 1*
	20 3	7 2	13.7	9.4	8.5		133.7	21.9	18.6	57.8	23.2
ALAT (U/L)	38 3	29.8	30	34	43.4	ALAT (U/L)	101.9	33.2**	31.2	39 1**	40.8**
	13.7	4 4	4.4	3 9	31.7		89 1	14 4	8	15	24.4
ALP (U/L)	135.6	149 1	134 4	166.4	155.5	ALP (U/L)	77 5	74 9	87.1	68 2	71.8
	27 7	28	25.8	35.7	34		12	11.4	22.5	12.9	15 1
CK (U/L)	166.8	153.3	179.8	473.7**	373.4**	CK (U/L)	235 4	149.4	133 9	218.4	162.4
	36	28.8	46	210.9	143.7		113.4	52.2	42	155	67
Sodium (mmol/L)	144.5	145	143 6	144 1	144.9	Sodium (mmol/L)	144.4	144.1	144.4	144.3	144.5
	0.9	0 7	1.1	1.1	0.9		1	0 8	1 1	0 9	1
Potassium (mmol/L)	3.73	3.75	3 66	4 32**	4.32**	Potassium (mmol/L)	4.03	4.17	3 98	4 27	3.86
	0 28	0.28	0.22	0.36	0.27		0.24	0.41	0.19	0 28	0 32
Chloride (mmol/L)	106	105.5	105.1	101.7**	101.9**	Chloride (mmol/L)	105.1	103.9	104.6	104.5	103.8
	1.3	0 9	1	1 6	1 3		1.2	0 9	1.2	1.6	1.2
Calcium (mmol/L)	2 62	2.7*	2.66	2.73**	2.69	Calcium (mmol/L)	2.49	2.6*	2 58	2.63**	2.63**
	0 06	0.04	0.09	0 07	0.07		0.08	0.08	0.11	0.12	0.08
Phosphorus (mmol/L)	2 11	2 39**	2 32*	2.48**	2.32*	Phosphorus (mmol/L)	1 59	1.9**	1.75	1 9**	1.91**
	0 18	0.09	0 11	0 27	0 13		0 16	0 21	0 15	0.12	0.17
Protein (g/L)	59 87	59 53	59.65	61.95	64.09**	Protein (g/L)	61 03	63 7	62.96	61 77	61.38
	2.7	2.18	2 48	2 73	2.37		3.38	2	2 8	2.77	2 1
Albumin (A) (g/L)	38 85	38.88	38.93	40 29	41.93**	Albumin (g/L)	37.78	39 39	39.57	38.07	38.92
	1.41	1.03	1 18	1 92	1.42		1 52	1 58	2 46	1.36	1.69

000080

Table 24. Effect of steviol glycosides on blood chemistry variables in male rats at interim (week 4) and terminal (week 13) study periods

Group	Week 4					Group	Week 13				
	Control	Supplier 1 (2.5%)	Supplier 1 (4.0 %)	Supplier 2 (2.5%)	Supplier 2 (4 0 %)		Control	Supplier 1 (2.5%)	Supplier 1 (4.0 %)	Supplier 2 (2.5%)	Supplier 2 (4.0 %)
	Group 1	Group 2	Group 3	Group 4	Group 5		Group 1	Group 2	Group 3s	Group 4	Group 5
Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation
Globulin (G) (g/L)	21.02	20.65	20.72	21.66	22.16	Globulin (g/L)	23.25	24.31	23.39	23.7	22.46
	1.65	1.75	1.81	1.67	2.17		2.36	1.83	2.08	2.26	1.83
A/G Ratio	1 86	1.89	1.89	1.87	1.91	A/G Ratio	1 64	1 63	1.71	1 62	1.75
	0 12	0 16	0.15	0.16	0 22		0.15	0.15	1.33	1.4	0.18

Values are mean ± SD; N=10 rats/group each used for 28-Day interim and 90-Day termination; *p < 0.05; **p< 0.01 as compared to control group. Abbreviations used: Creat-creatinine; BiliT- total bilirubin, Cholest- total cholesterol, Trigly -total triglycerides, ASAT- aspartate aminotransferase, ALAT- alanine aminotransferase, ALP -alkaline phosphatase, CK -creatinine kinase and A/G -albumin/globulin ratio.

Table 25. Reference range for relevant blood chemistry variables in male rats (Charles River 2006)

Glucose (mmol/L)	6.16-10 84	TG (mmol/L)	0 67-1.08	CK (U/L)	203.00-437 00	Phosphorus (mmol/L)	2.26-3 23
Urea (mmol/L)	3 57-5.71	ASAT (U/L)	77.0-114.0	Sodium (mmol/L)	141 0-150.0	Protein (g/L)	59 0-71 0
Creat (umol/L)	35 4-53.0	ALAT (U/L)	27.0-46 0	Potassium (mmol/L)	4.60-6.20	Albumin (g/L)	33 0-46 0
Bili-T (umol/L)	1 71-17 1	ALP (U/L)	104 0-188	Chloride (mmol/L)	101.0-107.0	A/G Ratio	1.10-2.70
Cholest (mmol/L)	1 4-2.3	GGT (U/L)	0.00-1.00	Calcium (mmol/L)	2.35-2.75		

Table 26. Effect of steviol glycosides on blood chemistry variables in female rats at interim (week 4) and terminal (week 13) study periods

Group	Week 4					Group	Week 13				
	Control	Supplier 1 2.5%	Supplier 1 4 %	Supplier 2 2 5%	Supplier 2 4 %		Control	Supplier 1 2.5%	Supplier 1 4 %	Supplier 2 2.5%	Supplier2 4 %
	Group 1	Group 2	Group 3	Group 4	Group 5		Group 1	Group 2	Group 3	Group 4	Group 5
Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation
Glucose (mmol/L)	8.01	6.89*	6.32**	6.54**	5 63**	Glucose (mmol/L)	8 73	8.03	7.64	8.11	8.04
	1.37	1.12	0.49	0 91	0 48		1 02	1.13	0.82	0.99	1.22
Urea (mmol/L)	4 66	4.92	4.91	5 25	5 29	Urea (mmol/L)	5 99	5 13*	5.98	5.35	4.45**
	0.92	0 81	0.82	0 62	0.47		1 13	0.61	0 68	0.48	0.54

Table 26. Effect of steviol glycosides on blood chemistry variables in female rats at interim (week 4) and terminal (week 13) study periods

Group	Week 4					Group	Week 13				
	Control	Supplier 1 2.5%	Supplier 1 4 %	Supplier 2 2 5%	Supplier 2 4 %		Control	Supplier 1 2.5%	Supplier 1 4 %	Supplier 2 2.5%	Supplier2 4 %
	Group 1	Group 2	Group 3	Group 4	Group 5		Group 1	Group 2	Group 3	Group 4	Group 5
Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation
Creat (µmol/L)	23 3	24.2	25.6	31.7**	33.6**	Creat (umol/L)	30.9	28.4	31 3	29.2	25.3*
	3.6	2.6	3.1	4.2	5.6		6.1	2.9	3.6	3 5	3.2
Bili-T (µmol/L)	2 76	2.4	2.24*	3 35*	3.53*	Bili-T (umol/L)	2.89	2.4	2.2	3.35	2.83
	0.4	0.39	0.16	0.39	0.77		0.73	1.24	0.86	1 88	0.9
Cholest (mmol/L)	1 81	2.11	1.78	1.94	1.78	Cholest (mmol/L)	2 05	2.15	2.41	2.46	2.38
	0.24	0.44	0.37	0.46	0.42		0.55	0.6	0 38	0 43	0 45
Trigly (mmol/L)	0.37	0.35	0.28	0.5*	0.44	Trigly (mmol/L)	0.71	0 47	0.6	0.68	0.35*
	0.11	0.09	0 08	0.08	0.17		0.27	0 12	0 34	0 34	0.09
ASAT (U/L)	62.7	62.1	64.4	70.6	83.7**	ASAT (U/L)	71.3	72.5	76.6	58.8	70.2
	10.6	5 8	7.7	11	11.5		9	38.9	26.7	11.3	16 4
ALAT (U/L)	26.1	25.7	24.3	26 6	32.3**	ALAT (U/L)	31 9	34.5	39.4	25.2	29 9
	4.5	3.1	3 8	1 6	3.7		5 8	27	27.4	5.1	12.5
ALP (U/L)	65 7	66.4	74 2	98.4*	92.9*	ALP (U/L)	34.3	41.3	41 2	33.4	31 2
	15 7	18 9	25 4	30.6	19.8		11.6	14	7 4	12.8	9.1
CK (U/L)	112.3	94 1	121.9	215.8**	270 2**	CK (U/L)	98.9	122.7	146 1	105	144.8
	15.7	9.2	24.3	31.5	101.9		27 7	63.6	47.5	39.3	42
Sodium (mmol/L)	141.1	141 7	141.8	144.2**	145.1**	Sodium (mmol/L)	144.3	143 6	142.5**	142.3**	142.5**
	1	0.7	0.9	1 1	1 1		0 6	0 7	0.6	1 3	0.9
Potassium (mmol/L)	3.45	3.44	3 62	3 98**	3.88*	Potassium (mmol/L)	3.18	3 46	3.42	3.43	3 91**
	0	0.16	0.22	0.41	0 39		0.17	0.3	0.34	0.41	0.31
Chloride (mmol/L)	103 6	104.2	104.8	102.4	103.5	Chloride (mmol/L)	104.1	103.4	102.9	102.4**	102**
	1.2	1	0 9	1	1.4		0.9	1.1	0.8	1.6	1
Calcium (mmol/L)	2 73	2.71	2.67	2 88	2 76	Calcium (mmol/L)	2 71	2.72	2.73	2.8	2.81
	0 07	0.08	0.06	0 11	0.09		0.11	0.09	0.08	0.12	0 08
Phosphorus (mmol/L)	1.95	2.05	2.09	1.92	2.04	Phosphorus (mmol/L)	1.15	1.56**	1.37	1.59**	1.9**
	0.14	0.25	0.14	0.26	0.32		0 22	0 27	0.24	0.26	0.13
Protein (g/L)	62.7	67 28	64.25	71.62*	69 96	Protein (g/L)	70.05	68.62	39 88	71.09	70.59
	2 8	3.77	1.93	4.15	3 89		5.43	3 48	3.42	4 49	3 07
Albumin (g/L)	47 96	48 06	45.66	50 59	49.28	Albumin (g/L)	49.08	48.64	49.09	51.01	49 24
	3 01	2.99	1.97	3 71	3 43		4.57	3 18	2.52	3.94	3.29
Globulin (g/L)	19.24	19.22	18.59	21 03*	20.68	Globulin (g/L)	20.97	19 98	20.79	20.08	21 35
	1 41	1.9	1.23	1.54	1 2		1.73	1.31	1 42	1 86	1.24
A/G Ratio	2.51	2.52	2 47	2 42	2.39	A/G Ratio	2.35	2 44	2.37	2 56	2 32
	0.27	0.28	0.22	0.23	0.18		1.88	0 22	2.17	0 3	0 25

000082
SPHERIX

Table 26. Effect of steviol glycosides on blood chemistry variables in female rats at interim (week 4) and terminal (week 13) study periods

Group	Week 4					Group	Week 13				
	Control	Supplier 1 2.5%	Supplier 1 4 %	Supplier 2 2.5%	Supplier 2 4 %		Control	Supplier 1 2.5%	Supplier 1 4 %	Supplier 2 2.5%	Supplier2 4 %
	Group 1	Group 2	Group 3	Group 4	Group 5		Group 1	Group 2	Group 3	Group 4	Group 5
Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Parameter	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation	Mean Standard Deviation

Values are mean ± SD; N=10 rats/group each used for 28-Day interim and 90-Day termination; *p < 0.05; **p< 0.01 as compared to control group. Abbreviations used: Creat-creatinine; BiliT- total bilirubin, Cholest- total cholesterol, Trigly -total triglycerides, ASAT- aspartate aminotransferase, ALAT- alanine aminotransferase, ALP -alkaline phosphatase, CK -creatinine kinase and A/G -albumin/globulin ratio.

Table 27. Reference range for relevant blood chemistry variables in female rats (Charles River 2006)

Glucose (mmol/L)	6.20-10.23	TG (mmol/L)	0 46-0.83	CK (U/L)	180.0-466.0	Phosphorus (mmol/L)	1.80-2.93
Urea (mmol/L)	3.92-6 97	ASAT (U/L)	72.0-123.0	Sodium (mmol/L)	141 0-149.0	Protein (g/L)	61 0-75 0
Creat (umol/L)	35.4-61 9	ALAT (U/L)	25.0-45.0	Potassium (mmol/L)	4 20-6.10	Albumin (g/L)	35.0-53.0
Bili-T (umol/L)	3.42-34 2	ALP (U/L)	65 0-147 0	Chloride (mmol/L)	101.0-108 0	A/G Ratio	1.10-2.90
Cholest (mmol/L)	1.72-2 52	GGT (U/L)	0.00-1.00	Calcium (mmol/L)	2 37-2.80		

There were no toxicologically adverse gross pathological findings observed during necropsy at the interim or final kill in any group given McNeil Stevia from Supplier 1 or Supplier 2. Organ weights evaluated at 28 days (rats given Supplier 1 product only) and in all groups of rats at 90 days (McNeil Stevia from Supplier 1 and Supplier 2) predominantly showed no remarkable differences from control values in absolute or relative (organ:body weight) weights in rats given either 2.5% or 4% dietary concentrations of both steviol glycoside products. The only statistically significant difference with possible toxicological relevance at 28 days was an indication of a decreased kidney:brain weight ratio in females in the 4% dietary group. However, this appeared to be a transient effect that was not seen in the same treatment group at the 90-day necropsy. In males, no significant differences in absolute or relative organ weights were seen following 28 to 90 days of ingestion of either steviol glycoside product. In females, slight (5 %) but statistically significant increases were seen at 90 days in brain weights at 4% levels of both steviol glycoside products. Brain to body weight ratios were significantly increased only in the group receiving 4% steviol glycosides from Supplier 1. The latter finding is not considered toxicologically significant since the groups of females receiving the same dietary concentrations of product from Supplier 2 did not show this same increase. The former is

also not supported by male results showing no statistically significant difference from control either in absolute or relative measurements of brain weights. The only other finding at 90 days, again unique to females, was a statistically significant indication of an increase in the liver:body weight ratio in all groups given steviol glycosides at 2.5% or 4% but there were no similar statistical differences from control values for liver: brain weight ratios. Because organ:brain weight ratios are considered more reliable than those calculated based upon body weight (because of possible greater variation in body weights), the statistical indicator, or a possible treatment effect is most likely a random event without toxicological significance.

Histopathological findings for tissues and organs prepared from samples taken at the 28-day kill from controls and rats fed McNeil Stevia from Supplier 1 showed no remarkable differences in treated and control groups, including liver and brain. Histopahtolgical findings for tissues and organs prepared from samples taken at the 90-day kill from controls and rats fed McNeil Stevia from Supplier 1 and Supplier 2 were within the range of normal background lesions that may be recorded in animlas of this strain and age. Therefore, there were no treatment-related adverse findings noted in the microscopic observations.

Under the conditions of this study, there were no target organs of toxicity identified with steviol glycosides in any of the organs and tissues examined. Based on the available data, the NOAEL in this study is considered to be 4% in the diet for McNeil Stevia, from both Suppliers 1 and 2; the NOAEL from this study is the highest amount tested.

c) Chronic toxicity

(1) Introduction

Chronic toxicity of steviol glycosides has been reported in the literature. Yamada et al. (1985) and Xili et al. (1992) reported NOAELs of 550 mg/kg bw/d and 600 mg/kg bw/d, respectively, the highest amount tested in each study. Toyoda et al. (1997) reported body weight reductions related to those administered high levels of steviol glycosides that were considered toxicologically significant in males (2000 mg/kg bw/d) and females (2,400 mg/kg bw/d) who were, along with a significantly reduced survival rate in high-level males. The authors considered the NOAEL in this study to be 970 mg/kg bw/d, based on the body weight gain and survival effects observed in males. Decreases in body weight gain in rodent toxicology studies examining the effects of high-intensity sweeteners are not unexpected. These findings are commonly associated with taste aversion of diets with such high sweetener concentrations and with overall reduced nutritional content of the feed on a weight-for-weight basis. The resulting minor body weight gain differences are considered of no toxicological significance. 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 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. The endpoints evaluated in the chronic studies included mortality rates, organ weight, feed consumption, hematological, clinical and biochemical variables, and histopathological observations. Results of a carcinogenicity study on steviol in hamesters did not show any signs of carcinogenic potential for steviol glycosides (Taskulkao et al. 2001).

(2) Review of studies

In a chronic toxicity study of dietary stevia extract, 480 F344/Du Crj rats of both sexes were fed 0, 0.3, and 1% stevia extract containing 74.54% stevioside and 16.27% rebaudioside A (other 9.9% unidentified) for 22 months in the case of males and 24 months in the case of females. Mortality at the end of the study in rats given the stevia extracts was not significantly different from that in the controls. General appearance and behavior were the same in all groups. Although a variety of changes were found at 6 months in urinary, hematological and blood chemistries, no such differences were found at 12 months or at the end of the study. The incidence and severity of non-neoplastic and neoplastic changes were unrelated to the level of the stevia extract in the diet. The highest level of stevia extract (1% in the diet) that caused no effects in the rats was 550 mg/kg (Yamada et al. 1985).

Xili et al. (1992) conducted a chronic oral toxicity and carcinogenicity study of stevioside (purity 85%) in Wister rats. Groups of 45 male and 45 female inbred animals were given diets containing stevioside at 0, 0.2, 0.6 or 1.2% for 2 years. After 6, 12 and 24 months, five rats from each group were killed for hematological and clinical biochemical tests. Growth, feed utilization and consumption, general appearance and mortality were similar in treated and control groups. The mean life span of rats given stevioside was not significantly different from that of the controls. No treatment-related changes were observed in hematological, urinary or clinical biochemical values at any stage of the study. The incidence and severity of non-neoplastic and neoplastic changes were unrelated to the level of stevioside in the diet. The maximum no observed effect level of stevioside was 1.2%, and an acceptable daily intake of stevioside for humans of 7.938 mg/kg bw /d was suggested by the authors.

The carcinogenic potential of stevioside (95.6% purity, added to a powdered diet at concentrations of 0, 2.5 and 5%) was evaluated in 50 male and 50 female F344 rats. Other than a significant decrease in final survival rate in the 5% treated males there were no other significant effects including development of neoplastic or non-neoplastic lesions attributable to the stevioside treatment (except a decreased incidence of mammary adenomas in females and a

reduced severity of chronic nephropathy in males). The authors (Toyoda et al. 1997) concluded that stevioside was not carcinogenic in F344 male and female rats.

Hagiwara et al. (1984) demonstrated the effects of stevioside and other sweeteners on urinary bladder carcinogenesis in rats initiated by N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN). The results of their study exhibited no preneoplastic or neoplastic lesions of the urinary bladder in rats treated with stevioside. Therefore, the authors concluded that stevioside does not promote bladder carcinogenesis.

Taskulkao et al. (2001) determined the carcinogenic potential of steviol (~90% purity) in groups of 55 male and 55 female hamsters fed the compound in the diet at 0.0, 100, 500 mg/kg. The calculated steviol intakes by these animals were approximately 38.7 and 199.8 mg/kg bw/d in 100 and 500 mg/kg diet in the males, respectively, 37.4 and 183.1 mg/kg bw/d in 100 and 500 mg/kg diet in the females, respectively. Males were on the diet for 22 months and females for 18 months. Body weight, general appearance and mortality were similar in treated and control groups. The authors stated that the decrease in body weight of hamsters in all groups observed in the females after 14 months and the males after 19 months was related to aging. However, a sharp increase in mortality rates that was observed after 18 months was related to pulmonary inflammation. The development of tumors, and/or general poor health was also attributed to the aging process. Histopathological findings revealed a variety of changes in various organs; however the incidences of non-neoplastic lesions were similar in steviol-treated hamsters and in controls. The mean life span of hamsters given steviol was not significantly different from that of the controls. No treatment-related changes were observed in hematological, urinary or biochemical values and there was no significantly altered development of neoplastic (Table 28) or non-neoplastic (benign) lesions attributable to steviol treatment in any organ or tissue.

Table 28. Incidence of neoplasms in hamsters fed steviol for up to 22 months (male) and 18 months (female)

Sex	Steviol (mg/kg/diet)	No. of hamsters examined	Benign tumors	Malignant tumors	Total no. (%) of hamsters with tumors
Male	0 (control)	45	2 (4.4)	0 (0)	2 (4.4)
	100	46	1 (2.2)	0 (0)	1 (2.2)
	500	45	1 (2.2)	1 (2.2)	2 (4.4)
Female	0 (control)	47	1 (2.1)	0 (0)	1 (2.1)
	100	48	1 (2.1)	1 (2.1)	1 (2.1)
	500	47	0 (0)	0 (0)	0 (0)

There were no significant differences between groups (chi-square test).

000086

A tabular summary of acute, subchronic and chronic toxicity studies is presented in Tables 29, 30 and 31.

Table 29. Acute Animal Toxicology Studies

Reference	Study Design/ Objective	Species & number/sex	Test Substance	Amount Administered	Duration	Results
Alvarez 2006	General non scientific article with information about toxicity of stevioside	Details not given	Purity varied	--	--	Oral LD 50: 8.2 to 15g/kg (93.5%) to 17 g/kg in mice and rats Reviews other studies
Medon 1982 (abstract only)	Acute oral and bacterial mutagenic potential	Male Swiss-Webster mice *Salmonella typhimurium* strain TM677 with and without metabolic activating system (Aroclor 1254 pretreated rats)	Pure stevioside; crude steviolbioside, dulcoside A, rebaudiosides A	Acute: single gastric intubation suspended in 1% carboxymethylellulose; followed 14 days Mutagenicity: No details	Acute: followed 14 days	No toxicity demonstrated in mice with any of the materials Not mutagenic

000087

Table 29. Acute Animal Toxicology Studies

Reference	Study Design/ Objective	Species & number/sex	Test Substance	Amount Administered	Duration	Results
Toskulkao et al. 1997	Acute toxicity study of stevioside;	Syrian golden hameters; 80-100g Swiss albino mice; 25-35g Wistar rats; 50-60g 10-15 animals used per level	Stevioside; Purity 96%	Hamsters: 8, 12 and 15g/kg bw stevioside orally 3, 4, 5, 6, 7 and 8 g/kg bw steviol by intubation Mice and Rats: 10 and 15g/kg bw of stevioside and 12, 14 and 15 g/kg bw of steviol Distilled water as vehicle and corn oil as negative control was used	14 days	Stevioside at a level of 15g/kg bw was not toxic to hamsters mice or rats. Hamsters were found to be more susceptible to steviol than rats or mice LD_{50} values of steviol in hamsters were 5.20 and 6.10 g/kg bw for males and females, respectively In rats and mice, LD_{50} values of steviol were higher than 15 g/kg bw in both sexes. Histopathological data in kidney of hamsters induced by steviol revealed severe degeneration of the proximal tubular cells.

000088

000089

Table 30. Subchronic Animal Toxicology Studies

Reference	Study Design/ Objective	Species & number/sex	Test Substance	Amount Administered	Duration	Results
Aze et al. 1991	Subchronic oral toxicity study	F344 rats, 10 males and 10 females per group	Purity of stevioside not given in abstract	feed at 0.0, 0.31, 0.62, 1.25, 2.5, & 5% in diet	13 weeks	5% in diet concluded to be suitable maximum tolerable level of stevioside for 2 year cancer study in rats
Curry and Roberts . 2008	Subchronic oral toxicity study	10 male and 10 female Han Wister rats for palatability study and 20 male and 20 female HsdRcc Han Wister rats for 13 week study	Rebaudioside A 97% purity	0, 25000, 50000, 75000, 100000 ppm rebaudioside A in diet	4 weeks for palatability study followed by 13 weeks in diet	In a 4-week study, the NOAEL determined to be 100000 ppm. In a 13-week study, reductions in body weight gain and lower caloric density of the diet were observed in the high level male and female groups. Inconsistent reductions in serum bile acids and cholesterol observed. Other hepatic function test results and liver histopathology were within normal limits. No significant changes in clinical pathology, organ weight and functional observational battery test observed. Macroscopic and microscopic examinations of all organs normal. The NOAEL determined to be 50,000 ppm in male and female rats for 1 13-week study.

Table 30. Subchronic Animal Toxicology Studies

Reference	Study Design/ Objective	Species & number/sex	Test Substance	Amount Administered	Duration	Results
Nikiforov and Eapen (2008)	Subchronic oral safety study	20 Male and 20 female Sprague-Dawley rats	Rebaudioside A purity 99.5%	500, 1000 and 2000 mg/kg/day	Subchronic- 90 days	No treatment related effects on the general condition, behavior of animals, functional observational battery, and locomotor activity assessments. No toxicologically relevant, treatment- related effects on hematology, serum chemistry or urinalysis. Normal micro and macro-scopic findings on tissues investigated. Results of this study demonstrated that dietary administration of high concentrations of rebaudiana A for 90 consecutive days was not associated with any signs of toxicity in rats.
Toskulkao et al. 2001	Subchronic oral administration of steviol to hamsters	Subchronic: 9 males/females Syrian golden hamsters (locally bred)	Steviol ~ 90% purity	0, 100, 500 mg/kg bw steviol in diet	13 weeks	Steviol at a level of 100 or 500 mg/kg bw in the diet had no significant effect on body weight gain or food conversion efficacy and showed no observable signs of toxicity or abnormal behavior. The maximum tolerated level of steviol given in the diet was estimated to be higher than 500 mg/kg for both sexes in hamsters.

000090

000091

Table 30. Subchronic Animal Toxicology Studies

Reference	Study Design/ Objective	Species & number/sex	Test Substance	Amount Administered	Duration	Results
McNeil, 2008	Subchronic oral toxicology study to corroborate the safety of ingestion of steviol glycoside extracts from two specific suppliers in rats	Male and female Sprague Dawley rats 160 rats (80 males and 80 female); 150-250g	Steviol glycoside extracts from Supplier 1 and Supplier 2 Source 1: purity 7.7% stevioside and 90.76% rebaudioside A (one lot) Source 2: purity 4.41-5.27% stevioside and 81.23-85.56% rebaudioside A (3 lots)	Gr 1: Control (rodent diet without steviol glycosides) Gr 2: Supplier 1, 2.5% in the diet Gr3: Supplier 1, 4% in the diet Gr 4: Supplier 2, 2.5% in the diet Gr 5: Supplier 2, 4% in the diet	90 days	Male and female rats given 2.5% and 4% steviol glycoside extracts from Supplier 1 and 2 experienced similar weight gains as compared to controls; body weights were not significantly different between treated and control groups. No adverse clinical signs were observed. No toxicologically significant differences in hematology, clinical chemistry, organ weights, gross or histopathology in treated compared to control groups. The NOAEL was determined to be the highest level tested.

Table 31. Chronic Animal Toxicology Studies

Reference	Study Design/ Objective	Species & number/sex	Test Substance	Amount Administered	Duration	Results
Hagiwara et al. 1984	Promotion study (stevioside) initiated by N-Butyl-N-(4-Hydroxybutyl)-Nitrosamine (BBN)	25 male F344/DuCrj rats from Charles River Japan	Stevioside, purity not given (Nikken Kagaku Co, Japan)	5% in diet with or with out BBN in drinking water (0.01%)	BBN, 4 weeks; followed by stevioside, 32 weeks	No preneoplastic or neoplastic lesions of the urinary bladder were observed; no promoting activity
Toskulkao et al. 2001	Chronic oral administration of steviol to hamsters	Subchronic: 9 males/females Syrian golden hamsters (locally bred) Chronic: 55 males/females (same animals)	Steviol ~ 90% purity	Chronic: 0, 100, 500 mg/kg steviol in diet (male = 200 mg/kg bw/d steviol; female = 183 mg/kg bw/d steviol) [200 mg steviol = 500 mg stevioside; 183 mg steviol = 458 mg stevioside]	Chronic: Males: 22 months Females: 18 months	Blood chemistry, urine analysis, feed consumption, general appearance, mortality and tumor formation: no difference between treated animals and controls. Conclusion: not carcinogenic under the experimental conditions in the hamster. NOAEL = highest level tested
Toyoda et al. 1997	Assessment of carcinogenicity of stevioside	50 male & 50 female F344/DuCrj rats from Charles River Japan	Stevioside from Stevia Industrial Association (Japan) 95.6% purity	2.5 & 5% stevioside in basal diet. Highest level in males = 2000 mg/kg bw/d; female = 2400 mg/kg bw/d	104 weeks treatment followed by 4 week without treatment for 108 weeks	No treatment effects; not carcinogenic at 5% (decrease in mammary adenomas at 2.5 & 5%). Final survival rate for 5% males significantly lower than controls. NOAEL = highest level tested

000092

000093

Table 31. Chronic Animal Toxicology Studies

Reference	Study Design/ Objective	Species & number/sex	Test Substance	Amount Administered	Duration	Results
Xili et al. 1992	Chronic oral toxicity & carcinogenicity study of stevioside in rats (90 day and 2 year)	Local weanling inbred Wistar rats 90 day: 3 groups of 10 male, 10 female chronic: 4 groups of 45 male, 45 females	Stevioside 85% purity	90 day: 3 or 5% stevioside in basal diet chronic: 0, 0.2, 0.6, 1.2 % in basal diet; highest level male = 749 mg/kg bw/d; female = 839 mg/kg bw/d.	90 days and 24 months	No treatment related changes observed in hematological, urinary or clinical biochemical values and the incidence of non-neoplastic and neoplastic changes was unrelated to stevioside NOAEL = highest level tested
Yamada et al. 1985	Chronic toxicity study of stevia extract (22 months males, 24 months in females)	480 in number , 4-week old , F344 Rats from Charles River, Japan	Stevia from Japan Stevia Association 95.98% purity	0.1, 0.3 and 1% stevia extract in compressed chow	22 months males, 24 months in females	General appearance and behavior same in control and experimental group; mortality in steviol glycosides group not significantly different from control group; No significant changes in biochemical, chemical and clinical variables; highest level of steviol glycosides that caused no effect in rats was 550mg/kg

C. Reproductive/Developmental Toxicity

1. Introduction

Studies with purified stevioside or steviol glycosides have not reported adverse reproductive or developmental effects (Mori et al., 1981; Yodyingyuad and Bunyawong, 1991; Usami et al., 1995; Curry and Roberts, 2008). However, two early studies conducted with crude extract of leaves of *S. rebaudiana* (Bertoni) Bertoni reported adverse effects on fertility (Mazzei-Planas and Kuc, 1968; Melis, 1999). The relevance of these two studies to the safety assessment of purified rebaudioside A, stevioside or stevioside glycosides are limited because of the lack of characterization of the crude steviol glycosides extracts and the use of protocols that do not meet regulatory guidelines for reproductive safety studies. The Joint Expert Committee on Food Additives (JECFA) reviewed the reproductive studies published prior to 1999 and then prior to 2004 and concluded that steviol glycosides met the JECFA monograph specifications and therefore, did not pose a reproductive hazard to humans based on the weight of the evidence from the safety data (JECFA 1999, 2005).

Curry et al. (2008) demonstrated no treatment-related effects of rebaudioside A in either the F_0 or F_1 generations on mating performance, fertility, gestation length, or estrous cycle. The results of this study corroborate the JECFA conclusion. Due to the similarities in metabolism, the results of this study can be generally extended to stevioside and high-purity steviol glycosides extracts (at least 95% steviol glycosides).

In addition, in chronic/carcinogenicity studies of stevioside and steviol in rats and hamsters, respectively, no test article related adverse effects on prostate or testis weights, spermatogenesis, proliferation of interstitial cells, or histopathology of the testis were seen which further corroborates the conclusion that steviol glycosides do not produce adverse reproductive effects (Yamada et al. 1985; Xili et al. 1992; Toyoda et al. 1997; Toskulkao 2001)

2. Review of studies

Only a few studies have been published that have evaluated the reproductive properties of steviol glycosides in animals. One of the earlier reproductive studies conducted on steviol glycosides was conducted on a water decoction extract (purity not stated) by Planas and Kuc in 1968. A water decoction of *S. rebaudiana* (Bertoni) Bertoni was supplied in the drinking water to virgin females and females of proven fertility from a locally bred colony of albino rats. The decoction was prepared daily by boiling the dry, powdered leaves in water for 10 minutes and filtering after cooling. In the first experiment, virgin females were divided into two groups of 14

000094
SPHERIX

each: one group was given 10 mLof a 5% decoction daily for the 6-day mating period (male rats did not receive the decoction solution) and the other group was the control group. In the 2nd experiment, 14 females were allowed to bear one litter. A week later, the same females were given 10 mL of the 5% decoction daily for the 6-day mating period (not stated if the males were treated or not). Each female served as her own control. In both experiments 1 and 2, the females were mated 50 to 60 days later (no treatment). In experiment 3, 14 females were allowed to mate under normal conditions (no treatment). One week after the litter was weaned, the females were treated as in experiments 1 and 2 except they were allowed to drink the decoction for 12 days and then mated again with the same male that fathered their first litter (not specified as to treatment of the male). Fertility was reduced 57 to 79% in the female rats drinking the decoction compared to controls. A 50 to 57% reduction in fertility was still evident 50 to 60 days after intake of the 5% decoction had ceased (Planas and Kuc 1968). This finding was not confirmed in subsequent work by other authors (cited in Oliveira-Filho et al. 1989). It was shown, however, that *in vitro*, stevioside can displace 5 alpha-dihydrotestosterone specifically bound to prostate androgen receptors (Oliveira-Filho et al. 1989). However, the high concentration used *in vitro* is not relevant to anti-androgenic effects *in vivo*. Therefore, subsequent work focused on studies designed to evaluate whether steviol glycosides extracts could produce effects on fertility in the male.

Melis et al (1999) conducted a study on prepubertal male Wistar rats (20/group) administrated a *S. rebaudiana* (Bertoni) Bertoni aqueous extract for 60 days. The extract corresponded to 66.7 g dried leaves/100 mL final solution that was given twice daily by gastric intubation at the rate of 2 mL/rat. Control animals received physiological saline on the same schedule. A decrease in final weight of testes, seminal vesicles and cauda epididimis was reported in the treated animals. In addition, the fructose content of the accessory sex glands and the epidiymal sperm concentration were decreased along with a slight decrease in plasma testosterone levels. No alteration of the lutenizing hormone level was reported. Melis et al (1999) speculated that one of the glycosides in the aqueous extract had an affinity for a certain androgen receptor. The authors concluded that the steva extract may have decreased the fertility of male rats.

Oliveidra-Filho et al. (1989) showed the effects of *S. rebaudiana* (Bertoni) Bertoni extract on endocrine parameters of Wistar male rats following administration (60 days) of a concentrated, crude extract of its leaves. Thirty animals were treated with the aqueous extract (66.7 g of dried leaves/100 mL final solution) twice daily via gastric tubing (2 mL/rat) over 60 days. Animals were deprived of feed and water 1 h before and 30 minutes after administration of the extract. Rats given the aqueous extract did not differ from the control group (with the

exception of seminal vesicle weight (down 60%), in glycemia, serum levels of T3 and T4 levels, T3 R index, binding of 3HR1881 to prostate cytosol, zinc content in the prostate, testis, submandibular salivary gland and pancreas, water content in testis or prostate.

Aritajat et al. (2000) performed a rodent dominant lethal test to evaluate a crude aqueous extract of stevia leaves for potential to cause chromosomal damage in the germ cells of male rats administered orally for 8 weeks at 2 mL/day prior to mating with untreated virgin females. No analysis of the extract was performed to quantify stevioside, steviol or any other possible terpene glycosides, thus the actual level of any particular components is unknown. However, with this crude decoction, there were no indications of an effect on the number of implantation sites or number of viable or dead fetuses that would indicate induction of lethal chromosomal events.

In contrast to results from many studies using crude extracts, studies with relatively pure stevioside have resulted in no adverse effects on fertility, reproductive indices or development of fetuses.

According to Mori et al. (1981, abstract only, article in Japanese) administration of 95.98% pure stevioside at dietary levels of 0.15, 0.75, or 3.00% to male rats for 60 days and to female rats for 14 days before mating had no adverse effect on pregnancy rate (fertility) or the development of the fetuses.

Yodyingyuad and Bunyawong (1991) studied 4 groups of 20 one-month old golden hamsters comprised of 10 males and 10 females each force-fed daily with stevioside (90% purity) at 0.0, 1.0, and 2.5 g/kg. Late pregnant and lactating females received stevioside in the drinking water. Each female was mated to produce a total of 3 litters. The same number of young F1 and F2 hamsters (40 of each sex from each generation) received stevioside via the drinking water until one month old and daily gavage afterwards at the same amounts as their parents. All generations showed normal growth and fertility. The authors concluded that stevioside at 2.5 g/kg affected neither growth or reproduction in hamsters.

Usami et al. (1995, abstract only) examined the teratogenicity of stevioside in pregnant Wistar rats following daily gavage of 0, 250, 500, and 1000 mg/kg/day on days 6 through 15 of pregnancy. The purity of stevioside and the number of pregnant rats examined was not given in the abstract. Following examination of fetuses on day 20 of pregnancy, the authors reported no increased incidences of fetal malformation and no toxic signs in the pregnant females or fetuses. The no observable adverse effect level was estimated to be over 1000 mg/kg/day for both pregnant females and fetuses.

The effect of stevioside (purity ~96% with ~3% steviolbioside and ~0.5% rebaudioside A) on 300 developing broiler embryo starting at day 7 of incubation with injections of fertile broiler eggs of 0.08, 0.8, or 4 mg stevioside/egg and then examined at day 21 (hatch) and 1 week later. No effect of the treatments was reported for embryonic mortality, body weight of the hatchlings or on deformities (bone, beak, and head malformations, abnormal feathering, open vent) or abnormal development of the gonads. No stevioside was detected in the blood of the hatchlings. The authors (Geuns et al. 2003a) concluded that prenatal exposure to stevioside was not toxic to the chicken embryo.

Sinchomi et al. (1989) as cited by Geuns 2000 investigated effects of stevioside on male fertility in male Wistar rats. Stevioside from a steviol glycosides extract was given daily for 31 days at a level of 0, 13, 65 and 260 mg/kg bw/d. The authors found no differences in weights of seminal vesicles, prostate, hypophysis, testicles, or body weight of rats. Anatomic-pathological examination of testicles showed no evidence of effects and no atrophy of seminal tubes was found. Leydig interstitial cells were not hyperplastic and did not show degenerative changes.

The effect of steviol (purity >98%) on the developing broiler embryo starting at day 7 of incubation with injections of 300 fertile broiler eggs at 0.025, 0.25, or 1.25 mg steviol/egg and then examined at day 21 (hatch) and 1 week later. No effect of the treatments was reported for embryonic mortality, body weight of the hatchlings or on deformities (bone, beak, and head malformations, abnormal feathering, open vent) or abnormal development of the gonads. No steviol was detected in the blood of the hatchlings. The authors (Geuns et al. 2003a) concluded that prenatal exposure to steviol was not toxic to the chicken embryo.

Wasuntarawat et al. (1998) evaluated the developmental toxicity of steviol (~90% purity) in virgin female golden Syrian hamsters. Pregnant hamsters were intubated with steviol at levels of 0.00, 0.25, 0.50, 0.75, and 1.0 g/kg/day on days 6-10 of gestation. Steviol at levels of 0.75 and 1.0 g/kg/day were highly toxic to both dams and fetuses. The number of live fetuses per litter and mean fetal weight also was significantly decreased in these animals. The animals treated with 0.50 mg/kg/day exhibited fewer signs of maternal and developmental toxicity than the two higher levels. No teratogenesis was detected. The authors concluded that an oral amount of 0.25 g steviol/kg/day was the no observable adverse effect level.

Curry et al. (2008) reported a two generation reproductive toxicity study in rats treated with rebaudioside A. Male and female Han Wister rats were administered with rebaudioside A at 0, 7500, 12500 and 25000 ppm for two generations. Rebaudioside A treated animals did not exhibit any signs of clinical toxicity or adverse effects on body weight, body weight gain, or feed

consumption. 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, estrous cycles, or sperm motility, concentration, or morphology. The survival and general condition of the F_1 and F_2 offspring, their pre-weaning reflex development, overall body weight gains, and the timing of sexual maturation, were not adversely effected by rebaudioside A treatment. The NOAEL for reproductive effects was 25000 ppm and the NOAEL for the survival, development, and general condition of the offspring also was considered to be 25000 ppm or 2048 to 2273 mg/kg bw/d.

Data from the Curry et al. (2008) study depicting mating performance and fertility, gestation length and gestation index, litter size and offspring survival, and sperm analysis and morphology is presented in tables 32, 33, 34, and 35, respectively.

A tabular summary of reproductive and developmental toxicity studies is presented in Tables 36.

Table 32. Mating performance and fertility

		F_0 generation[a]				F_1 generation			
		0 ppm	7,500 ppm	12,500 ppm	25,000 ppm	0 ppm[b]	7,500 ppm[c]	12,500 ppm[b]	25,000 ppm[b]
Number paired	Male	30	30	30	30	25	24	25	25
	Female	30	30	30	30	25	24	25	25
Number mating	Male	30	30	30	30	25	24	25	25
	Female	30	30	30	30	25	24	25	25
Number achieving pregnancy	Male	30	29	30	30	25	24	24	25
	Female	30	29	30	30	25	24	24	25
Percentage mating	Male	100	100	100	100	100	100	100	100
	Female	100	100	100	100	100	100	100	100
Conception rate (%)	Male	100	97	100	100	100	100	96	100
	Female	100	97	100	100	100	100	96	100
Fertility index (%)	Male	100	97	100	100	100	100	96	100
	Female	100	97	100	100	100	100	96	100

[a] n = 30/group.
[b] n = 25/group.
[c] n = 24/group.

Table 33. Gestation length and gestation index

		F_0 generation[a]				F_1 generation			
		0 ppm	7,500 ppm	12,500 ppm	25,000 ppm	0 ppm[b]	7,500 ppm[c]	12,500 ppm[c]	25,000 ppm[b]
Gestation length in days[d]	22	0	0	5 (17)	6 (20)	4 (17)	2 (8)	0	4 (16)
	22.5	13 (45)	16 (55)	11 (37)	11 (37)	7 (29)	10 (42)	12 (50)	14 (56)
	23	12 (41)	10 (34)	10 (33)	13 (43)	12 (50)	12 (50)	12 (50)	7 (28)
	23.5	4 (14)	3 (10)	4 (13)	0	1 (4)	0	0	0
Number of live litters born		29	29	30	30	24	24	24	25
Gestation Index (%)		97	100	100	100	96	100	100	100

[a] n = 30/group except for the 7500 ppm group where n = 29.
[b] n = 25/group.
[c] n = 24/group.
[d] Presented as n (%); percentage distribution of gestation lengths calculated from 29 and 24 animals for F_0 and F_1generation, respectively (one pregnant female failed to litter in each generation).

Table 34. Litter size and offspring survival

		F_0 generation				F_1 generation			
		0 ppm[a]	7,500 ppm[b]	12,500 ppm[c]	25,000 ppm[c]	0 ppm[d]	7,500 ppm[e]	12,500 ppm[d]	25,000 ppm[f]
Implantations[g]		12.5 ± 3.7	12.9 ± 2.9	13.6 ± 2.8	13.7 ± 2.6	12.8 ± 1.8	13.7 ± 2.6	13.2 ± 2.6	13.1 ± 2.1
Total litter size[g]	Day 1	11.4 ± 3.6	11.3 ± 3.7	12.4 ± 3.0	12.4 ± 2.7	11.8 ± 2.0	12.7 ± 2.6	11.9 ± 2.9	12.0 ± 2.2
Live litter size[g]	Before cull								
	Day 1	11.2 ± 3.6	11.0 ± 3.6	12.0 ± 2.9	12.0 ± 2.7	11.6 ± 1.9	12.4 ± 2.5	11.7 ± 3.0	11.7 ± 2.2
	Day 4	11.0 ± 3.8	11.0 ± 3.6	11.8 ± 2.9	11.8 ± 2.6	11.6 ± 1.9	12.4 ± 2.5	11.6 ± 3.1	11.6 ± 2.2
	After Cull								
	Day 4	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.9 ± 0.7	7.9 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
	Day 7	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.8 ± 0.7	7.9 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
	Day 14	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.8 ± 0.8	7.9 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
	Day 21	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.8 ± 0.8	7.9 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
	Day 25	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.8 ± 0.8	7.8 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
Post Implantation Survival Index (%)		91.2	86.4	91.2	90.8	90.6	92.7	90.6	91.6
Live Birth Index (%)		97.7	98.3	96.6	97.0	98.8	97.9	98.3	97.2
Viability Index (%)		98.3	99.5	99.0	98.5	100.0	100.0	98.6	99.7
Lactation Index (%) on Day 21		100.0	100.0	100.0	99.2	100.0	100.0	100.0	100.0

Statistical analysis performed on values before day 4 cull only. $p \geqslant 0.05$, no statistical significance.
[a] $n = 29$/group except day 7, where $n = 28$/group.
[b] $n = 27$/group.
[c] $n = 30$/group.
[d] $n = 24$/group.
[e] $n = 23$/group.
[f] $n = 25$/group.
[g] Data presented as mean ± standard deviation.

000100
SPHERIX

Table 35. Sperm analysis and morphology

		F_0 generation[a]				F_1 generation			
		0 ppm[a]	7,500 ppm[a]	12,500 ppm[b]	25,000 ppm[b]	0 ppm[c]	7,500 ppm[d]	12,500 ppm[e]	25,000 ppm[c]
Motile sperm (%)		91 ± 9	89 ± 11	91 ± 5	90 ± 9	90 ± 7	90 ± 9	89 ± 11	93 ± 5
Progressively motile sperm (%)		56 ± 10	54 ± 13	57 ± 11	56 ± 9	56 ± 9	55 ± 11	54 ± 12	56 ± 11
Cauda epididymis	Weight (g)	0.205 ± 0.026	NR	NR	0.205 ± 0.025	0.219 ± 0.030	NR	NR	0.225 ± 0.027
	Sperm count (millions/g)	790 ± 182	NR	NR	790 ± 171	735 ± 157	NR	NR	688 ± 149
	Total (million)	162 ± 41	NR	NR	161 ± 35	160 ± 35	NR	NR	153 ± 32
Testis	Weight (g)	1.91 ± 0.18	NR	NR	1.85 ± 0.15	1.94 ± 0.13	NR	NR	1.96 ± 0.18
	Sperm count (millions/g)	217 ± 34	NR	NR	213 ± 31	174 ± 38	NR	NR	178 ± 44
	Total (million)	414 ± 69	NR	NR	394 ± 64	336 ± 74	NR	NR	346 ± 72
Total number of sperm examined		5800	NR	NR	6000	5000	NR	NR	5008
Normal	Number	196 ± 6	NR	NR	196 ± 3	193.8 ± 10.3	NR	NR	194.4 ± 3.1
	%	97.8 ± 2.9	NR	NR	97.8 ± 1.4	96.9 ± 5.2	NR	NR	97.0 ± 1.7
Decapitate	Number	1 ± 2	NR	NR	1 ± 1	3.0 ± 9.4	NR	NR	1.4 ± 1.6
	%	0.7 ± 1.0	NR	NR	0.6 ± 0.6	1.5 ± 4.7	NR	NR	0.7 ± 0.8
Abnormal	Number	3 ± 4	NR	NR	3 ± 2	3.2 ± 2.0	NR	NR	4.6 ± 2.8
	%	1.6 ± 1.9	NR	NR	1.7 ± 1.2	1.6 ± 1.0	NR	NR	2.3 ± 1.4

Presented as mean ± standard deviation; NR = not reported.
[a] *n* = 29/group.
[b] *n* = 30/group.
[c] *n* = 25/group.
[d] *n* = 23/group.
[e] *n* = 24/group.

Summary of studies identified in this section are presented in Table 36.

000101

Table 36. Reproductive and Developmental toxicity studies

Reference	Study Design/Objective	Species and number/sex	Test Material	Amount Administered	Duration	Results (only safety endpoints summarized; effect compared to control)
Curry et al. 2008	Two- generation reproductive toxicity	29 female HsdBrl: Han Wister rats and their litters for preliminary palatability study and 128 male and female HsdBrl: Han Wister rats (32 litters of 4 males and 4 females each) randomized into groups of 30/sex	Rebaudioside A purity 97%	25000, 37500 and 50000 ppm rebaudioside A in a preliminary study and 7500, 12500 and 25000 ppm in the two-generation study	The F0 females for the preliminary study treated from days 14-21 of lactation and for the two-generation reproductive study F0 rats received diets appropriate for their group for 10 weeks prior to mating	No signs of clinical toxicity or adverse effects on body weight, body weight gain, feed consumption. No adverse effects on reproductive performance parameters including mating performance, fertility, gestation lengths, estrous cycles, or sperm motility, concentration, or morphology. The survival and general condition of the F_1 and F_2 offspring, their pre-weaning reflex development, overall body weight gains, and the timing of sexual maturation not adversely effected. The NOAEL was 25000 ppm and the NOAEL for the survival, development, and general condition of the offspring was 25000 ppm.

000103

Table 36. Reproductive and Developmental toxicity studies

Reference	Study Design/Objective	Species and number/sex	Test Material	Amount Administered	Duration	Results (only safety endpoints summarized; effect compared to control)
Geuns 2003a	Effect of stevioside & steviol on developing broiler embryos	600 fertile broiler eggs (Ross) from local hatchery (Avibel, Zoersel, Belgium)	Stevioside (Stevita Co, Arlington, TX) purified by repeated crystallization from MeOH [> 96%, stevilbioside (3%), rebaudioside A (0.5%)] Steviol (98%)	Injected into yolk sac Stevioside: 0.08, 0.8, 4 mg/egg Steviol: 0.025, 0.25, 1.25 mg/egg	Single injection; on day 18, eggs were candled and then allowed to hatch	Neither stevioside or steviol, at the levels tested, were toxic to the chicken embryo
Melis 1999	Effects on fertility	20 male Wistar rats	Crude aqueous extract of leaves of *S. rebaudiana* (Bertoni) Bertoni (3.8 kg extract yielded 1 kg of dry concentrated extract)	Orally an aqueous extract of *S. rebaudiana* (Bertoni) Bertoni corresponding to 66.7 g of dried leaves/100 mL final solution	twice daily for 60 days via gastric tubing (2 mL/rat)	Decrease in final relative weights of testis, seminal vesicle and cauda epididymidis; fructose content of accessory sex glands and epididymal sperm concentration were decreased; plasma testosterone level was down; no effect on luteinizing hormone level; steviol glycosides extracts may decrease fertility of male rats

Table 36. Reproductive and Developmental toxicity studies

Reference	Study Design/Objective	Species and number/sex	Test Material	Amount Administered	Duration	Results (only safety endpoints summarized; effect compared to control)
Mori et al. 1981 (abstract only)	Fertility in rats	Male and female rats: 22 mated pairs, 19 or 20 pregnant dams per group	Stevioside 95.98% pure	0.15, 0.75 and 3.0% in diet	males: 60 days before and during mating; females: 14 days before mating & for 7 days during gestation	No adverse effect on fertility or on the development of fetuses; 3%, slight retardation of body weight increase in early period of administration in both sexes
Oliveira-Filho et al. 1989	Chronic administration of water extract of *S. rebaudiana* (Bertoni) Bertoni in rats: endocrine effects	50 Wistar 25-30 day old male rats	Concentrated aqueous extract of *S. rebaudiana* (Bertoni) Bertoni	66.7 g dried leaves/100 mL final solution; 2 mL per rat	30 rats treated twice a day by means of gastric tubing (2 mL/rat) for 60 days; no feed 1 h before and 30 min after	*S. rebaudiana* (Bertoni) Bertoni treated rats did not significantly differ from controls with exception of the seminal vesicle weight, (down 60%). Treatment did not modify Zn content in testis and other androgen target organs. It also did not alter the number of binding sites for androgens of the apparent affinity constant in prostate. Therefore, anti-androgenic activity of the extract was not demonstrated.
Planas & Kuc 1968	To determine contraceptive properties in females	Strain of albino rats derived from 28 years of inbreeding the descendents of one pair; groups of 14 females	Water decoction of *S. rebaudiana* (Bertoni) Bertoni	Varied by experiment: typically 10 mL of 5% decoction; females only	6 (during mating period) to 12 days (prior to mating)	Fertility was reduced 57 to 79% with 50 to 57% reduction still evident 50 to 60 days after intake of the decoction had ceased

000104

000105

Table 36. Reproductive and Developmental toxicity studies

Reference	Study Design/Objective	Species and number/sex	Test Material	Amount Administered	Duration	Results (only safety endpoints summarized; effect compared to control)
Sinchomi et al. 1989 (as cited in Geuns 2000)	Investigate effects of stevioside on male fertility	Male Wistar rats	Stevioside from a stevia extract Purity not given	0, 13, 65, 260 mg/kg bw/d stevioside	Extract given daily for 31 days	No differences in weights of seminal vesicles, prostate, hypo-physis, testicles, or body weight. Anatomic-pathological examination of testicles showed no evidence of effects. No atrophy of seminal tubes was found, and generation of semen was present. Leydig interstitial cells were not hyperplastic and did not show degenerative changes. No evidence of inflammatory infiltration or signs of intolerance.
Aritajat et al. 2000	Dominant Lethal assay	Male Norwegian rats	Aqueous extract of stevia leaves	2 mL/day oral (crude extract, purity not given)	8 weeks	Negative
Usami et al. 1995	Teratology study (fetuses examined for malformation but not stated by what methods)	Pregnant Wistar rats, number not given in abstract	Stevioside, purity not given in abstract	0, 250, 500, & 1000 mg/kg/day	Days 6- 15 of pregnancy; killed on day 20 of pregnancy	No teratogenicity in rats (>1000 mg/kg/day consider NOAEL for both pregnant rats and rat fetuses)

Table 36. Reproductive and Developmental toxicity studies

Reference	Study Design/Objective	Species and number/sex	Test Material	Amount Administered	Duration	Results (only safety endpoints summarized; effect compared to control)
Wasuntarawat et al. 1998	Developmental tox of steviol in hamsters	20 virgin female golden hamsters (*M. auratus*)/ group (another group of 12 dams)	Steviol obtained by oxidation of stevioside as described by Ogawa et al ~90% purity	Each of 20 dams was gavaged with 0, 250, 500, 750 mg/kg bw; 12 dams received 1000 mg/kg bw	daily on day 6 to day 10 of gestation	No level-related teratogenesis was detected; steviol at levels of 750 & 1000 mg/kg bw/d were highly toxic to both dams and fetuses
Yodyinguad and Bunyawong 1991	Effect of stevioside on growth and reproduction hamsters; 3 generation study	10 male & 10 female golden hamsters (*Mesocricetus auratus*) per group; total of 80 hamsters; same number for F1 & F2 generations	Stevioside (90%, white crystalline powder) dissolved in distilled water	concentration of 0.5 g/mL and 0.5, 1.0, & 2.5 g/20 mL water	Orally force-fed daily, with either distilled water or stevioside syrup; late pregnant and lactating females received stevioside in drinking water	Stevioside at a level as high as 2.5 g/kg body wt/day affected neither growth or reproduction in hamsters

D. GENOTOXICITY AND MUTAGENICITY

1. Introduction

Stevioside and steviol have been subjected to extensive testing for genotoxicity. Steviol, the aglycone metabolite of stevioside, in the presence of a rat liver, S9 metabolic activation system, produced positive effects in both Salmonella strain TM677 and an *in vitro* chromosome damage test in Chinese hamster lung cells. No positive effects were seen in the absence of S9 activation, in any of the other strains of Salmonella tested or in *in vivo* tests for chromosome damage. The significance of these two positive effects for steviol for extrapolating to carcinogenic risk *in vivo* is questionable because positive effects were found only under *in vitro* testing conditions.

The majority of the findings show no evidence of genotoxic activity for stevioside. Neither stevioside nor its aglycone steviol have been shown to react directly with DNA or demonstrate genotoxic damage in assays relevant to human risk. The mutagenic activity of steviol and some of its derivatives, exhibited in strain TM677, was not reproduced in the same bacteria having normal DNA repair processes. The single positive *in vivo* study measuring single strand DNA breaks in Wister rat tissues by stevioside, was not confirmed in experiments in mice and appear to be measuring processes other than direct DNA damage in the Wister rat. Neither stevioside nor steviol induced clastogenic effects at extremely high levels *in vivo*. Therefore, the conditions under which positive genotoxicity is seen *in vitro* appear to have limited relevance to *in vivo* exposures. Negative results in so many sensitive test systems, that used a range of metabolic activation parameters, supports the fact that stevioside *per se* is a non-genotoxic substance. This is corroborated by negative results in four carcinogenicity assays conducted in rats and hamsters; stevioside levels ranged up to 458 mg/kg bw/d (calculated from steviol intake) in the hamster and up to 2400 mg/kg bw/d in the rat.

2. Review of studies

a) Bacterial Mutagenicity

Stevioside and its aglycone derivative steviol, as well as various related diterpene glycosides found in stevioside preparations, have been evaluated for genotoxic activity in a range of bacterial test systems. Klongpanichpak et al. (1997) reported that both stevioside and steviol as fine white powders of 96% purity were non-mutagenic in *Salmonella typhimurium* strains TA98 and TA100 in a pre-incubation assay conducted in the presence and absence of a liver homogenate metabolic activation system. Liver homogenates were prepared from Swiss mice, Wistar rats, Syrian golden hamsters and albino guinea pigs that were treated with sodium

phenobarbital plus 5,6-benzoflavone to induce metabolic activity prior to preparation of the liver homogenates. Test concentrations of stevioside (96%), dissolved in distilled water, ranged between 12.5 to 50 mg/plate, while concentrations of steviol (96%), dissolved in DMSO, were tested up to a level that was cytotoxic at 2.0 mg/plate. The authors concluded that neither steviol nor stevioside was mutagenic in strains TA98 and TA100 in the Ames assay conducted, with or without metabolic activation.

Several other investigators have confirmed absence of mutagenic activity of stevioside and steviol in the Ames assay. Matsui et al. (1996) reported on evaluation of stevioside (83% purity) and steviol (99% purity) with 4 different bacterial test systems that involved different bacterial strains capable of detecting distinct types of mutations. A reverse mutation test of steviol or stevioside dissolved in DMSO was conducted with *Salmonella typhimurium* strains TA97, TA98, TA100, TA102 and TA104. Tests were conducted in the presence and absence of a rat-liver homogenate metabolic activation system prepared from Fischer rats induced with polychlorinated biphenyls. No increases in mutants were observed at levels up to 5 mg stevioside/plate in the presence or absence of metabolic activation, respectively. Steviol at levels up to 5 mg/plate was also negative for mutagenic activity in assays employing these same strains tested either with or without metabolic activation. In addition, the authors reported that stevioside was not mutagenic to *Bacillus subtilis* in the DNA damage rec assay, in the *Escherichia coli* WP2 *uvrA/pKM101* polymerase ± DNA repair test, or in the *umu* test [an indirect test for DNA damage employing an enzyme reporter gene] with *S. typhimurium* strain TA1535/pSK1002. Steviol was reported to be weakly active in the *umu* test, but not positive in any of the other bacterial mutation test systems, either with or without metabolic activation. Suttajit et al. (1993) reported steviol (purity not specified) to be non-mutagenic to Salmonella strains TA98 and TA100 in tests both with and without rat liver metabolic activation at levels from 1-20 mg/plate. Stevioside (99% purity) was non-mutagenic in the same strains up to concentrations of 25 mg/plate both with and without metabolic activation. However, at a level of 50 mg/plate (10X higher than required by standard international test protocols), stevioside produced increases in mutations of approximately 4-fold over the negative control values. The authors suggested that this single positive result at such a high test concentration "might be due to contamination by impurities" in the test material.

Pezzuto et al. (1985) evaluated the mutagenic potential of stevioside and steviol (purity not specified for either) using Salmonella strain TM677, a strain that detects forward mutations involving loss of activity of the enzyme guanine phosphoribosyl-transferase (gpt). Unlike the standard Ames assay, this test system detects mutations by quantifying bacteria with resistance to 8-azaguanine, a toxic purine analogue that is incorporated into DNA in the presence of a functional *gpt* enzyme. Concentrations of stevioside and 5 other structurally-related diterpene

glycosides from 0.1 to 10 mg/mL were not mutagenic to *S. typhimurium* strain TM677 when tested in the absence or presence of an Aroclor 1254-induced S9 metabolic activation system. Steviol, in contrast, produced dosage-related increases in the numbers of mutants within this same range of concentrations, but only in tests that incorporated a metabolic activation system. Thus, the authors suggested that a metabolic derivative of steviol is the likely mutagenic agent rather than steviol *per se*. They did not detect mutagenic activity of potential metabolic intermediates that included isosteviol, steviol-16α, 17-epoxide or dihydrosteviol A or B. Mutagenic activity for stevioside derivatives appeared to be a function of an unsaturated bond at the hydroxyl group on position 13 of the diterpene molecule which was evident by the absence of mutagenic activity to TM677 for stevioside or any of the related glycosides found in stevioside samples that had a glycoside residing at position 13, including steviobioside, dulcoside A, and rabaudisoside A, B and C. Pezzuto et al. (1986) also noted acetylation of the hydroxyl group at position 13 of steviol, or replacement by a proton or glycoside at position 13 of steviol totally negated the mutagenic activity to TM677.

In a subsequent paper, Pezzuto (1986) confirmed the findings noted in 1985 and reported that epoxide hydrolase did not inhibit the mutagenic activity of steviol to TM677 tested in the presence of a liver homogenate metabolic activation system. This result suggested that the mutagenic metabolite is either not an epoxide or that the epoxide metabolites produced from steviol are not substrates for this particular enzyme that eliminates the mutagenicity of other epoxide mutagens. However, 19-O-β-D-glucopyranosyl steviol, a potential hydrolysis product of stevioside, was reported to be a mutagen to TM677 in the presence of S9 metabolic activation, indicating that it may be one of the metabolites responsible for genotoxicity.

Matsui et al. (1996) also reported positive mutagenic effects of steviol using *Salmonella typhimurium* strain TM677. These investigators reported that steviol (purity not specified) produced no significant increases in mutation incidence in this strain in tests without a rat liver metabolic activation system. However, positive and dosage-related increases in mutations were seen in the test with metabolic activation over a range of 7 concentrations from 0.1 to 10 mg/mL.

Eight chemically synthesized derivatives of steviol were reported to be mutagenic to Salmonella strain TM677 in the presence of S9 activation by Terai et al. (2002). The synthesized derivatives of steviol included: steviol-16-17-epoxide, 15α-hydroxysteviol, 15-oxo-steviol, steviol methylester, 16-oxo-steviol methylester, 13,16-seco-13-oxosterviol methylester, 13,16-seco-13α-hydroxy-oxosterviol methylester and steviol methlyester 8,13-lactone. However, the mutagenicity data were presented only in summary form and there was no indication if these synthesized steviol derivatives are formed as metabolites either *in vitro* or *in vivo* to support the authors' suggestion that these synthesized derivatives may provide a basis for the genotoxicity of

steviol. One derivative, 15-oxosteviol, reported to be mutagenic to Salmonella strain TM677 with S9 activation by both Compadre et al. (1988) and by Terai et al. (2002), was found to be non-mutagenic in the same test system by Procinska et al. (1991). These latter authors claimed that if inadequate numbers of bacteria are plated or if incorrect methods are used to calculate data in the TM677 test system, spurious conclusions can be made concerning mutagenicity.

In summary, stevioside has been found to be consistently non-mutagenic in bacterial assays using standardized tests with *Salmonella typhimurium*, *B. subtilis* and *E. coli tester* strains. The single exception reporting a postive mutagenic effect was for some synthetic metabolites of the steviol when tests were conducted by incorporating a liver S9 activation system in the forward mutation test procedure involving *Salmonella* strain TM677, but not in any other Ames assay *Salmonella* strains. None of these synthetic metabolites has been reported to be produced either *in vitro* or *in vivo*. Furthermore, if inadequate numbers of bacteria are plated or if incorrect methods are used to calculate data in the TM677 test system, spurious conclusions can be made concerning mutagenicity.

b) Mammalian *In Vitro* and *In Vivo* Genotoxicity

(1) Stevioside

Mammalian cell assays of stevioside for production of mutations or chromosome damage in mouse lymphoma cells, Chinese hamster lung (CHL) cells and human lymphocytes have all shown consistently negative results. Although some of the studies were conducted with poorly defined test material, even crude extracts did not lead to positive responses in the test systems employed, which is a problem often encountered with biological extracts that contain impurities.

More recently Brusick (2000) summarized the earlier studies and suggested that neither stevioside nor steviol-induced clastogenic effects are observed at extremely high levels *in vivo*. Based on the application of a Weight-of-Evidence approach he suggested that steviol glycosides are safe and do not pose a risk of genetic damage following human consumption.

Oh et al. (1999), in an English abstract from a Chinese publication, reported that concentrations of stevioside from 1.25 to 5 mg/mL (purity not stated) were not mutagenic to L5178Y mouse lymphoma cells following a 3 hour exposure period to the test substance in the presence or absence of an S9 metabolic activation systems. Stevioside was also negative for mutagenicity following a 24hour exposure period without S9 activation (not tested with S9 activation).

Absence of clastogenic potential of stevioside was seen in several studies. Matsui et al. (1996) found no increases in incidence of chromosome aberrations in CHL cells within a range of concentrations of stevioside (83% purity) from 2 to 8 mg/mL without S9 or 2 to 12 mg/mL with S9 activation. Exposure times to stevioside were 6, 24 or 48 hourr for –S9 and 6 hour for +S9. Ishidate et al. (1984) also reported in a summary table that stevioside (85% purity) was not clastogenic to CHL cells tested up to 12 mg/mL. These tests were apparently conducted only in the absence of S9 metabolic activation, and experimental results for lower levels were not presented. No clastogenic effects were produced by stevioside (99% purity) in cultures of human lymphocytes exposed at concentrations of 1, 5 and 10 mg/mL (exposure period not stated) (Suttajit et al. 1993). In a recent JECFA evaluation of steviol glycosides, results of an unpublished study (Nakajima 2000a cited in WHO 2006) indicated that rebaudioside A (unspecified purity) was not clastogenic to CHL fibroblasts.

Oral administration of a non-characterized "stevia extract" at levels of 250, 500, 1000 and 2000 mg/kg to male BDF1 mice did not show DNA-damage using the comet assay with cells isolated from mouse stomach, colon or liver examined 3 and 24 h after administration (Sekihashi et al. 2002). The composition of the test substance in the Japanese article was stated to be 52% stevioside by JECFA (WHO 2006). No DNA damage was detected following one oral administration of 2000 mg/kg (limit level, (undefined composition)) to ddy male mice by examination of DNA breakage in the comet assay from single cells from stomach, colon, liver, kidney, bladder, lung, brain and bone marrow tissues at 3 or 24 h after administration (Sasaki et al. 2002). Most recently, an additional Comet assay of stevioside was reported (Nunes et al. 2007). Stevioside at a level of 4 mg/mL was given orally to Wister rats and was reported to induce lesions in peripheral blood, liver, brain, and spleen cells, organs that have not shown any adverse histopathology in subchronic or chronic studies conducted with steviol glycosides (Toyoda et al. 1997; Yamada et al. 1985; Sili et al 1992; Curry et al. 2008; McNeil 2008). The data reported by Nunes et al. (2007) is inconsistent and conflicting since both control and treated animals exhibited a high degree of DNA damage. The Comet assay is only a screening assay for DNA damage. It does not indicate heritable genetic effects and is only considered an aid to detect the toxic effect of a compound to the genetic material.

Oh et al. (1999) reported no increases in micronuclei indicative of chromosome damage from levels of 62.5, 125 and 250 mg/kg stevioside (purity not provided) given once orally to ICR mice with evaluation of the incidence of micronuclei in bone marrow polychromatic erythrocytes (PCE) and in hepatocytes. The ratio of PCEs to total erythrocytes in the bone marrow decreased in relation to increasing level, which provided evidence that the test substance entered the systemic circulation as determined by mild cytotoxicity to the target cells in this test system.

Aritajat et al. (2000) performed a rodent dominant lethal test to evaluate a crude aqueous extract of stevia leaves for potential to cause chromosomal damage in the germ cells of male rats administered orally for 8 weeks at 2 mL/day prior to mating with untreated virgin females. No analysis of the extract was performed to quantify stevioside, steviol or any other possible terpene glycosides, thus the actual amount of any particular components is unknown. However, with this crude decoction, there were no indications of an effect on the number of implantation sites or number of viable or dead fetuses that would indicate induction of lethal chromosomal events. This negative result is consistent with other tests that show that stevioside and other related components lack genotoxic activity *in vivo*.

Additional information from several brief reports also indicate that stevioside and related terpene glycosides are not genotoxic. Kerr et al. (1983) found that stevioside (purity not stated) was not mutagenic when fed at 2% of the diet to Muller-5 strain of *Drosophila melanogaster*. Based on an unpublished study (Nakajima 2000b cited in WHO 2006), JECFA noted that rebaudioside A was negative for production of chromosome damage in the BDF_1 mouse bone marrow assay within a range of levels from 500to 2000 mg/kg per day for 2 days. The route of administration was not specified but was likely to be via the drinking water or diet to allow for this period of exposure.

(2) Steviol

Steviol (99% purity) produced positive increases in chromosome aberrations in Chinese hamster lung (CHL) cells *in vitro,* but only in tests conducted with addition of a rat-liver S9 homogenate metabolic activation system (Matsui et al. 1996). However, these authors did not find significant increases in chromosome damage from steviol tested *in vivo* in the mouse micronucleus test with levels from 125 to 1000 mg/kg bw. The same authors observed significant increases related to the amount of steviol administered in mutant CHL cells resistant to diphtheria toxin in tests conducted with S9 activation (-S9 not tested), with an increase of approximately 3-fold over control values at the highest level of 400 μg/mL. Stevioside (83% purity), tested in these same test systems, did not produce increases in mutations or chromosome damage (Matsui et al. 1996).

Oh et al. (1999) did not find any increases in the numbers of mutations in the mouse lymphoma L5178Y gene mutation assay with levels of steviol (purity not stated) from 42.7 to 341 μg/mL tested both with and without a rat liver metabolic activation system. Similarly, they found no increases in chromosome damage relative to control values by quantifying incidence of micronuclei in the bone marrow or hepatocytes from groups of 5 ICR mice given a range of levels from 50 to 200 mg/kg. Suttajit et al. (1993) reported similar negative clastogenicity for

steviol in a summary of a limited test conducted with human lymphocytes. No positive effects were seen in 4 of 5 blood samples treated with 0.1 or 0.2 mg/mL "except in one case" [which they did not discuss further]. They indicated addition of S9 mix in the cultures "did not alter the result" [lack of clastogenicity as concluded in the report]. In another study showing lack of genotoxicity of steviol, Sekihashi et al. (2002) reported that oral levels of 250, 500, 1000 or 2000 mg/kg steviol (purity not available Japanese report) given orally to mice did not produce increases in DNA damage as determined by the comet assay in cells from the stomach, colon, liver, kidney and testis.

Temcharoen et al. (2000) evaluated clastogenicity of steviol in the bone marrow micronucleus assay using 5 male and 5 female rats, mice and hamsters per each evaluation time interval. Rats and mice were given single oral levels of 8 g/kg b.w. and hamsters were given orally 4 g/kg b.w.; these single levels are above the limit level of 2 g/kg typically used in protocols as the maximum level for this test. The incidence of micronucleated polychromatic erythrocytes (MNPCEs) was quantified to determine clastogenic effects and the polychromatic (PCE) to normochromatic erythrocyte (NCE) ratio was determined to evaluate potential cytotoxicity to the bone marrow from steviol or its metabolites. In evaluations of these parameters in animals terminated at 0, 24, 30, 48 and 72 h after administration, no significant increases in numbers of MNPCEs were seen in any of the groups of males or females of the 3 species evaluated. Steviol was clearly not clastogenic in this well conducted study. A slight degree of cytotoxicity to the bone marrow indicative of effects from steviol or a steviol metabolite was evident by decreases in the PCE:NCE ratio of the bone marrow preparations from female rats, hamsters and mice at evaluation intervals of 48 and 72 h. This finding is typically used as evidence that the test substance was absorbed and that it, or its metabolites, were systemically available to the bone marrow target cells. This is an important parameter that supports the results of an *in vivo* clastogenicity test in cases when negative clastogenic results are obtained.

Since steviol has been reported to be genotoxic in a few bacterial and cellular *in vitro* tests in the presence of an Aroclor 1254-induced rat liver homogenate activation system it has been suggested that a steviol metabolite produced under *in vitro* conditions is the genotoxic component. However, there have been no findings of genotoxicity when steviol has been evaluated using *in vivo* test systems, including the micronucleus test in rats, mice and hamsters or using the comet assay to detect DNA damage in cells from multiple organs from mice treated orally with steviol levels up to a 2000 mg/kg "limit level" and higher. The applicability of the artificial metabolism conditions used for the *in vitro* tests for assessing dietary risks from steviol are problematic in view of the convincing *in vivo* studies that showed no genotoxic effects.

Studies evaluating the genotoxicity of steviol glycosides are summarized in Table 37.

Table 37. Studies of genotoxicity with purified stevia extract and its major components, stevioside and redaubioside A

End-point	Test system	Material	Purity (%)	Concentration or Amount Administered	Result	Reference
In vitro						
Reverse mutation	*S. typhimurium* TA98, TA100	Stevioside	99	50 mg/plate	Negative[a]	Suttajit et al. 1993
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, TA1537	Stevioside	83	5 mg/plate (without metabolic activation) 1 mg/plate (with metabolic activation)	Negative	Matsui et al. 1996
Forward mutation	*S. typhimurium* TM677	Stevioside	83	10 mg/plate	Negative[a]	Matsui et al. 1996
Forward mutation	*S. typhimurium* TM677	Stevioside	NS	10 mg/plate	Negative[a]	Pezzuto et al. 1985
Gene mutation (umu)	*S. typhimurium* TA1535/ pSK1002	Stevioside	83	5 mg/plate	Negative[a]	Matsui et al. 1996
Gene mutation	*S. typhimurium TA 98 and TA 100*	Stevioside	96%	2 mg to 50 mg/plate	Negative	Klongpanichpak et al. 1997
Gene mutation	B. subtilis H17 *rec* , M45 *rec*$^-$	Stevioside	83	10 mg/disk	Negative[a]	Matsui et al. 1996
Gene mutation	Mouse lymphoma L5178Y cells, *TK* $^-$ locus	Stevioside	NS	5 mg/m	Negative[a,b]	Oh et al. 1999
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	83	8 mg/m[e] 12 mg/mL[f]	Negative	Matsui et al. 1996
Chromosomal aberration	Human lymphocytes	Stevioside	NS	10 mg/mL	Negative	Suttajit et al. 1993
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	85	12 mg/mL	Negative	Ishidate et al. 1984
Chromosomal aberrations	CHL/IU Chinese hamster lung fibroblasts	Rebaudioside A	NS	1.2-55 mg/mL	Negative[a]	Nakajima et al. 2000a
Reverse mutation	*S. typhimurium* TA98 and TA100	Steviol	NS	20 mg/plate	Negative[a]	Suttajit et al. 1993
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, TA1537	Steviol	99	5 mg/plate	Negative[a]	Matsui et al. 1996
Forward mutation	*S. typhimurium* TM677	Steviol	NS	10 mg/plate[f] 0.5-10 mg/plate[g]	Negative Positive	Matsui et al. 1996

000114

000115

Table 37. Studies of genotoxicity with purified stevia extract and its major components, stevioside and redaubioside A

End-point	Test system	Material	Purity (%)	Concentration or Amount Administered	Result	Reference
Forward mutation	*S. typhimurium* TM677	Steviol	NS	10 mg/plate[f] 10 mg/plate[f]	Negative	Pezzuto et al. 1985
Forward mutation	*S. typhimurium* TM677	Steviol	NS	NS	Positive[g]	Terai et al. 2002
Forward mutation	*S. typhimurium* TM677	Steviol-16, 17-epoxide	NS	NS	Positive[g]	Terai et al. 2002
Forward mutation	*S. typhimurium* TM677	15α-hydroxysteviol	NS	NS	Negative	Terai et al. 2002
Forward mutation	*S. typhimurium* TM677	15-oxo-steviol	NS	NS	Positive[g]	Terai et al. 2002
Forward mutation	*S. typhimurium* TM677	Steviol methylester	NS	NS	Positive[g]	Terai et al. 2002
Forward mutation	*S. typhimurium* TM677	16-oxo-steviol methylester	NS	NS	Negative[a]	Terai et al. 2002
Forward mutation	*S. typhimurium* TM677	13,16-seco-13-oxo-steviol methylester	NS	NS	Positive[g]	Terai et al. 2002
Forward mutation	*S. typhimurium* TM677	13,16-seco-13α-hydroxy-steviol methylester	NS	NS	Negative[a]	Terai et al. 2002
Forward mutation	*S. typhimurium* TM677	Steviol methylester 8,13-lactone	NS	NS	Positive (the presence of metabolic activation decreased mutagenicity)	Terai et al. 2002
Forward mutation	*S. typhimurium* TM677	15-Oxosteviol	NS	0.025-0.2 mg/mL	Positive in the absence of metabolic activation	Compadre et al. 1987
Forward mutation	*S. typhimurium* TM677	15α-hydroxyteviol	NS	00.31-7.5 mg/mL	Negative in the presence of metabolic activation	Compadre et al. 1987

Table 37. Studies of genotoxicity with purified stevia extract and its major components, stevioside and redaubioside A

End-point	Test system	Material	Purity (%)	Concentration or Amount Administered	Result	Reference
Forward mutation	*S. typhimurium* TM677	Ent-Kaurenoic acid; Steviol; Steviol acetate; steviolbioside, stevioside; Rebaudioside B; Rebaudioside A; Rebaudioside C	NS	Various between 0.31 to 15 mg/mL depending on level of toxicity for the individual compounds	Negative with metabolic activation	Pezzuto et al. 1986
Forward mutation	*S. typhimurium* TM677	19-O-β-D-glucopyranosyl steviol	NS	1.6-15 mg/mL	Positive with metabolic activation	Pezzuto et al. 1986
Gene mutation (umu)	*S. typhimurium* TA1535/pSK 1002	Steviol	99	625-1250μg/plate[f] 1259-2500μg/plate[g]	Positive Positive	Matsui et al. 1996
Gene mutation	*B. subtilis* H17 *rec* , M45 *rec*$^-$	Steviol	99	10 mg/disk	Negative[a]	Matsui et al. 1996
Gene mutation	Chinese hamster lung	Steviol	99	400μg/mL[g]	Positive	Matsui et al. 1996
Gene mutation	Mouse lymphoma L5178Y cells, *TK* $^-$ locus	Steviol	NS	340μg/mL	Negative[a,b]	Oh et al. 1999
Gene mutation	*S. typhimurium* TM677	15-oxosteviol	NS	0.07 – 96 mg/mL	Negative	Procinska et al. 1991
Chromosomal aberration	Chinese hamster lung fibroblasts	Steviol	NS	0.5g/mL[g] 1-1.5mg/mL[g]	Negative Positive	Matsui et al. 1996
Chromosomal aberration	Human lymphocytes	Steviol	NS	0.2mg/mL	Negative	Suttajit et al. 1993
DNA damage (comet assay)	TK6 and WTK1 cells	Steviol	NS	62.5-500μg/mL	Negative[a]	Sekihashi et al. 2002
In vivo						
Mutation	D. melanogaster Muller 5 strain	Stevioside	NS	2% in feed	Negative[b]	Kerr et al. 1983
DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver	Stevia extract	Stevioside, 52; rebaudioside A, 22	250-2000 mg/kg	Negative[c]	Sekihashi et al. 2002

000116

000117

Table 37. Studies of genotoxicity with purified stevia extract and its major components, stevioside and redaubioside A

End-point	Test system	Material	Purity (%)	Concentration or Amount Administered	Result	Reference
DNA damage (comet assay)	Male ddY mouse stomach, colon, liver, kidney, bladder, lung, brain, bone marrow	Stevia	NS	2000 mg/kg	Negative[c]	Sasaki et al. 2002
DNA damage (comet assay)	Male Wistar rats, liver, red blood cells, brain and spleen	Stevioside	88.62%	4 mg/mL for 45 days	Positive	Nunes et al. 2007
Micronucleus formation	ddY mouse bone marrow and regenerating liver	Stevioside	NS	62.5-250 mg/kg	Negative[b]	Oh et al. 1999
Micronucleus formation	BDF1 mouse bone marrow	Rebaudioside A	NS	500-2000 mg/kg bw /d for 2 days	Negative[d]	Nakajima et al. 2000b, cited in WHO 2006
DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver; male CRJ: CD1 mouse liver kidney, colon and testes	Steviol	>99	250-2000mg/kg	Negative	Sekihashi et al. 2002
Micronucleous formation	MS/Ae mice	Steviol	99	1000mg/kg bw	Negative	Matsui et al. 1996
Micronucleous formation	Swiss mouse bone	Steviol	About 90	8000mg/kg	Negative[h]	Temcharoen et al. 2000
Micronucleous formation	Wistar rat bone marrow	Steviol	About 90	8000mg/kg	Negative[h]	Temcharoen et al. 2000
Micronucleous formation	Syrian golden hamster bone marrow	Steviol	About 90	4000mg/kg	Negative[h]	Temcharoen et al. 2000
Micronucleous formation	ddY Mouse regenerating liver	Steviol	NS	50-200mg/kg	Negative	Oh et al. 1999
Dominant Lethal Assay	Norwegian Rats *Rattus norvergicus*	Aqueous extract of stevia leaves	NS (crude extract)	2 mL/day oral	Negative	Aritajat et al. 2000

Table 37. Studies of genotoxicity with purified stevia extract and its major components, stevioside and redaubioside A

End-point	Test system	Material	Purity (%)	Concentration or Amount Administered	Result	Reference

Source: Adapted from WHO 2006
NS, not specified
[a] With or without metabolic activation (source not specified in original monograph).
[b] Inadequate detail available.
[c] Killed 3hour and 24hour.
[d] Killed 30h after second administration
[e] The presence of metabolic activation decreased the mutagenicity.
[f] Without metabolic activation.
[g] With metabolic activation.
[h] Without metabolic activation.

E. Physiological Effects

1. Introduction

Stevia extracts have been used for many years as high-intensity sweeteners in several countries; the extracts contain steviodside and rebaudioside A which differ by one additional glucose moiety on rebaudioside A. Both are metabolized to steviol in the gastrointestinal tract (JECFA, 2005). Because of the similar metabolic fate of steviol glycosides, it is expected that these components would not only be toxicologically equivalent, but would be expected to produce similar physiological effects. Leaves of *S. rebaudiana* (Bertoni) Bertoni have been used as a traditional medicine in South America for treatment of diabetes (Ferri et al. 2006). In addition, some animal and human studies of steviol glycosides have reported hypotensive and hypoglycemic effects. Several human studies have suggested that stevioside may offer therapeutic benefits for subjects with hypertension and type 2 diabetes (Carakostas et al. 2008; Chan et al. 2000; Gregersen et al. 2004). The results of these studies investigating potential beneficial effects in diabetic subjects after short term usage raised questions about untoward effects in subjects with normal blood pressure or with safety concerns for subjects with diabetes after long term repeated exposure to steviol glycosides. In 2004, JECFA noted that additional scientific data should be developed to evaluate the question of whether there were physiological effects of the glycosides that could lead to adverse effects on blood pressure and glucose homeostasis in individuals with hypertension or with type-2 diabetes. Since that time, additional studies have been conducted and published that resolve these issues.

Steviol glycosides have been reported in some studies to produce antihypertensive and hypoglycemic effects in animals and humans (Melis and Sainati, 1991a, Melis and Sainati, 1991b, Melis, 1992a, Melis, 1992b, Chan et al., 1998, 2000, Hsieh et al., 2003, Jeppesen et al., 2003, Liu et al., 2003; Dyrskog et al. 2005a,b). More recent human trials of normotensive and hypertensive individuals, as well as individuals with type 2 diabetes, do not provide evidence to support a finding that any adverse effects on glucose homeostasis or blood pressure would be expected with ingestion of steviol glycosides as a sweetener and resolves the question of safety of repeat quantity consumption of steviol glycosides in these subpopulations (Ferri et al., 2006; Jeppesen et al., 2006; Geuns et al. 2007; Maki et al. 2008 a,b).

Investigations into the effects of steviol glycosides on glucose homeostasis have been conducted with stevioside, steviol and rebaudioside A. Animal and *in vitro* studies suggest that steviol glycosides may have a hypoglycemic effect through enhanced secretion of insulin from the pancreas when there is impaired response to glucose stimulation. Increased insulin secretion, resulting from decreased insulin sensitivity, is a hallmark response in type 2 diabetes. Stevioside

and the aglycone steviol stimulated glucose-dependent insulin secretion from islets in an *in vitro* mouse model (Jeppesen et al. 2000). Animal studies of normal or diabetic rats given steviol glycosides were conducted using intravenous, intraperitoneal or oral administration; studies with dietary or gavage administration ranged from single administration to 9 weeks. Results from acute studies show decreases in blood plasma glucose and an enhanced insulin response. In a 6-week study of stevioside ingestion by diabetic GK rats, an intra-arterial catheter was inserted and an arterial glucose tolerance test was conducted at week 6. Stevioside enhanced first phase insulin response and concomitantly suppressed glucagon levels (Jeppesen et al. 2003). In this study, bolus injections of stevioside in the fasting state did not induce hypoglycemia in normal or diabetic GK rats. These data from an animal model support the view that stevioside ingestion would not produce an adverse hypoglycemic event in human diabetic subjects. In studies of human subjects, hypoglycemic effects were observed in the two short studies (bolus amount and 3 days of treatment. In the first study Curi et al. (1986) administered aqueous extract of 5g of stevia leaves to volunteers at regular 6-h intervals for 3 days. The result of this study demonstrated increased glucose tolerance and decreased plasma glucose levels during the test and after overnight fasting in subjects. In the second study, Gregersen et al. (2004) demonstrated the acute effects of stevioside in type 2 diabetic patients. The results showed that stevioside supplementation caused a reduction in postprandial blood glucose levels indicating beneficial effects on the glucose metabolism. In studies ranging from 3 montyhs to 2 years showed no effects of stevioside on glucose or insulin levels in individuals with mild hypertension or hyperlipidemia (WHO 2006; Ferri et al. 2006; Chan et al. 2000; Hsieh et al. 2003). In a three-month study of subjects with type 2 diabetes, fasting blood glucose and glycosylated hemoglobin (HbA1c) were not significantly lowered by intake of 1500 mg/d of stevioside compared with placebo (Jeppesen et al. 2006). Additionally, the incremental area under the glucose concentration curve following test meal administration at the end of the treatment period was also unaltered relative to placebo. In another recent study measuring HbA1c, the standard accepted measure of chronic glycemic control, 16 weeks of consumption of rebaudioside A did not produce significant changes in this endpoint in subjects with type 2 diabetes compared with placebo administration (Maki et al. 2008a). Rebaudioside A was well-tolerated, and records of hypoglycemic episodes showed no excess vs. placebo. Thus, the weight of the evidence from human studies evaluating glucose homeostatis supports the safety of chronic use of steviol glycosides.

Investigations to address the effects of steviol glycosides on blood pressure have been done primarily with stevioside; studies to address the effects of rebaudioside A on blood pressure lowering are limited (Carakostas et al. 2008). Animal and *in vitro* studies investigating the antagonistic effects of stevioside on blood pressure have shown that in studies designed to look

at mechanism, this effect may be produced through vasorelaxation likely mediated through inhibition of calcium influx into the blood vessels. Hypertensive rat models, using primarily intraperitoneal and intravenous administration of stevia extracts, have demonstrated reductions in blood pressure with stevioside administration. Studies of ingestion of steviol glycosides in human studies provide the most relevant information from which to evaluate the safety of steviol glycoside ingestion from its use as a sweetener. In particular, safety must be addressed from the perspective of possible untoward effects on blood pressure in sensitive subpopulations. Results from two longterm human trials (1-2 years) in China studying men and women with mild to moderate essential hypertension have suggested antihypertensive effects of stevioside (purity undefined) at intakes of 750 and 1500 mg/day (approximately 300 and 600 mg steviol equivalents/day) (Chan et al., 2000, Hsieh et al., 2003). However, other studies have not shown measurable effects of steviol glycosides on blood pressure in humans. Geuns and colleagues (2007) reported that administration of stevioside (750 mg/day (approximately 300 mg steviol equivalents/day) for three days) failed to significantly alter blood pressure in nine subjects with normal blood pressure. Ferri and colleagues (2006) also reported a lack of any antihypertensive effect of stevioside in patients with untreated, mild hypertension following six weeks of stevioside administration at dosages up to 15.0 mg/kg/day. In a study designed to investigate the effects of chronic consumption of rebaudioside A on glucose homeostasis in subjects with type 2 diabetes, 1000 mg/day of rebaudioside A (approximately 330 mg steviol equivalents/day) for 16 weeks did not significantly alter blood pressure (Maki et al. 2008a). In a separate trial of men and women with type 2 diabetes, more than half of whom were taking antihypertensive medications, consumption of as much as 1000 mg/day of rebaudioside A (approximately 330 mg steviol equivalents/day) produced no clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure (Maki 2008b).

In the study by Chan et al. (2000) and Hsieh et al. (2003) the subjects had higher initial baseline blood pressures as they were mild to moderately hypertensive to start with, compared to normal or normal/hypotensive subjects in the other studies (Geuns et al. 2007; Maki et al. 2008a,b). This may explain why reductions were observed in studies of subjects with higher blood pressures compared to those with normal blood pressure or mild hypertension (FSANZ 2008).

- Although the database is extensive many of the key studies in humans which investigated the effects of stevioside on blood glucose and blood pressure did not report the purity of stevioside or mixtures of stevioside/rebaudioside (Chan et al 2000, Hseih et al 2003, Ferri et al 2006, Barriocanal et al. 2008). Thus, in these studies, it is unknown if the purity of the steviol glycoside extract would be representative of the expected specifications of steviol glycoside extract that humans would be exposed to in the diet. In addition, as only

one dose level was used in the pivotal human studies (Gregersen et al 2004, Temme et al 2004, Chan et al 2000, Hseih et al 2003), it precluded the establishment of a NOAEL on which an ADI could be established. Therefore, it is more appropriate to use the chronic animal study data to establish an ADI, and this was the approach recently taken by FSANZ and JECFA to establish an ADI for steviol glycosides.

2. Effect of Stevioside on Blood Glucose and Insulin levels

a) Studies in Animals:

(1) Introduction

Numerous studies have been conducted in normal and diabetic animal models to study the effects of steviol glycosides on blood glucose and insulin levels, measures of insulin resistance and glucose tolerance. Summaries of the pivotal studies are presented in Table 38. As shown in Table 38, stevioside was the most frequently used test article, though several investigators also examined the effects of steviol and rebaudioside A. *In vitro* work suggests that steviol glycosides may have a hypoglycemic effect through enhanced secretion of insulin from the pancreas. Results from some acute studies show decreases in blood plasma glucose and an enhanced insulin response in both healthy and diabetic animals (Jeppesen et al. 2003; Chen et al. 2005), but not all studies showed this effect (Dyrskog et al. 2005a,b). In other studies, effects were seen only with a subset of the quantities, test articles, or routes of administration (Ferreira et al. 2006a; Jeppesen et al. 2003; Lailerd et al. 2004; Suanarumsawat and Chaiyabutr 1997; Hubler et al., 1994). A study using bolus injections of stevioside in the fasting state demonstrated no induction of hypoglycemia in normal or diabetic GK rats.

(2) Review of studies

The influence of stevioside on the glycogen levels of fasted rats was demonstrated by Hubler et al. (1994). In one set of experiments, single amounts of stevioside (200 μmol) or steviol (200 μmol) were given orally to 24-h fasted rats, either alone or simultaneously with fructose. The authors reported increased glycogen deposition in the liver of rats. In another set of experiments, stevioside was given to the rats in the drinking water at the beginning of the fasting periods of 24 and 48 h. Increased hepatic glycogen levels were found at 48-h with stevioside (1.0 mM) and at 24-h with stevioside (2.0 mM). Steviol had no effect on hepatic glycogen levels when given in the drinking water. Therefore, it was concluded that stevioside exerts a stimulatory action on hepatic glycogen synthesis under gluconeogenic conditions.

Toskulkao et al. (1995) demonstrated the effects of stevioside and steviol on intestinal glucose absorption in hamster jejunum. By using the everted sac technique, the authors found that stevioside (1 and 5 mM) had no inhibitory effect on glucose absorption. In contrast, glucose absorption was inhibited 29% by 1 mM steviol. The inhibition of glucose absorption by steviol was related to steviol concentration and incubation time. It was found that reductions in the intestinal mucosal ATP content and absorptive surface area were responsible for the inhibition of glucose absorption by steviol. In addition, the decrease in the intestinal mucosal ATP content was accompanied by a decrease in the activities of mitochondrial NADH cytochrome c reductase and cytochrome oxidase. Moreover, no inhibitory effects of steviol on the activity of intestinal Na+,K(+)-ATPase and glucose uptake in the intestinal brush-border membrane vesicles were observed in the study. The results of the study suggested that inhibition of intestinal glucose absorption by steviol in isolated hamster jejunum is due to the reduction in mucosal ATP content and an alteration of the morphology of the intestinal absorptive cells. The results of this *in vitro* system cannot, however, be extrapolated to the *in vivo* situation, since steviol is produced in the colon by microflora activity on the steviol glycosides. The jejunum would not be exposed to steviol.

Suanarunsawat and Chaiyabutr (1997) demonstrated the effect of stevioside on glucose metabolism. Wister rats were treated with stevioside either by intravenous infusion or feeding at different quantity levels. The results of their study showed a significant increase in the plasma glucose levels both during and after stevioside infusion, whereas the plasma glucose levels were not affected by feeding stevioside at a level of 13.3 mL/kg bw. The glucose turnover rate (GTR) of radiolabelled glucose was not significantly different between control and stevioside infused animals. The plasma insulin level did not change, whereas the plasma glucose level changed significantly during stevioside infusion. Animals treated with angiotensin II and arginine vasopressin showed no significant effect, while animals treated with prazosin (α1-adrenergic antagonist) had an attenuated hyperglycemic effect of stevioside infusion. In addition, pretreatment with indomethacin or N-nitro-L- arginine methyl ester (L-NAME; 1 mg/mL) alleviated the plasma glucose level during the second period of stevioside infusion. Pretreatment with the combination infusion of indomethcin and L-NAME reduced the plasma glucose level significantly and normalized the plasma glucose level in the second period of stevioside infusion. In addition, insulin infusion inhibited the hyperglycemic effect of stevioside infusion. The results of the study showed that the elevation of the plasma glucose level during stevioside infusion is not due to the reduction of the insulin level, but rather a possible effect of stevioside on glucose transport across the cell. Also, insulin response to high plasma glucose is suppressed during stevioside infusion.

Malaisse et al. (1998) investigated the effects of both bitter and non-bitter artificial sweeteners on insulin release and cationic fluxes in isolated rat pancreatic islets. It was shown that sodium saccharin, sodium cyclamate, stevioside and acesulfame-K, all of which display a bitter taste, augmented insulin release from islet cells incubated in the presence of D-glucose. In contrast, aspartame, which is devoid of bitter taste, failed to affect insulin secretion. It was proposed that the insulinotropic action of some artificial sweeteners and, possibly, that of selected hexose pentaacetate esters, may require G-protein-coupled receptors similar to those operative in the recognition of bitter compounds by taste buds.

In earlier studies, Jeppesen et al. (1996, 2000) demonstrated a direct insulinotropic effect in isolated mouse islets and the clonal beta cell line INS-1 of stevioside. Later they reported that stevioside *in vivo* exerts an antihyperglycaemic effect in a nonobese animal model of type 2 diabetes (Jeppesen 2002). This study was conducted in type 2 diabetic Goto-Kakizaki (GK) and normoglycemic Wistar rats. Rats were anaesthetized and administered stevioside (0.2 g/kg bw) and D-glucose (2.0 g/kg bw) as i.v. bolus injections. The results of the study exhibited significant suppression of the glucose response to the glucose tolerance test in the GK rats and a concomitant increase in the insulin response between the stevioside-treated and control rats. Interestingly, the authors observed a suppressed glucagon level by stevioside during the glucose tolerance test between both the groups. In the normal Wister rats, stevioside enhanced insulin levels above basal during the glucose tolerance test. In conclusion, stevioside exerted antihyperglycaemic, insulinotropic, and glucagonostatic actions in the type 2 diabetic GK rat. The authors proposed that stevioside may be effective as a novel antidiabetic drug for use in type 2 diabetes.

In another study by the same author, antihyperglycaemic and blood pressure-lowering effects were examined in a long- term study in the type 2 diabetic GK rats (Jeppessen 2002). Rats were fed 0.025 g/kg/d of stevioside for 6 weeks. An intra-arterial catheter was inserted into the rats after 5 weeks, and conscious rats were subjected to arterial glucose tolerance test (2.0 g/kg) during week 6. Stevioside had an antihyperglycaemic effect in rats when compared to the control group. It also enhanced the first phase insulin response and concomitantly suppressed the glucagons levels. It was concluded that stevioside acts as an antihyperglycaemic compound and has a therapeutic potential in the treatment of type 2 diabetes and the metabolic syndrome.

Raskovic et al. (2004a) reported in an abstract on the effect of two commercial products (purity not stated) of *S. rebaudiana* (Bertoni) Bertoni, one from Stevita Co, Arlington ,TX and the other from Clear Steviosides Liquid, Herbal Supplement, Brazil and sodium monoketocholate (purity not stated) on blood glucose concentration in mice (sex and strain not reported). One group of animals was treated subcutaneously (s.c.) for four days with 4 mg/kg

000124
SPHERIX

mkc; the second group was administered 200 mg/kg of the Texas-source of steviol glycosides intraperitoneally (i.p.); the third group received a Clear Stevioside Liquid from Brazil at 20 mg/kg, i.p.; the fourth group was administered a combination of steviol glycosides (Texas) and mkc; and the fifth group with steviol glycosides (Brazil) and sodium monoketocholate. Blood glucose concentration was measured before treatment, after the first and fourth administration, as well as after subjecting animals to a glucose-tolerance test (500 mg/kg, p.o.) or provoking glycemia by injecting adrenaline (0.2 mg/kg, s.c.). It was reported that administration of one of steviol glycosides from either source combined with sodium monoketocholate caused a significant increase in glycemia compared to mkc alone. As for the interaction of mkc and steviol glycosides, it was reported that the combined pretreatment yielded lower values of glycemia compared with that measured after treatment with steviol glycosides alone

Another abstract by the same author (Raskovic et al. (2004b) reported the effect of the same two commercial products (purity not given) on blood glucose concentration in mice (sex and strain not given). One group of mice was pretreated for four days with 200 mg/kg steviol glycosides (Stevita Co, Arlington, TX) and the other with 20 mg/kg of clear steviol glycoside liquid (Stevita Co, Herbal Supplement, Brazil), whereas the animals in the control group received physiological saline. Blood glucose concentration was measured before pretreatment and four days later. Changes in the glucose level were provoked by a glucose-tolerance test (500 mg/kg, p.o.) and subcutaneous injection of adrenaline (0.2 mg/kg). The same procedure of measuring blood glucose was applied on mice with alloxan-induced diabetes mellitus (two administrations of 100 mg/kg with a 24-h interval). Blood glucose levels in mice pretreated with either commercial product were lower compared with the control group for animals provoked by a glucose tolerance test or by adrenaline. Pretreatment with the Brazilian material was reported to be more effective. Pretreatment with both source materials protected test animals from the toxic action of alloxan.

Lailerd et al. (2004) determined the effect of stevioside treatment on skeletal muscle glucose transport activity in both insulin-sensitive lean and insulin-resistant obese Zucker rats. Stevioside was administered at a level of 500 mg/kg bw 2 h before an oral glucose tolerance test. Whereas the glucose incremental area under the curve was not affected by stevioside in lean rats, the insulin incremental area under the curve and the glucose-insulin index were decreased significantly. The basal glucose transport in epitrochlearis muscles was not altered by stevioside. Acute oral stevioside increased whole-body insulin sensitivity, while low concentrations of stevioside modestly improved *in vitro* insulin action on skeletal muscle glucose transport in both lean and obese rats.

Abudula et al. (2004) demonstrated the influence of rebaudioside A on insulin release from mouse islets. The results of their study showed rebaudioside A (10^{-16} to 10^{-6} mol/L) dependently stimulated insulin secretion in the presence of 16.7 mmol/L glucose. In addition, rebaudioside A stimulated insulin secretion in a glucose dependent manner and potentiated insulin secretion at glucose >6.6 mmol/L. The effect of rebaudioside was critically dependent on the presence of extracellular calcium.

Chen et al. (2005) studied the effects of stevioside (99% purity; 0.5, 1.0, 5.0 mg/kg given by gastrogavage twice daily) on glucose and insulin metabolism in 2 rat models of diabetes: STZ-induced diabetic rats and rats with type 2 diabetes induced by feeding with fructose. Stevioside (0.5 mg/kg) lowered blood glucose levels in both STZ-induced diabetic rats and in the fructose induced model as early as 60 minutes post treatment. Stevioside also demonstrated a quantity-dependent effect in lowering glucose levels in both diabetic rat models. Stevioside reduced the rise in glucose during glucose tolerance testing in normal rats. In another experiment, stevioside quantity-dependently decreased protein levels of phosphoenol pyruvate carboxykinase (PEPCK) and PEPCK mRNA after 15 days of treatment twice daily with 0.5, 1.0, 5.0 mg/kg by gastrogavage. In addition, stevioside also reduced insulin resistance in the diabetic animals as shown by the glucose lowering effects of tolbutamide. It was concluded by the authors that stevioside regulated blood glucose levels by enhancing not only insulin secretion, but also insulin utilization in insulin-deficient rats; the latter was associated with decreased PEPCK gene expression in rat liver by slowing down gluconeogenesis.

Chang et al. (2005) showed that stevioside (purity not given), when administered as a single oral administration of 5.0 mg/kg for 90 minutes, decreased plasma glucose concentrations in a quantity-dependent manner in rats receiving fructose-rich chow for four weeks. In addition, stevioside also reversed the value of the glucose-insulin index, indicating that stevioside has the ability to improve insulin sensitivity in the proposed insulin-resistant animal model. Tolbutamide was also utilized in their experiment as an indicator of insulin-resistance development. Compared to control, stevioside treatment markedly delayed the abatement of the plasma glucose lowering effects of tolbutamide (10 mg/kg; i.p.) The authors concluded that oral administration of stevioside improves insulin sensitivity, and seems suitable as a potential adjuvant for diabetic patients and/or those that consume large amounts of fructose.

Dyrskog et al. (2005a) investigated whether the combination of stevioside (91% stevioside, 4% rebaudioside A, and 5% other glycosides) and a dietary supplement of soy protein possess beneficial qualities in the treatment of type 2 diabetes and the metabolic syndrome in Zucker diabetic fatty rats. Male Zucker diabetic fatty rats were divided into 4 groups and fed different test diets for 10 weeks: Standard carbohydrate-rich laboratory diet (chow),

chow+stevioside (0.03 g/kg bw/d), 50% soy (Abalon)+50% chow (adjusted for vitamins and minerals), and 50% soy (Abalon)+50% chow+stevioside 0.03 g/kg bw/d. Plasma glucose, blood pressure, weight, and feed intake was measured once weekly. The animals were equipped with an intra-arterial catheter, and at week 10, conscious rats underwent an intra-arterial glucose tolerance test. Chow plus stevioside treatment exhibited a blood glucose lowering effect of 19% as compared to chow alone. In addition, treatment with the 50% soy +50% chow+ stevioside diet exhibited a blood glucose lowering effect of 12% as compared to the 50% soy +50% chow diet in rats. No effects on insulin or glucagon responses were detected.

One other study by the same group (Dyrskog et al. 2005b) evaluated if longterm administration of rebaudioside A could improve glycemic control and lower blood pressure in an animal model of type 2 diabetes. Male Zucker diabetic fatty rats were divided into 2 groups and fed a standard chow diet for 8 weeks. The diet was supplemented with oral rebaudioside A (0.025 g/kg bw/d) in the experimental group. Blood glucose, weight, blood pressure and food intake was measured weekly. Animals were equipped with an intra-arterial catheter, and at week eight the conscious rats underwent an intra-arterial glucose tolerance test (IAGTT). During the IAGTT, the level of glucose, glucagon, and insulin response did not differ significantly between the two groups. Fasting levels of glucose, glucagon, insulin or levels of blood lipids did not differ between the groups throughout the study period. The authors concluded that oral supplementation with rebaudioside A (0.025 g/kg bw/d) for eight weeks had no effect on blood pressure or glycemic control in GK rats.

Ferreira et al. (2006a) compared the effects of oral treatment of steviol glycosides on glycaemia and gluconeogenesis in 15-h fasted rats. Rats received *S. rebaudiana* (Bertoni) Bertoni dried powdered leaves (20 mg/kg/day) or stevioside produced from the leaves (purity not given) (5.5 mg/kg/day), or an equal amount of water (control) for 15 days. The results of the study demonstrated decreased glycemia and gluconeogenesis after pre-treatment with the powdered leaves. However, the treatment with stevioside did not influence either glycemia or gluconeogenesis.

A recent study by Jeppesen et al. (2006) reported the beneficial effect of stevioside in combination with a soy-based dietary supplement against type 2 diabetes in diabetic Goto-kakizaki rats. Over the course of 4 weeks, rats were fed one of the following four test diets: (1) standard carbohydrate- rich laboratory diet (chow), (2) stevioside in combination with chow, (3) soy protein isolate (SPI) plus chow and (4) SPI plus chow plus stevioside. Compared to normal chow diet, stevioside in combination with SPI demonstrated a significant 56% reduction in plasma glucose, a 118% increase in first-phase insulin levels, a 20% reduction in glucagon, a 28% reduction in total cholesterol levels, a 13% reduction in FFA (free fatty acids), a 49%

reduction in TG (triglycerides) and a 11% reduction in the systolic pressure. It was concluded by the authors that the combination of stevioside and SPI has synergistic positive effects on hyperglycemia, hypertension and dyslipidemia.

(3) In Vitro Studies:

Ishii et al. (1987) evaluated the influence of stevioside and two of its derivatives on the transport and metabolism of D-glucose and D-fructose in the isolated perfused rat liver. Stevioside and its derivatives inhibited D-glucose and D-fructose transport across the cell membrane. The results of the study indicated isosteviol to be more potent than stevioside, whereas steviolbioside was less active. Stevioside did not exhibit any effect on D-glucose metabolism, except for transient changes in D-glucose release, reflecting changes in the intracellular concentration in liver cells. D-Fructose consumption affected D-glucose production, L-lactate and pyruvate production, and extra oxygen uptake. In addition, livers releasing D-glucose from endogenous glycogen increased the intracellular to extracellular D-glucose concentration ratio (Ci/Ce).

It was further suggested that potential mechanisms for the hypoglycemic effects may be due to increased insulin production in beta-cells (Jeppsen et al. 2000, 2003) or increased glucose transport into muscle (Lailerd et al. 2004). The antihypertensive effects of stevioside have been attributed to prostaglandin activity (Melis and Sainati 1985 as cited in Chan et al. 1998), a calcium antagonistic mechanism that is similar to that of verapamil (Melis and Sainnati 1991 as cited by Chan et al. 1998), and inhibition of calcium influx in smooth muscle cells (Lee et al. 2001).

Chen et al. (2006) demonstrated the beneficial effects of stevioside in preventing glucotoxic effects by regulating acetyl –CoA carboxylase (ACC) activity. They also confirmed that ACC plays a pivotal role in glucose-stimulated insulin secretion (GSIS). During lipotoxicity, stevioside did not alter ACC protein levels, phosphorylated-ACC or glucose uptake during lipotoxicity induced by palmitate. The results showed that SVS counteracts the impaired insulin secretion during lipotoxicity in rat islets as well as in INS-1E cells without affecting ACC activity.

Several recent studies support the view that the acetyl-CoA carboxylase (ACC) plays a pivotal role for glucose-stimulated insulin secretion (GSIS). Chen et al. (2007) have recently shown that stevioside enhances insulin secretion and ACC gene expression. Isolated mouse islets as well as clonal INS-1E beta-cells were exposed for 48 h to 27 or 16.7 mM glucose, respectively. It was found that 48-h exposure to high glucose impairs GSIS from mouse islets

and INS-1E cells, an effect that is partly counteracted by stevioside. In addition, ACC dephosphorylation inhibitor okadaic acid, and 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside, an activator of 5'-AMP protein kinase that phosphorylates ACC, eliminated the beneficial effect of stevioside. Also, 5-tetrade-cyloxy-2-furancarboxylic acid, the specific ACC inhibitor, blocked the effect of stevioside as well. During glucotoxity, ACC gene expression, ACC protein, and phosphorylated ACC protein were increased in INS-1E beta-cells. Stevioside pretreatment further increased ACC gene expression with strikingly elevated ACC activity and increased glucose uptake accompanied by enhanced GSIS. The authors concluded that glucose is a potent stimulator of ACC and that stevioside, to some extent, counteracts glucotoxicity via increased ACC activity.

In one of their recent studies, Abudula et al. (2008) reported a cellular signaling mechanism of rebaudioside A in an *in vitro* system. Isolated mouse islets were used in the radiolabelled cAMP (cyclic AMP) scintillation proximity assay to measure total cAMP level, and in a luminometric method to measure intracellular ATP and ADP concentrations. The results of their study demonstrated the insulinotropic effect of rebaudioside A is mediated via inhibition of ATP-sensitive K^+channels and requires the presence of high glucose. The inhibition of ATP-sensitive K^+channels is probably induced by changes in the ATP/ADP ratio. They proposed a distinct therapeutic advantage of rebaudioside A over sulphonylureas because of less risk of causing hypoglycemia.

(4) Other related studies

Other *in vitro* studies have shown that at high concentrations (0.8-0.97 mg/mL), stevioside weakly inhibits oxidative phosphorylation (Kelmer-Bracht et al. 1985 and Vignais et al. 1966, both as cited in WHO 1999). At stevioside concentrations of 2 mg/mL, ketogenesis was inhibited by 66 percent (Constantin et al. 1991 as cited in WHO 1999), and at a concentration of 0.7 mM, stevioside affected renal tubular function through inhibition of *p*-aminohippurate (PAH) transepithelial transport (Jutabha et al. 2000). Additional potential effects of stevioside or steviol glycosides extracts include anti-inflammatory and immunomodulatory activities (Boonkaewwan et al. 2006) and bactericidal activity toward food-borne pathogenic bacteria (Tomita et al. 1997).

A summary of the pivotal results from studies investigating the physiological effects of stevioside (>95% pure), steviol, steviol glycosides extracts containing mixtures of stevioside and rebaudioside A, and unspecified extracts from *S. rebaudiana* (Bertoni) Bertoni from human, animal, and *in vitro* studies is presented in Table 38.

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
In vivo				
Chang et al. 2005	Evaluate the effect of stevioside in conjunction with a high fructose diet on insulin resistance.	M Wistar rats with induced IDDM (n not specified)	5.0 mg/kg stevioside 3x/d 4 weeks; oral 98.6% pure stevioside powder	Stevioside delayed time for loss of plasma glucose lowering response to tolbutamide in fructose-rich chow, indicating that repeated administration of stevioside delayed the development of insulin resistance.
	Stevioside induced insulin sensitivity in fructose –rich chow fed rats	STZ-diabetic rats (n not specified)	0.2 mg/kg stevioside, 3x/d 10 days, oral 98.6% pure stevioside powder	Stevioside increased the response to exogenous insulin.
Chan et al. 1998	To determine the effect of stevioside on blood pressure and plasma catacholamines in spontaneously hypertensive rats.	Spontaneously hypotensive rats; 8 rats/group; weighing	Intravenous (IV) injection of 50, 100, 200 mg/kg in conscious hypotensive rats Stevioside (purity >95% pure)	The hypotensive effect on both systolic and diastolic blood pressure was quantity dependent for intravenous levels of 50, 100 and 200 mg/kg in conscious SHR. Maximum reduction in systolic and diastolic blood pressure was 31.4 and 40.8 % respectively Hypotensive effect lasted for more than 60 min with a level of 200 mg/kg.

000130

0001131

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Chen et al. 2005	Study hypoglycemic effects of stevioside in streptozotocin (STZ)-induced diabetic rats (a model for IDDM) and rats with induced NIDDM based on blood glucose and insulin levels, measures of insulin resistance, glucose tolerance tests, and detection of protein levels of phosphoenol pyruvate caboxykinase.	M Wistar rats, 10 per group	0.5, 1.0, or 5.0 mg/kg, twice daily single administration or 15 d; by gavage 99% pure stevioside from extract of dried *S. rebaudiana* (Bertoni) Bertoni leaves	In normal fasting rats and STZ-induced diabetic rats, stevioside decreased blood glucose levels in a quantity-dependent manner. Stevioside increased insulin levels in the normal rats. In STZ-induced diabetic rats and diet-induced NIDDM rats given stevioside for 15 d, blood glucose levels decreased after 1 day of treatment and blood glucose levels continued to decrease in a quantity-dependent manner. In a glucose tolerance test given to normal rats following administration of stevioside or the control, plasma glucose responses were lower in rats given stevioside. Stevioside decreased PEPCK mRNA expression and PEPCK protein production in STZ-induced diabetic rats fed stevioside for 15 d. In NIDDM rats, stevioside reduced insulin resistance.
Chen et al. 2007	Role of stevioside in pancreatic beta-cell function during glucotoxicity.	Adult female NMRI mice, 20-25 g	Isolated mouse islets and clonal INS-1E beta-cells exposed for 48 h to 27 or 16.7 mM glucose Purity not specified	48-h exposure to high glucose impairs GSIS from mouse islets and INS-1E cells, this effect was counteracted by stevioside Glucose stimulates acetyl-CoA carboxylase activity *in vitro* and that stevioside to some extent counteracts glucotoxicity via increased ACC activity.

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Dyrskog et al. 2005a	Investigate if the combination of stevioside and a soy protein supplement possess beneficial qualities in the treatment of type 2 diabetes and metabolic syndrome.	M obese ZDF rats, 12 per group	0.03 g/kg bw/d stevia supplement 9 weeks, then anesthetized; in feed 91% stevioside, 4% rebaudioside A, 5% other glycosides (Stevitafarm, Maringa, Brazil)	The stevia supplement did not affect insulin or glucagon response.
Dyrskog et al. 2005b	Effect of rebaudioside A on glycemic control or blood pressure	24 eleven week old male Goto-Kakizaki (GK) rats (n=12 animals/group)	Divided into two groups (n=12 animals/group) which were fed a standard laboratory chow diet for eight weeks.after eight weeks treatment in the Goto-Kakizaki rat. 97.8% purity	Fasting levels of glucose, glucagon, insulin or levels of blood lipids did not differ between the groups throughout the study period. No effect on blood pressure or weight development.
Ferreira et al. 2006a	Compare effects of daily intake of *S. rebaudiana* (Bertoni) Bertoni leaves (SRB) and stevioside (STV) on glycemia and gluconeogenesis of rats fasted for 15 h. Liver perfusion experiments were completed, and isolated hepatocytes were incubated.	M Wistar rats (n not specified)	SRB leaves: 20 mg/kg/d powdered leaves in water STV: 5.5 mg/kg/d (purity not specified) in water Control: water only 15 d; by gavage Dried powdered leaves from Steviafarma (Maringa, Brazil); standardized stevioside obtained from a leaf extract containing a mixture of stevioside and rebaudioside	Fasting glycemia and hepatic gluconeogenesis were decreased after SRB leaf intake as compared to the control group; the stevioside treatment had no effects on glycemia or gluconeogenesis. Hypoglycemic effect of SRB was not mediated by peroxisome-proliferator-activated receptors (PPAR)-gamma activation.

000132

000133

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Hübler et al. 1994	Study the influence of stevioside on glycogen levels of fasted rats.	M Wistar rats fasted 24 h, 6 to 9 per group	1.0 or 2.0 mM aqueous stevioside or steviol, or water ad libitum for 24 or 48 h; oral (substances not characterized)	Hepatic glycogen was elevated above the control group levels only in the 2.0 mM stevioside group, while hepatic glycogen was elevated only in the 1.0 mM stevioside group. No effects on hepatic glycogen were seen in rats fed steviol in drinking water.
		M Wistar rats fasted 24 h (n not specified)	200 µmol fructose, stevioside, steviol, fructose + stevioside, or fructose + steviol (in 2 mL water) single administration; oral (test substances not characterized)	Glycogen production was higher in the fructose group compared to the control group, and glycogen production was further enhanced in rats fed fructose + stevioside or fructose + steviol.
Jeppesen et al. 2002	Determine the effects of stevioside on circulating glucose, insulin, and glucagon during an i.v. glucose tolerance test in anaesthetized diabetic and normal rats.	Goto-Kakazaki (GK) rats (type 2 diabetes), 6 per group Wistar rats, 12-14 per group	200 mg/kg bw stevioside with 2.0 g/kg bw glucose or 2.0 g/kg bw glucose only single administration; i.v. 95% pure stevioside (Sigma Chemical Co. Mo. USA)	In diabetic (GK) rats, stevioside reduced the glucose response, increased the insulin response, and suppressed glucagon levels. In normal, healthy (Wistar) rats, stevioside had no effect on glucose or glucagon response; stevioside did induce an increase in insulin levels in the healthy animals, but levels returned to control levels after 90 min.

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Jeppesen et al. 2003 (*In vivo*)	Determine if longterm administration of stevioside has antihyperglycemic and blood pressure lowering properties in rats with type 2 diabetes. Glycemic response data was collected from an intra-arterial glucose tolerance test in conscious rats. Blood pressure was measured weekly.	Goto-Kakazaki (GK) rats (type 2 diabetes), 10 per group	25 mg/kg/d stevioside in tap water or 16.7 mg/kg/d glucose 6 weeks; oral >99.6% pure stevioside (Sigma Chemical Co. Mo. USA)	Fasting glucose and insulin levels were not different between groups. Stevioside animals had a lower glucose response, a higher first phase insulin response, and a lower first phase glucagon response. Systolic/diastolic blood pressure was lower in the stevioside group. Stevioside had no effect on body weight.
Jeppesen et al. 2006	Beneficial effect of stevioside in combination with a soy-based dietary supplement against type 2 diabetes in the diabetic Goto-kakizaki rats.	Goto-Kakizaki (GK) rats (type 2 diabetes).	Over the course of 4 weeks, the rats were fed with the following four test diets which consisted of standard carbohydrate- rich laboratory diet (chow), stevioside in combination with chow, soy protein isolate (SPI) diet plus chow and the last group comprised of SPI plus chow plus stevioside.	Stevioside in combination with SPI demonstrated a significant ($p<0.001$) 56% reduction in plasma glucose, a 118% increase in first-phase insulin levels ($p<0.005$), a 20% reduction in glucagons ($p<0.05$), a 28% reduction in total cholesterol levels ($p<0.001$). Also a 13% reduction in FFA ($p<0.01$), a 49% reduction in TG ($p<0.001$) and a 11% reduction in the systolic pressure ($p<0.001$) was also observed. It was concluded that the combination of stevioside and SPI has synergistic positive effects on hyperglycemia, hypertension and dyslipidemia.

000134

000135

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Lee et al. 2001	Inhibitory effects of stevioside on calcium influx to produce antihypertension	Spontaneously hypertensive rats (SHR), 8 per group	Single IP injection of stevioside at a level 25 mg/kg Stevioside prepared from dried *S. rebaudiana* (Bertoni) Bertoni leaves 95% purity	Stevioside caused vasorelaxation via an inhibition of Ca^{2+} influx into the blood vessel. The phenomenon was also confimed in cultured aortic smooth muscle cells (A7r5) The data showed that the vasorelexation effect of stevioside was mediated through Ca^{2+} influx inhibition.
Lailerd et al. 2004	Determine effects of stevioside treatment on skeletal muscle glucose transport activity in insulin-sensitive and insulin-resistant rats.	F lean and F obese Zucker rats, 5 per group	200 or 500 mg/kg bw stevioside single administration; gavage (test substance not characterized)	Acute administration of stevioside had no effect on plasma glucose, insulin, or free fatty acids. Treatment with 200 mg/kg bw stevioside did not alter plasma glucose and insulin responses during the OGTT in either lean or obese rats. Treatment with 500 mg/kg bw stevioside had no effect on glucose response in lean rats during the OGTT, but reduced plasma insulin levels (compared to control) at 15 and 30 min. Plasma insulin response was not altered by stevioside in the obese rats while plasma glucose was lower in stevioside animals at 30 min; overall insulin sensitivity was increased.

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Raskovic et al. 2004 a	Determining the glucose concentration in the blood of intact and alloxan-treated mice after pretreatment with commercial preparations of Stevia.	Mice (Strain and species information not available)	One group of mice pretreated four days with 200 mg/kg of steviol glycosides. Other group pretreated with 20 mg/kg of clear stevioside liquid Control animals received same time physiological solution Route of administration : Information not available First preparation of stevita from (Stevia Co., Inc, Arlington, TZ) Second preparation of stevioside from Stevita Co., Inc, Herbal supplement, Brazil.	Blood glucose levels in mice pretreated with steviol glycosides and stevioside were lower as compared to control. Blood glucose level increased observed with pretreated mice in the glucose tolerance test, pretreatment with stevioside was more effective. Pretreatment with stevioside caused no significant increase in blood glucose concentration after administering adrenaline, which was not the case with the animals pretreated with steviol glycosides and control. Pretreatment with steviol glycosides, and to a greater extent with stevioside, protected test animals from the toxic action of alloxan compared with controls.

000136

000137

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Raskovic et al. 2004 b	To determine the combined effect of two commercial products of *S. rebaudiana* (Bertoni) Bertoni and sodium monoketocholate (mkc) on blood glucose concentration in mice.	Mice (Strain and species information not available)	Group 1: Treated four days with MKC, 4 mg/kg, s.c. Group 2: 200 mg/kg, i.p., of Stevita (Stevita Co, INC, Arlington, Texas) (steviol glycosides), Group 3: 20 mg/kg, i.p., of Clear Steviosides Liquid (Stevita Co, INC, Herbal supplement, Brazil) (stevioside) Group 4: Combination of steviol glycosides and MKC Group 5: Stevisode and MKC	Stevioside combined with mkc caused a significant increase of glycemia with respect of mkc alone and control When repeated four days, the same pretreatment resulted in a significant decrease of glycemia compared with single-quantity pretreatment . Glucose-tolerance test was significantly lower compared to that in mice that were pretreated four days only with mkc before receiving glucose. Analogous difference was observed between the animals given mkc alone and mkc plus stevioside after injecting adrenaline. Combined effect of MKC and steviol glycosides yielded lower values of glycemia compared with that measured after treatment with stevia alone.

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Suanarunsawat and Chaiyabutr 1997	Determine effects of stevioside on glucose metabolism in anaesthetized rats.	M Wistar rats, 8 per group	100, 150, or 200 mg/kg bw/h stevioside or saline continuous infusion for 1 h; i.v. infusion or oral feeding 95% pure stevioside	Plasma glucose increased following i.v. administration of stevioside at all levels; no change in plasma glucose in rats fed stevioside. Glucose carbon recycling and glucose clearance were increased in stevioside infused rats compared to control rats; no effect was seen on insulin response.
In Vitro				
Abudula et al. 2004	Examine the influence of rebaudioside A on insulin release from mouse islets using static incubations and perfusion experiments.	Islet cells from adult F NMRI mice	10^{-16} to 10^{-6} mol/L rebaudioside A 6-step perifusion including 2 30 min perifusions with rebaudioside A rebaudioside from Wako Pure Chemical industries, Tokyo, Japan Purity not given	Rebaudioside A caused a quantity-dependent and glucose-dependent increase in insulin secretion. The effect is dependent on presence of extracellular Ca2+.

000138

000139

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Abudula et al. 2008	Cellular signaling mechanism of rebaudioside A in isolated mouse islets cells.	Islet cells from adult F NMRI mice and β-cell line MIN6	10^{-13} to 10^{-7} M rebaudioside A Quantity dependent stimulation of rebaudioside A on glucose –stimulated insulin release and secretion from MIN6 cells, effect of rebaudioside A on c-AMP, ATP/ADP level and membrane conductance Rebaudioside from Wako Pure Chemical industries, Tokyo, Japan	Isulanotropic effect of rebaudioside A mediated via inhibition of ATP-sensitive K^+ -channels and requires the presence of high glucose. The inhibition of ATP-sensitive K^+ -channels is probably induced by changes in the ATP/ADP ratio. Therapeutic advantage of rebaudioside A over sulphonylureas because of less risk of causing hypoglycemia.
Chen et al. 2006	Evaluated whether stevioside stimulates basal insulin secretion or causes desensitization of beta-cells.	Isolated mouse islets from adult F NMRI mice.	10^{-8} to 10^{-5} M stevioside 2 h or 24 h stevioside, Wako Pure Chemical industries, Osaka, Japan	Pretreatment of cells with stevioside prior to glucose incubation reduced intracellular insulin content.
	Whether stevioside can alleviate impaired beta-cell function by regulating Acetyl CoA carboxylase (ACC) activity.	Isolated rat islets from adult male Wister rats.	10^{-6} M stevioside 24 to 120 hour stevioside, Wako Pure Chemical industries, Osaka, Japan	Stevioside counteracts the impaired insulin secretion during tipotoxicity in rat islets as well as in INS-1E cells without affecting ACC activity.

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Ishii et al. 1987	Investigate the influence of stevioside and its derivatives on the transport and metabolism of D-glucose and D-fructose in the isolated perfused rat liver.	Livers of M albino Wistar rats perfused with stevioside and derivatives (n not specified)	0.5 - 5.0 mM stevioside or derivatives of stevioside in perfusion fluid; monosaccharide transport (test substance not characterized)	Stevioside did not affect D-glucose metabolism, but transport of D-fructose was inhibited.
Jeppesen et al. 2003 *(In vitro)*	Study mechanism of the hypoglycemic effect in the clonal pancreatic beta-cell line, INS-1, and effects of stevioside on gene expression profile in INS-1 cells.	Beta-cell line INS-1 cells	1 µmol/L stevioside or steviol and 11 mmol/L glucose 24 hours >99% pure stevioside	Stevioside and steviol increased insulin content of the clonal beta-cell line. Proinsulin gene, liver type pyruvate, and acetyl-coenzyme A carboxylase, were upregulated; fatty acid oxidation genes were downregulated.

000140

000141

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Jeppesen et al. 2000	Determine if stevioside and steviol stimulate insulin release from normal mouse islets and the beta-cell line INS-1.	Islets from F NMRI mice and beta-cell line INS-1 cells.	5-step perfusion process including 10 min perifusion with glucose + 1 mmol/L stevioside or 1 μmol/L steviol 95% pure stevioside (Sigma) 90% pure steviol	Stevioside and steviol quantity-dependently enhanced insulin secretion in the presence of glucose. Stevioside and steviol potentiated insulin secretion from the beta-cell line INS-1. Stevioside and steviol did not influence plasma K^{+}_{ATP}-sensitive channel activity or alter cAMP levels in islets.
Lailerd et al. 2004	Determine the effects of stevioside treatment on skeletal muscle glucose transport activity.	Soleus and epitrochelearis muscles from F lean and obese Zucker rats, 5 per group	Rats were treated with 200 or 500 mg/kg bw stevioside by gavage; *in vitro* glucose transport activity of muscle was measured. (test substance not characterized)	Low concentrations of stevioside improved *in vitro* insulin action on skeletal muscle glucose transport in lean and obese rats.

Table 38. Animal and *In Vitro* Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Malaisse et al. 1998	Investigate the effects of artificial sweeteners on insulin release and cationic fluxes in isolated rat pancreatic cells.	Isolated rat pancreatic islets	1.0 mM stevioside + glucose (test substance not characterized)	Stevioside augmented insulin release from islets incubated with glucose.
Toskulkao et al. 1995	Study effects of stevioside and steviol on intestinal glucose absorption in the hamster jejunum using an everted sac technique.	Everted sacs of intestines from M Syrian golden hamsters	0.1 – 2 mM steviol 10 min 95% pure stevioside 90% pure steviol	Absorption of glucose was not affected during incubation with stevioside, though absorption of glucose was inhibited by steviol, and degree of inhibition increased with incubation time. A shortening of jejunal villi and undulating epithelial lining was remarkable in the steviol-treated jejunum and there was degeneration of crypts. Intestinal mucosal ATP content decreased over time in the steviol group vs. the control group. Intestinal mucosal mitochondrial NADH cytochrome c reductase and cytochrome oxidase activities were reduced in the presence of steviol.

b) Studies in Humans:

(1) Introduction

Several studies have measured glucose homeostasis after ingestion of stevioside and stevia extracts (Barriocanal et al. 2008; Curi et al. 1986; Chan et al. 2000; Jeppesen et al. 2006; Gregersen et al., 2004; Chen et al., 2005; Maki et al. 2008a). Steviol glycosides were reported to reduce post-prandial glucose response in some trials (Curi et al. 1986; Gregersen et al. 2004). However, in a three-month study of subjects with type 2 diabetes, Jeppesen and colleagues (2006) reported that fasting blood glucose and glycosylated hemoglobin (HbA_{1c}) were not significantly lowered by intake of 1500 mg/d of stevioside compared with placebo. Maki et al. (2008a) also reported that no significant changes in HbA1c were noted between rebaudioside A and placebo groups in type 2 diabetic subjects. Additionally, fasting glucose, insulin and C-peptide did not differ significantly.

(2) Review of Studies

The effect of aqueous extracts of *S. rebaudiana* (Bertoni) Bertoni leaves of unknown purity on glucose tolerance was assessed in 16 healthy adults (Curi et al. 1986). Each aqueous extract was prepared from 5 g stevia leaves. A group of 6 healthy subjects served as a control and ingested an aqueous arabinose solution. Arabinose was selected as the control because of its high content in the stevia extract. Study participants completed an oral glucose tolerance test (OGTT) prior to ingestion of the test articles. After the OGTT, a total of 13 study quantities were consumed at 6-h intervals for approximately 3 days, and a second OGTT was administered. Each OGTT followed an overnight fast; after intake of 100 g glucose and then plasma glucose was measured at 0, 30, 60, 90, 120 and 180 min. Glycosuria was measured with glucostest tapes. Plasma glucose levels were significantly lower after the steviol glycosides treatment as compared to baseline levels at each time point during the OGTT. One subject exceeded the renal threshold for glycosuria in the first 90 min; however, after steviol glycosides treatment, no glycosuria was observed. The arabinose treatment had no effects on plasma glucose response following the OGTT.

Chan et al. (2000) conducted a double- blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in hypertension. The results of their study demonstrated oral stevioside was well tolerated in humans and blood biochemistry parameters, including blood glucose level, did not exhibit any significant changes as compared to the control group.

The effect of stevioside on postprandial glucose response was studied in 12 patients with type 2 diabetes (Gregersen et al. 2004). The mean age of subjects was 65.8 ± 1.6 years. Each patient consumed either 1000 mg stevioside or 1000 mg cornstarch with a test meal in this crossover study. The stevioside used in the study was reported to be 91% pure stevioside, 4% rebaudioside A, and 5% other stevioside derivatives. Blood samples were drawn at time points – 30, 0 (time of meal), 10, 20, 30, 45, 60, 90, 120, 180, and 240 minutes for measurement of plasma glucose, insulin, triglyceride, free fatty acids, total cholesterol, LDL-cholesterol, HDL-cholesterol, glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP), glucagon, glycated hemoglobin A_{1C}, sodium, potassium, creatinine, and hemoglobin. Urine volume was determined during the experiment as well as urinary excretion of albumin, glucose, sodium, and potassium. Blood pressure was also measured. The postprandial blood glucose response was significantly reduced following stevioside intake versus cornstarch intake; glucose response area under the curve ($AUC_{glucose}$) values were 522 ± 64 and 638 ± 55 mmol/L x 240 min, respectively. The insulinogenic index (ratio of $AUC_{insulin}$: $AUC_{glucose}$) was increased following intake of stevioside, with values in the treatment and control groups of 84 ± 11 and 60 ± 8, respectively. Stevioside reduced glucagon AUC (281 ± 33 vs. 348 ± 46 pmol/L x 240 min). No other differences in measurements were observed, and no adverse effects were noted.

Maki et al. (2008a) evaluated the effects of 16 weeks of consumption of 1000 mg rebaudioside A (n=60) compared to placebo (n=62) in men and women (33-75 years of age) with type 2 diabetes mellitus. The results of the trial exhibited no significant changes in glycosylated hemoglobin levels between the rebaudioside A and placebo groups. In addition, changes in fasting glucose, insulin and C-peptide did not differ significantly for rebaudioside A and placebo controlled groups. Assessment of changes in blood pressure, body weights, and fasting lipids also demonstrated no difference by treatment. The results of this study demonstrated that rebaudioside A was well tolerated, and chronic use of 1000 mg rebaudioside A did not alter glucose homeostasis or blood pressure in individuals with type 2 diabetes mellitus. Data showing glycosylated hemoglobin levels, physiological and chemical variables and serum chemistry and hematology in both treated and control groups are presented in Tables 39, 40 and 41, respectively.

Table 39. Glycosylated hemoglobin at baseline and changes from baseline to weeks 4, 8, 12, and 16[a]

Glycosylated hemoglobin	Rebaudioside A	Placebo	P-value[b]
	(*n* = 60)	(*n* = 62)	
	Mean ± SEM		
Baseline (%)	6.71 ± 0.11	6.70 ± 0.10	0.964
Week 4 Δ (%)	0.02 ± 0.03	0.02 ± 0.03	0.982
Week 8 Δ (%)	0.11 ± 0.05	0.04 ± 0.04	0.574
Week 12 Δ (%)	0.11 ± 0.05	0.07 ± 0.05	0.248
Week 16 Δ (%)	0.11 ± 0.06	0.09 ± 0.05	0.355

[a]Baseline = the average of values at weeks -2 and 0. Change from baseline is signified by Δ.
[b]P-values for the change from baseline are for the analysis of covariance with baseline value as the covariate. All values were ranked prior to analysis.
Abbreviation: SEM = standard error of the mean
From: Maki et al. 2008a

Table 40. Fasting Glucose, Insulin, C-Peptide, Body Weight, Blood Pressure, and Lipids at Baseline and Following Treatment[a]

Parameter	Rebaudioside A	Placebo	P-value[b]
	(*n* = 60)	(*n* = 62)	
	Mean ± SEM or median (min, max)		
Baseline Glucose (mg/dL)	135.8 ± 3.6	134.3 ± 4.1	0.433[d]
Δ (mg/dL)	7.5 ± 3.7	11.2 ± 4.5	0.966[d]
Baseline Insulin (μU/mL)	17.3 ± 1.5	14.6 ± 1.4	0.046[d]
Δ (μU/mL)	1.0 ± 0.6	3.3 ± 1.5	0.979[d]
Baseline C-Peptide (ng/mL)	3.9 ± 0.2	3.5 ± 0.2	0.102[d]
Δ (ng/mL)	0.1 ± 0.1	0.4 ± 0.1	0.313[d]
Baseline Body Weight (kg)	97.8 ± 2.1	98.4 ± 2.3	0.841
Δ (kg)	0.0 ± 0.3	0.2 ± 0.3	0.832[d]
Baseline Systolic Blood Pressure (mmHg)	121.6 ± 1.4	126.0 ± 1.6	0.051[d]
Δ (mm Hg)	−0.2 ± 1.0	−0.9 ± 1.1	0.775
Baseline Diastolic Blood Pressure (mmHg)	72.5 ± 1.0	71.3 ± 1.1	0.429
Δ (mm Hg)	0.2 ± 0.8	−1.1 ± 0.7	0.132
Baseline Total-C (mg/dL)	170.7 ± 4.2	168.7 ± 4.1	0.737
Δ (mg/dL)	−1.5 ± 3.4	−3.0 ± 2.0	0.833
Baseline LDL-C (mg/dL)	92.9 ± 3.5	92.3 ± 3.4	0.874[d]
Δ (mg/dL)	−2.1 ± 3.0	−5.8 ± 1.8	0.132[d]
Baseline HDL-C (mg/dL)	46.5 ± 1.5	46.7 ± 2.2	0.543[d]
Δ (mg/dL)	0.3 ± 0.7	0.7 ± 0.7	0.676[d]
Baseline Non-HDL-C (mg/dL)	124.2 ± 4.4	122.0 ± 4.1	0.662[d]
Δ (mg/dL)	−1.8 ± 3.3	−3.7 ± 2.0	0.240[d]
Baseline Triglycerides (mg/dL)	128.8 (62.0, 904.0)	133.5 (39.0, 478.5)	0.992[d]
Δ (mg/dL)	8.3 (−158.0, 352.5)	12.0 (−83.5, 1050.0)	0.387[d]

[a]Baseline = the average of values at weeks -2 and 0 and change (signified by Δ) is the change from baseline to the average of values at weeks 12 and 16.
[b]P-values for the change from baseline are for the analysis of covariance with baseline value as the covariate.
[c]Triglycerides were not normally distributed, therefore median (minimum, maximum) values are shown as a measure of central tendency.
[d]Values were ranked prior to analysis.
Abbreviations: HDL-C high-density lipoprotein cholesterol, LDL-C = low-density lipoprotein cholesterol, Max = maximum, Min = minimum, non-HDL-C = non-high-density lipoprotein cholesterol, SEM = standard error of the mean, total-C = total cholesterol
From: Maki et al. 2008a

000146
SPHERIX

Table 41. Serum Chemistry and Hematology Parameters at Screening (Week -2) and the Changes from Screening to Week 16[a]

Parameter	Rebaudioside A	Placebo	P-value[b]
	(*n* = 59)	(*n* = 62)	0.906[c]
Mean ± SEM			
Screening Alanine Transaminase (U/L)	24.0 ± 1.3	24.4 ± 1.5	0.005[c]
Δ (U/L)	1.7 ± 1.2	−1.5 ± 0.8	0.508[c]
Screening Aspartate Transaminase (U/L)	21.6 ± 0.9	22.6 ± 1.0	0.105[c]
Δ (U/L)	0.6 ± 0.9	−0.9 ± 0.7	0.759[c]
Screening Alkaline Phosphatase (U/L)	73.0 ± 2.3	76.0 ± 2.8	0.234[c]
Δ (U/L)	0.3 ± 1.4	−1.7 ± 1.6	0.313[c]
Screening Blood Urea Nitrogen (mg/dL)	16.6 ± 0.6	16.1 ± 0.7	0.447
Δ (mg/dL)	0.1 ± 0.5	0.6 ± 0.5	0.334[c]
Screening Creatinine (mg/dL)	0.9 ± 0.0	0.9 ± 0.0	0.971[c]
Δ (mg/dL)	0.0 ± 0.0	0.0 ± 0.0	0.910[c]
Screening Gamma Glutamyl Transferase (U/L)	27.7 ± 2.4	29.0 ± 3.0	0.020[c]
Δ (U/L)	2.0 ± 1.8	−0.7 ± 1.1	0.075
Screening Red Blood Cell Count (10^6/μL)	4.7 ± 0.1	4.5 ± 0.1	0.768
Δ (10^6/μL)	−0.1 ± 0.0	−0.1 ± 0.0	0.731
Screening White Blood Cell Count (10^3/μL)	6.6 ± 0.2	6.5 ± 0.2	0.934[c]
Δ (10^3/μL)	0.0 ± 0.2	−0.1 ± 0.2	0.241
Screening Basophil[c] (%)	0.36 ± 0.04	0.42 ± 0.04	0.029
Δ (%)	0.09 ± 0.06	−0.04 ± 0.05	0.440
Screening Hemoglobin (g/dL)	13.7 ± 0.2	13.5 ± 0.2	0.746
Δ (g/dL)	−0.2 ± 0.1	−0.2 ± 0.1	0.456
Screening Hematocrit (%)	41.4 ± 0.5	40.9 ± 0.5	0.772[d]
Δ (%)	−0.3 ± 0.3	−0.4 ± 0.3	0.906[c]

[a]Change from Baseline to week 16 is indicated by Δ.
[b]P-values for the change form baseline are for the analysis of covariance with baseline value as the covariance.
[c]Basophil (%) is presented because it was the only subset of white blood cells that showed a significant difference in treatment responses.
[d]Values were ranked prior to analysis.
Abbreviations: SEM = standard error of the mean
From: Maki et al. 2008a

Two studies conducted to assess the effects of stevioside on hypolipidemic effects in humans have been summarized by the JECFA in the 2006 summary of steviol glycosides (Anonymous 2004a, 2004b as cited in WHO 2006). One study was conducted with 48 hyperlipidemic volunteers to investigate hypolipidemic and hepatotoxic potential of a steviol glycoside extract. Participants consumed capsules containing steviol glycoside extract (purity not stated) or a placebo twice daily for a period of 3 months. Clinical measures included total cholesterol, low-density lipoproteins, triglycerides, liver-derived enzymes, and glucose. At the

end of the study, both groups showed decreased serum concentrations of total cholesterol and low-density lipoproteins. A total of 4 subjects (1 control group, 3 steviol group) did not complete the study due to personal reasons that were not related to adverse reactions. Treatment was reported to have no adverse effects on triglycerides, liver-derived enzymes, or glucose. As reported by WHO, the authors question the subjects' compliance based on the similarity of effect between treatment and placebo groups. The second study was a follow-up study in which 12 patients were given 3.25, 7.5, or 15 mg/kg of the steviol glycoside extract daily for 30 days at each administration. It was reported that preliminary results indicated no adverse responses in blood and urine biochemical parameters measured.

One of the more recent studies by Barriocanal et al. (2008) demonstrated the effects of oral steviol glycosides (≥ 92% purity) on blood glucose and other clinical and biochemical variables in human subjects. In this study, the effects of steviol glycosides were evaluated on blood glucose and on blood pressure in 3 groups of individuals. Groups 1 and 2 were comprised of subjects with type 1 and 2 diabetes, respectively. Group 3 contained subjects without diabetes and with normal/low-normal blood pressure levels. Subjects in each group were randomly allocated to receive treatment with either 250 mg stevioside or placebo, taken three times daily, and followed for 3 months. Results of this study demonstrated post-treatment systolic blood pressure, diastolic blood pressure, glucose and glycated hemoglobin were not significantly different from baseline measurement. In the placebo type 1 diabetic group significant difference was observed for systolic blood pressure and glucose. In addition, no differences in these measures or in reported side effects were observed between the two treatment groups.

Summary of studies identified in this section is presented in Table 42.

000149

Table 42. Human Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Test Material	Subjects	Methods	Result and Discussion
Anonymous 2004a (as cited in WHO 2006)	Steviol glycoside extract: 73 ± 2% stevioside, 24 ± 2 rebaudioside A and 3% other plant polysaccharides	48 adults; hyperlipidemic volunteers	100 mg steviol glycoside extract 2x/d (total of 200 mg/d) Placebo Steviol glycoside extract: 73 ± 2% stevioside, 24 ± 2 rebaudioside A and 3% other plant polysaccharides 3 months	At the end of the study, both groups showed decreased serum concentrations of total cholesterol and low-density lipoproteins. A total of 4 subjects (1 control group, 3 steviol group) did not complete the study due to personal reasons that were not related to adverse reactions. No adverse effects on triglycerides, liver-derived enzymes, of glucose were observed.
Anonymous 2004b (as cited in WHO 2006)	Steviol glycoside extract	12 adults; hyperlipidemic volunteers	3.25, 7.5, and 15 mg/kg bw/d steviol glycoside extract 30 day per administration	Preliminary results indicated no adverse responses in blood and urine biochemical parameters.

Table 42. Human Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Test Material	Subjects	Methods	Result and Discussion
Barriocanal et al., 2008	Steviol glycosides which was ≥ 92% pure (measured three times) was provided by Steviafarma Industrial S.A., Maringa, Brazil	Randomized, double-blind, placebo-controlled, longterm study in three groups of patients. 76 Subjects (30 with type 2 diabetes; 16 with type 1 diabetes; 30 without diabetes)	Group 1: 16 subjects (8 on treatment and 8 on placebo) with Type 1 diabetes (normotensive or hypertensive); Group 2: 30 subjects (15 on treatment and 15 on placebo) with Type 2 diabetes (normotensive or hypertensive); and Group 3: 30 healthy subjects (13 on treatment and 17 on placebo) with normal/low-normal blood pressure levels. Blood pressures and a full panel of blood analysis were conducted at the beginning and at the end of the study The subjects in each group were randomly allocated to active treatment (the steviol glycoside stevioside: 250mg tds) or to placebo treatment and followed-up for 3 months.	Post-treatment systolic blood pressure, diastolic blood pressure, glucose and glycated hemoglobin (HbA_{1c}) were not significantly different from baseline measurements, except for the placebo Type 1 diabetics group where a significant difference was observed for systolic blood pressure (decreased after treatment) and glucose (increased after treatment). No side effects were observed in the two treatments for all three subject groups. This study shows that oral steviol glycosides, taken as sweetener are well tolerated and have no pharmacological effect.

000150

Table 42. Human Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Test Material	Subjects	Methods	Result and Discussion
Chan et al. 2000	Stevioside (250 mg) capsules	60 M+F (stevioside) 46 M+F (placebo) Hypertensive subjects, ages 28-75 y	250 mg stevioside 3x/d (total of 750 mg/d) Placebo not specified Purity not stated (from Nan Kai Chemical Factory, Tien Jing, China) 1 y	After 3 mo, systolic and diastolic blood pressure values were significantly decreased in stevioside group and the effects persisted throughout the study. No significant changes observed in the blood glucose levels.
Curi et al. 1986	Aqueous extract of *S. rebaudiana* (Bertoni) Bertoni	12 M+F Diabetic adults, mean age of 65.8 y	1000 mg stevioside; 91% pure stevioside, 4% rebaudioside A, 5% other stevioside derivatives Placebo = 1000 mg cornstarch (Steviafarm Industrial S/A, Marina, Parana, Brazil) single administration	The postprandial blood glucose response was significantly reduced following stevioside intake, the insulinogenic index was increased, and glucagon decreased.
Gregersen et al. 2004	Extract containing 91% stevioside, 4% rebaudioside A, and 5% of other stevioside derivatives	12 M+F Diabetic adults, mean age of 65.8 y	1000 mg stevioside; Placebo = 1000 mg cornstarch (Steviafarm Industrial S/A, Marina, Parana, Brazil) single administration	The postprandial blood glucose response was significantly reduced following stevioside intake, the insulinogenic index was increased, and glucagon decreased.

000151

Table 42. Human Studies of the Effects of Steviol Glycosides on Blood Glucose and Insulin

Reference	Test Material	Subjects	Methods	Result and Discussion
Maki et al. 2008a	Rebaudioside A Purity 97%	60 M+F; age 33-75 yrs; (rebaudioside A) 62 M+F; age 33-75 yrs; (placebo) All subjects have type 2 diabetes mellitus one year prior to screening with HbA_{1C} ≤9.0% at screening.	250 mg rebaudioside A 4x/d (total of 1000 mg/d); rebaudioside A 250 mg microcrystalline cellulose 4x/d (total of 1000 mg/d) 16 week double blind treatment period	No significant changes in glycosylated hemoglobin levels between the rebaudioside A and placebo groups. In addition, changes in fasting glucose, insulin and C-peptide did not differ significantly for rebaudioside A and placebo controlled groups. Assessment of changes in blood pressure, body weights and fasting lipids also demonstrated no difference by treatment. Rebaudioside A was well tolerated and chronic use of 1000 mg rebaudioside A did not alter glucose homeostasis or blood pressure in individuals with type 2 diabetes mellitus.

000152

3. Effect of Stevioside on Blood Pressure

a) Studies in Animals:

(1) Introduction

Several studies using shortterm administration of steviol glycosides, primarily stevioside, on blood pressure in different animal models, including hypertensive rat models, using primarily intraperitoneal or intravenous administration, have demonstrated reductions in blood pressure. Animal and *in vitro* studies investigating the antagonistic effects of stevioside on blood pressure suggested that that reduction in blood pressure is produced through vasorelaxation likely mediated through inhibition of calcium influx into the blood vessels.

(2) Review of studies

(Melis and Sainati, 1991a), showed that stevioside from *S. rebaudiana* (Bertoni) Bertoni leaves produced a fall in systemic blood pressure, as well as diuresis and natriuresis per milliliter of glomerular filtration rate in rats. Verapamil tended to increase the renal and systemic effects of stevioside. In contrast, an infusion of calcium chloride prepared with stevioside induced a marked attenuation of the vasodilating responses of stevioside in rats. These authors suggested the possibility of stevioside acting as a calcium antagonist, as is the case for verapamil.

Melis (1992 a,b,c) also evaluated the effect of stevioside on renal function in both normal and hypertensive rats (GII) using clearance techniques. Stevioside provoked hypotension, diuresis and natriuresis in both the normal and hypertensive rats. Normal rats showed an increase in renal plasma flow (RPF) and glomerular filtration rate (GFR) following stevioside administration. The later effect infers vasodilation of both the afferent and efferent arterioles. Moreover, stevioside infusion in hypertensive rats caused an increase in RPF and GFR. The author concluded that the data were consistent with impairment of a renal autoregulation mechanism in this hypertensive model.

The effects of administration of *S. rebaudiana* (Bertoni) Bertoni extracts for 20, 40 and 60 days on renal function and mean arterial pressure in normal Wistar rats were evaluated by Melis (1995). Results showed that rats treated with the *S. rebaudiana* (Bertoni) Bertoni extract for 20 days did not significantly differ from the control group. Chronic administration of a crude extract for 40 and 60 days induced hypotension, diuresis and natriuresis with the glomerular filtration rate (GFR) remaining constant. An increase of the renal plasma flow (RPF) was observed only for the group treated for 60 days. The results suggest that oral administration to

rats of an aqueous extract of dried stevia leaves induces systemic and renal vasodilation, causing mild hypotension, diuresis and natriuresis.

Melis et al. (1996) evaluated the effect of *S. rebaudiana* (Bertoni) Bertoni extract on renal function in normotensive and renal hypertensive rats (GII) using clearance techniques. Oral-administration of stevia extract, corresponding to 2.67 g dry leaves/day for 30 days, resulted in a significant decrease in mean arterial pressure in both the normo-(N) and hypertensive rats (H). Glomerular filtration rate was constant in the N rats and increased significantly in the H rats after steviol glycosides treatment. Normo- and hypertensive rats showed an increase in renal plasma flow following oral steviol glycosides administration. The authors concluded that the stevia extract, at levels higher than used for sweetening purposes, is a vasodilator agent in normo- and hypertensive animals.

Chan et al. (1998) studied the effect of intravenous stevioside on blood pressure in spontaneously hypertensive rats (SHR). The hypotensive effect on both systolic and diastolic blood pressure was quantity-dependent for intravenous quantities of 50, 100 and 200 mg/kg in conscious SHR. The maximum reductions in systolic and diastolic blood pressure were 31.4 +/- 4.2% and 40.8 ± 5.6% (mean ± SEM), respectively and the hypotensive effect lasted for more than 60 min with an administration of 200 mg/kg. The data suggested that stevioside when given intravenously to conscious SHR was effective in blood pressure reduction with no change in serum catecholamine levels in anaesthetized animals.

Lee et al. (2001) demonstrated the antihypertensive effect of stevioside in spontaneously hypertensive rats (SHRs) when injected intraperitoneally at an administration of 25 mg/kg bw. In isolated aortic rings from normal rats, stevioside could quantity-dependently relax vasopressin-induced vasoconstriction in both the presence and absence of endothelium. However, stevioside had no effect on phenylephrine- and KCl-induced phasic vasoconstriction. The results indicated that stevioside caused vasorelaxation via an inhibition of Ca(2+) influx into the blood vessel. This phenomenon was also confirmed in cultured aortic smooth muscle cells (A7r5) through an event that is independent of the effects of nitric oxide.

Hsu et al. (2002) evaluated the antihypertensive effect of stevioside in different strains of hypertensive rats and confirmed its effect on the blood pressure lowering capability. Stevioside administered intraperitoneally at concentrations of 50, 100 and 200 mg/kg to normotensive Wistar-Kyoto rats (NTR), SHR, deoxycorticosterone acetate-salt (DOCA-NaCl) sensitive hypertensive rats (DHR) and renal hypertensive rats (RHR) led to a significant hypotensive effect at 50 mg/kg in all of the rat models. Stevioside at an increased concentration of 100 and

200 mg/kg, ip also caused slow and persistent lowering of blood pressure in SHR and NTR and resulted in lowering of blood pressure in SHR quantity-dependently at 100, 200, and 400 mg/kg. Relatively higher levels of stevioside were used in these experiments (up to 400 mg/kg) to demonstrate its safety at higher levels. In addition, blood pressure returned to previous levels after stevioside was discontinued for 2-3 days, thereby confirming the hypotensive effects of stevioside, which was more prominent in hypertensive rats.

Jeppesen et al. (2003) showed insulinotropic effects of *S. rebaudiana* (Bertoni) Bertoni in vitro. Its potential antihyperglycemic and blood pressure-lowering effects were examined in a longterm study in the type 2 diabetic Goto-Kakizaki (GK) rat. Rats were fed 0.025 g/kg bw/d stevioside (purity > 99.6%) for 6 weeks. Stevioside exhibited an antihyperglycemic effect and concomitantly suppressed the glucagon levels when compared to the control group. In addition, stevioside caused a pronounced suppression of both systolic (135 +/- 2 v 153 +/- 5 mm Hg; P <.001) and diastolic blood pressure (74 +/- 1 v 83 +/- 1 mm Hg; P <.001). The results of the study support the hypothesis that stevioside acts an antihyperglycemic and a blood pressure-lowering substance, and the authors propose its use as a potential therapeutic agent in treating type 2 diabetes and the metabolic syndrome.

Liu et al (2003) demonstrated the antihypertensive effects of stevioside in anesthetized dogs. This study evaluated the hypotensive effect of stevioside in dogs and a potential underlying mechanism. Briefly, stevioside powder (200 mg/kg, purity not stated) was administered through the nasogastric route in healthy mongrel dogs, which resulted in a significant decrease in blood pressure after 60 min, which returned to baseline level at 180 min. The reduction of blood pressure was more rapid (at 5-10 min) and effective after intravenous injection. In addition, no significant change of blood pressure was observed after injection through the left vertebral artery, implicating that the hypotensive effect is not related to the central nervous system. Stevioside also showed significant hypotensive effects in renal hypertensive dogs, in a quantity-dependent manner. The authors proposed the mechanism of action was via inhibition of the Ca(2+) influx.

Dyrskog et al. (2005a) reported that longterm administration of rebaudioside A, could improve glycemic control and lower blood pressure in an animal model for type 2 diabetes. Male Goto-Kakizaki (GK) rats were divided into two groups, which were fed a standard laboratory chow diet for eight weeks. The diet was supplemented with oral rebaudioside A (0.025 g/kg bw/d) in the experimental group. Blood glucose, weight, blood pressure and feed intake were measured weekly. Animals were equipped with an intra-arterial catheter, and at week eight the conscious rats underwent an intra-arterial glucose tolerance test (IAGTT) (2.0 g/kg bw). During

the IAGTT, the level of glucose, glucagon, and insulin responses did not differ significantly between the two groups. Fasting levels of glucose, glucagon, insulin or levels of blood lipids did not differ between the groups throughout the study period. The authors concluded that oral supplementation with rebaudioside A (0.025 g/kg bw/d) for eight weeks did not influence blood pressure or glycemic control in GK rats.

In another study conducted by the same group, Dyrskog et al. (2005b) demonstrated beneficial effects of a commercial dietary supplement consisting of soy protein, isoflavones, and cotyledon fiber on cardiovascular risk markers in type 2 diabetes. The aim of this study was to investigate if the combination of stevioside and a dietary supplement of soy protein possesses beneficial qualities in the treatment of type 2 diabetes and the metabolic syndrome. Male Zucker diabetic fatty rats were divided into 4 groups and fed different test diets for 10 weeks: (A) standard carbohydrate-rich laboratory diet (chow), (B) chow+stevioside (0.03 g/kg bw/d), (C) 50% soy +50% chow (adjusted for vitamins and minerals), and (D) 50% soy+50% chow+stevioside 0.03 g/kg bw/d. Plasma glucose, blood pressure, weight, and feed intake was measured once weekly. After 2 weeks of treatment, a decrease in the systolic blood pressure was observed in the stevioside-treated groups ($P<0.01$). The authors concluded that stevioside and soy supplementation may prove effective in treating a number of the characteristic features of the metabolic syndrome, including hypertension.

Jeppesen et al. (2006) reported the beneficial effect of stevioside in combination with a soy-based dietary supplement against type 2 diabetes in the diabetic Goto-kakizaki rats. Over the course of 4 weeks, the rats were fed with the following four test diets which consisted of standard carbohydrate- rich laboratory diet (chow), stevioside in combination with chow, soy protein isolate (SPI) plus chow and the last group comprised of SPI plus chow plus stevioside. Compared to normal chow diet, stevioside in combination with SPI demonstrated a significant reduction of 11% in the systolic pressure ($p<0.001$), noting a combined beneficial effect of stevioside and SPI on hyperglycemia, hypertension and dyslipidemia.

Summary of studies identified in this section is presented in table 43.

000156
SPHERIX

000157

Table 43. Animal Studies of the Effects of Steviol Glycosides on Blood Pressure

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Chan et al. 1998	To determine the effect of stevioside on blood pressure and plasma catacholamines in spontaneously hypertensive rats.	Spontaneously hypotensive rats; 8 rats/group; weighing 230-300g	Stevioside Intravenous (IV) injection of 50, 100, 200 mg/kg in conscious hypotensive rats (purity >95% pure)	The hypotensive effect on both systolic and diastolic blood pressure was quantity dependent for intravenous quantities of 50, 100 and 200 mg/kg in conscious SHR. Maximum reduction in systolic and diastolic blood pressure was 31.4 and 40.8 % respectively Hypotensive effect lasted for more than 60 min with an administration of 200 mg/kg.
Dyrskog et al. 2005a	Preventive effects of soy based diet supplemented with stevioside on the development of the metabolic syndrome and type 2 diabetes in Zucker diabetic fatty rats	Forty-eight, 8 week old male M obese ZDF rats 4 groups of 12 rats	4 groups of 12 rats each fed with four different diets for 10 weeks. 1. Standard Chow 2. Chow +stevioside in drinking water 3. 50% soy protein + 50% chow 4. Soy Protein + stevioside in drinking water Stevioside supplement (91% stevioside, 4% rebaudioside A and 5% other glycosides) from Stevitafarm Industrial, S/A, Brazil	After 2 weeks of treatment, a decrease in the systolic blood pressure was observed in the stevioside-treated groups (P<.01).

Table 43. Animal Studies of the Effects of Steviol Glycosides on Blood Pressure

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Dyrskog et al. 2005b	Effect of rebaudioside A on glycemic control or blood pressure	24 eleven week old male Goto-Kakizaki (GK) rats (n=12 animals/group)	Divided into two groups (n=12 animals/group) which were fed a standard laboratory chow diet for eight weeks.after eight weeks treatment in the Goto-Kakizaki rat. Purity 97.8%	Fasting levels of glucose, glucagon, insulin or levels of blood lipids did not differ between the groups throughout the study period. No effect on blood pressure or weight development.
Hsu et al. 2002	To determine the antihypertensive effects of stevioside in different strains of hypertensive rats	Normotensive Wistar-Kyoto rats (NTR), spontaneous hypertensive rats (SHR), DOCA-NacL sensitive hypertensive rats (DHR), and renally hypertensive rats (RHR) 10 rats per group	Stevioside administered intraperitoneally (IP) at a concentration of 50, 100 and 200 mg/kg to normotensive Wistar-Kyoto rats (NTR), spontaneous hypertensive rats (SHR), DOCA-NacL sensitive hypertensive rats (DHR), and renally hypertensive rats (RHR Noninvasive tail-cuff method employed to measure blood pressure Stevioside was provided as agift from Dr. Hsu, Teipai Medical University. Purity not given	Stevioside resulted in quantity-dependent decreases in systolic blood pressure in normal and hypertensive rats.
Jeppesen et al. 2003	Anti-hyperglycemic and blood pressure effects of stevioside in the diabetic Goto-kakazaki rats	Goto-Kakazaki (GK) rats , age 20 weeks; 200-320g and Wistar rats n=20/group	0.025 g/kg/d stevioside in tap water for 6 weeks Stevioside (purity 99.6%); Sigma chemicals, St. Louis, MO, USA	Systolic/diastolic blood pressure was lower in the stevioside group. Stevioside enjoys a dual positive effect by acting as an antihyperglycaemic and a blood pressure lowering substance.

000158

Table 43. Animal Studies of the Effects of Steviol Glycosides on Blood Pressure

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Jeppesen et al. 2006	Effectiveness of stevioside in combination with a soy –based dietary supplement in treating type 2 diabetes in Goto-Kakizaki rats	Adult male GK rats weighing 200-230 g at the age of 20 week	Four groups (n = 12 in each group) and fed for 4 week with four different pellet diets: Group A received a standard carbohydrate-rich laboratory diet (chow). Group B received chow + stevioside (0.03 g per kg bw/d). Group C received 80% SPI + 20% chow. Group D received 80% SPI + 20% chow + stevioside (0.03 g/kg bw/d). All diets contained similar amounts of vitamins and minerals. Fasting blood glucose, blood pressure, body weight and food intake were measured every week during the 4 week period. At week 3 an intra-arterial glucose tolerance test was performed (see below). Water was given ad libitum and a light cycle of 12 h/12 h was used.	Compared to normal chow diet, stevioside in combination with SPI showed the following beneficial effects in GK rats with mild type 2 diabetes: 1. a 56% reduction in plasma glucose ($p < 0.001$), 2. a 118% increase in first-phase insulin ($p < 0.005$), 3. a 20% reduction in glucagons ($p < 0.05$), 4. a 28% reduction in total cholesterol ($p < 0.001$), 5. a 13% reduction in FFA ($p < 0.01$), 6. a 49% reduction in TG ($p < 0.001$) and 7. a 11% reduction in the systolic blood pressure ($p < 0.001$). In conclusion, the combination of stevioside and SPI has synergistic positive effects on the characteristic features of the metabolic syndrome, i.e. hyperglycemia, hypertension and dyslipidemia.

000159

Table 43. Animal Studies of the Effects of Steviol Glycosides on Blood Pressure

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Liu et al. 2003	Antihypertensive effect of stevioside on anesthetized dogs	Healthy mongrel dogs, 8 per group	200 mg/kg stevioside powder 30 min; nasogastric feeding while anesthetized 50 mg/kg stevioside powder i.v. feeding Stevioside (purity not mentioned); Nangkang Chemicals, Shanghai, China	In nasogastric feeding, systolic, diastolic, and mean arterial blood pressure decreased at 60 min and returned to baseline levels at 180 min. Blood pressure decreased within 5-10 min with intravenous feeding. Hypotensive effect was quantity-dependent in renal hypertensive dogs. Hypotensive effect was not related to central nervous system. Antagonistic effects of stevioside on blood pressure appear to be through inhibition of calcium influx.
Lee et al. 2001	Inhibitory effects of stevioside on calcium influx to produce antihypertension	Spontaneously hypertensive rats (SHR), 8 per group	Single IP injection of stevioside at an administration of 25 mg/kg Stevioside prepared from dried *S. rebaudiana* (Bertoni) Bertoni leaves (95% purity)	Stevioside caused vasorelaxation via an inhibition of Ca^{2+} influx into the blood vessel. The phenomenon was also confimed in cultured aortic smooth muscle cells (A7r5) The data showed that the vasorelexation effect of stevioside was mediated through Ca^{2+} influx inhibition.

000160

000161

Table 43. Animal Studies of the Effects of Steviol Glycosides on Blood Pressure

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Melis, 1995	Renal effects of the chronic administration of aqueous extract of stevia in rats	Wistar rats 60 in number; 80-100 g bw	Animals divided into 6 groups of 10 animals each. Treated animals (3 groups) were given orally an aqueous extract of stevia corresponding to 66.7 g of dried leaves/10 mL final solution) twice daily by means of gastric tubling. Crude aqueous extract of leaves	Chronic administration of a crude extract for 40 and 60 days induced hypotension, diuresis and natriuresis with glomerular filtration rate (GFR) constant. An increase of the renal plasma flow (RPF) observed for the group treated for 60 days. The results suggests that oral administration to rats of an aqueous extract of stevia dried leaves induce systemic and renal vasodilation, causing hypotension, diuresis and natriuresis.
Melis, 1996	Effect of a crude extract of *S. rebaudiana* (Bertoni) Bertoni increases the renal plasma flow of normal and hypertensive rats.	Renal hypertensive rats (GII) Male Wistar rats weighing 300-330 g n=10 animals/group	Oral-administration of stevia extract, corresponding to 2.67 g dry leaves/day for 30 days Stevia (purity not mentioned)	Significant decrease in mean arterial pressure in both the normo-(N) and hypertensive rats (H) (N rats: 113 +/- 3.0 mmHg in the control (C) group vs 69.5 +/- 4.0 mmHg in the stevia (S) group; H rats: 155 +/- 3.0 mmHg in C vs 108 +/- 4.0 mmHg in S; $P < 0.05$). Stevia extract, at levels higher than used for sweetening purposes, is a vasodilator agent in normo- and hypertensive animals.

Table 43. Animal Studies of the Effects of Steviol Glycosides on Blood Pressure

Reference	Study objective	Species and Number or *In Vitro* Test System	Methods: Amount Administered, Duration, Administration Route and Test Substance Characterization	Results
Melis, 1992	Effect of stevioside on renal function of normal and hypertensive rats	Wister rats; weighing 250-320g; Two groups for clearance studies with 10 animals/group	Intravenous administration of 16 mg/kg at a rate of 16/mg/kg/h Stevioside (purity 90%)	Stevioside provoked hypotension, diuresis and natriuresis in both normal and hypertensive rats Stevioside infusion in hypertensive rats caused an increase in RPF and GFR. Impairment of renal autoregulation mechanism in the presented experimental model
Melis and Sainati, 1991	Effect of calcium and verapamil on renal function of rats during treatment with stevioside.	Wister rats; weighing 250-320g; 4 group treatment	Intravenous infusion of stevioside Group 1: Stevioside 8 mg/kg Group 2: Stevioside 16 mg/kg Group 3: Stevioside 16 mg/kg + Verapamil Group 4: Stevioside 16 mg/kg + Verapamil + $CaCl_2$	Fall in systemic blood pressure

000162

b) Studies in Humans:

(1) Introduction

A number of studies have examined the effect of oral steviol glycosides on normal and hypertensive humans. Results from two longterm human trials (1-2 years) in China studying men and women with mild to moderate essential hypertension have suggested antihypertensive effects of stevioside (purity undefined) at intakes of 750 and 1500 mg/day (Chan et al., 2000, Hsieh et al., 2003). However, other studies have not shown measurable effects of steviol glycosides on blood pressure in humans (Geuns et al. 2007; Ferri et al. 2006; Maki et al. 2008a,b). In a trial of healthy adults with normal and low blood pressure, consumption of as much as 1000 mg/day rebaudioside A produced no clinically important changes in blood pressure. In a separate trial of men and women with type 2 diabetes, more than half of whom were taking antihypertensive medications, consumption of as much as 1000 mg/day of rebaudioside A produced no clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure (Maki 2008a). Barriocanal et al. (2008) also reported no production of hypotension in persons with type 1 or 2 diabetes taking 250 mg tds stevioside for 3 months. Various safety, biochemical and clinical variables were evaluated in these studies, including body weight, blood pressure, serum chemistry, lipids, fasting glucose, insulin and c-peptide at baseline and after treatment.

(2) Review of studies

Chan et al. (2000) evaluated the effect of stevioside (undefined purity) in human hypertension in a multicenter, randomized, double-blind, placebo-controlled study. This study group consisted of 106 Chinese hypertensive subjects with diastolic blood pressure between 95 and 110 mmHg and ages ranging from 28 to 75 years with 60 subjects allocated to active treatment and 46 to placebo treatment. Each subject was given capsules containing stevioside (250 mg) or placebo thrice daily and followed-up at monthly intervals for 1 year. The results of this study reported a significant decrease in systolic and diastolic blood pressure of the stevioside group after 3 months. The effect persisted during the whole year. Blood biochemistry parameters including lipid and glucose showed no significant changes. Oral stevioside was well tolerated in the study and was proposed as an alternative or supplementary therapy for patients with hypertension.

A similar study was conducted to assess the longterm effects of stevioside (undefined purity) on adults with mild hypertension (Hsieh et al. 2003). In this 2-year study, 85 patients

consumed 500 mg stevioside capsules 3 times daily (total of 1500 mg/d), and 89 patients consumed placebo capsules 3 times daily. Clinical visits at baseline, 6 months, 1 year and 2 years included evaluations of blood pressure, heart rate, weight, glucose, cholesterol (total, HDL, LDL), triglycerides, renal function (uric acid, blood urea nitrogen, creatinine), alanine aminotransferase, aspartate aminotransferase, electrolytes (sodium, potassium, chloride), and occurrence of adverse events. Echocardiographic examinations (for determination of left ventricular mass index (LVMI)) were completed at baseline and after 1 and 2 years of treatment. Blood pressure measures were significantly decreased in the stevioside group reportedly as early as 1 week after initiation of treatment. Systolic blood pressure (mean ± SD) in the stevioside and placebo groups at 2 years was 140 ± 6.8 vs. 150 ± 7.0 mmHg, and diastolic blood pressure was 95 ± 4.8 vs. 89 ± 3.2 mmHg. After 2 years, 6/52 patients (11.5%) in the stevioside group had left ventricular hypertrophy vs. 17/50 patients (34.0%) in the placebo group ($P < 0.001$). Compliance was evaluated by tablet counting at each visit; subjects in the placebo and stevioside groups consumed an average of 92 ± 3% and 93 ± 3%, respectively, of the tablets provided during the treatment phase. Six patients (3 in each group) were withdrawn before the last scheduled study visit for loss to follow-up (1 in each group) and adverse events (2 in each group, nausea, dizziness, asthenia). These 6 subjects were not included in the analysis. Overall, stevioside was well tolerated; eight patients in each group reported minor adverse events. The types and incidences of of adverse effects (abdominal fullness, nausea, asthenia, dizziness, headache and myalgia) were similar between the active-treatment and the placebo groups. Only two in each group discontinued treatment and were lost to the study. There were no significant changes in body weight or biochemical parameters and the results of laboratory tests were similar in the two groups throughout the study. No cardiovascular events or mortality occurred in either group. After 2 years, subjects in the stevioside group reported higher scores vs. the placebo group on quality of life assessments (all indices but mental health). No differences in other measures were observed and no significant adverse events were noted. The authors concluded that "oral stevioside significantly decreased systolic blood pressure and diastolic blood pressure compared with placebo" in these patients with mild hypertension.

Ferri et al. (2006) examined the antihypertensive effect of a crude stevioside extract on previously untreated mild hypertensive patients. Patients with essential hypertension were submitted to a placebo phase for 4 weeks and then received either capsules containing placebo during 24 weeks or crude stevioside 3.75 mg/kg/day (7 weeks), 7.5 mg/kg/day (11 weeks) and 15.0 mg/kg/day (6 weeks). After the placebo phase and after each administration of crude stevioside, body mass index, electrocardiogram and laboratory tests were performed. During the investigation, blood pressure was measured biweekly and the remaining data were collected at the end of each stevioside administration step. The results of the study demonstrated no major

adverse clinical effects during the trial. Systolic and diastolic blood pressure decreased ($p < 0.05$) during the treatment with crude stevioside, but a similar effect was also observed in the placebo group. It was concluded that crude stevioside up to 15.0 mg/kg/day did not show an antihypertensive effect, and is safe and supports the well-established tolerability of this material during longterm use as a sweetener.

In one of the recent studies conducted by Geuns et al (2007), Stevioside (250mg capsules) was given thrice daily for 3 days to 10 healthy subjects. Blood samples were collected and blood pressure was measured after nocturnal fasting at different time points. No significant differences were observed in blood pressure or various other biochemical variables between the control and Stevioside group. In addition, no significant difference was observed in 24-h urinary excretion of electrolytes and mean blood glucose and insulin levels between both the groups. It was concluded that oral Stevioside is not directly effective as a hypotensive or hypoglycemic agent in healthy subjects at the level administered in this study. Besides the biochemical and blood variables, no uptake of Stevioside by the GI tract was observed. All the Stevioside reaching the colon was degraded by micro-organisms into steviol, the only metabolite found in feces. Also, no stevioside or free steviol was found in the blood plasma.

More recently, Barriocanal et al. (2008) demonstrated that oral steviol glycosides ($\geq$ 92% purity) are safe and well tolerated. In this study, effects of steviol glycosides were evaluated on blood glucose and on blood pressure in 3 groups of individuals. Group 1 and 2 were comprised of subjects with type 1 and 2 diabetes, respectively. Group 3 contained subjects without diabetes and with normal/low-normal blood pressure levels. Subjects in each group were randomly allocated to receive treatment with either 250 mg stevioside or placebo treatment, taken three times daily, and followed for 3 months. Results of this study demonstrated post-treatment systolic blood pressure, diastolic blood pressure, glucose and glycated hemoglobin were not significantly different from baseline measurement, except for the placebo type 1 diabetic group where a significant difference was observed for systolic blood pressure and glucose. In addition, no differences in these measures or in reported side effects were observed between the two treatment groups.

The study by Barriocanal et al. (2008) carries significant importance as the study is well-planned and conducted and utilized steviol glycosides material that was well-characterized and demonstrated to be at least 92% pure steviol glycosides. The results of the study demonstrate that steviol glycosides are safe and that they do not lead to hypotension in diabetic subjects. The effect of steviol glycoside and placebo treatment at baseline and post-treatment in type 1 and type 2 diabetes on measured parameters are shown in Tables 44 and 45 respectively.

Table 44. Characteristics of the steviol glycosides and placebo groups at baseline and post-treatment: group 1 (type 1 diabetes)

	Steviol glycosides (n = 8)		Placebo (n = 8)	
	Baseline	**Post-treatment**	**Baseline**	**Post-treatment**
BMI (kg/m^2)	23.2 (3.3)	23.1 (3.1)	22.4 (1.0)	22.4 (1.0)
24-h SBP (mmHg)	117.1 (6.6)*	115.9 (8.6)	108.3 (3.0)	105.7 (2.8)**
24-h DPB (mmHg)	72.6 (6.9)	68.9 (7.2)	70.7 (4.4)	69.7 (3.3)
Laboratory				
Glucose (mg/dL)	144.9 (95.1)	155.3 (78.3)	219.3 (74.1)	298.3 (58.8)**
HbA_{1c} (%)	7.1 (1.6)	7.3 (1.1)	8.2 (1.4)	8.3 (1.6)
Total cholesterol (mg/dl)	148.9 (25.9)	159.5 (36.7)	144.6 (16.4)	150.6 (10.0)
HDL-C (mg/dl)	46.3 (6.3)	44.6 (8.0)	53.0 (7.7)	48.7 (11.4)
LDL-C (mg/dl)	85.4 (21.8)	96.3 (27.6)	81.6 (20.6)	88.8 (7.4)
Triglycerides (mg/dl)	86.8 (33.6)*	86.8 (41.5)	51.3 (11.5)	67.3 (15.1)
Creatinine (mg/dl)	0.9 (0.1)	0.9 (0.1)	0.8 (0.2)	0.9 (0.1)
CPK (U/L)	34.4 (4.2)	33.3 (13.5)	37.6 (5.8)	40.8 (13.2)
AST (U/L)	21.4 (8.1)	18.6 (9.4)	19.3 (7.4)	23.3 (3.0)
ALT (U/L)	18.6 (7.2)	16.5 (6.9)	16.3 (5.8)	19.3 (6.2)
γGT (U/L)	22.0 (12.3)	29.3 (20.3)	26.3 (5.3)	30.6 (7.6)
Sodium (mEq/L)	138.8 (3.3)	140.4 (3.3)	138 (1.7)	138.3 (1.6)
Potassium (mEq/L)	4.2 (0.4)	4.5 (0.5)	4.5 (0.5)	4.9 (0.3)
Chloride (mEq/L)	100.0 (2.0)	100.9 (2.4)	99.3 (1.0)	99.8 (1.6)

Values are mean (SD).
BMI, body mass index; 24-h SBP, 24-h systolic blood pressure; 24-h DPB, 24-h systolic blood pressure; CPK, creatinine phosphokinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, gamma glutaryltransferase; HbA_{1c}, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein.
* p <0.05 versus placebo baseline.
** p <0.05 post-treatment versus baseline (within treatment group).

From: Barriocanal et al. 2008

000166

Table 45. Characteristics of the steviol glycosides and placebo groups at baseline and post-treatment: group 2 (type 2 diabetes)

	Steviol glycosides (n = 15)		Placebo (n = 15)	
	Baseline	**Post-treatment**	**Baseline**	**Post-treatment**
BMI (kg/m^2)	28.7 (3.4)	29.2 (2.9)	30.1 (3.3)	30.2 (3.5)
24-h SBP (mmHg)	127.3 (15.1)	124.3 (13.5)	127.9 (13.7)	124.9 (13.3)
24-h DPB (mmHg)	77.3 (9.1)	74.7 (8.3)	76.7 (5.6)	77.4 (9.5)
Laboratory				
Glucose (mg/dl)	151.2 (54)	133.8 (34.5)	131.3 (46.7)	118.9 (34.0)
Insulin (μUl/mL)	13.3 (15.3)	11.6 (11.1)	14.7 (10.2)	15.3 (9.6)
HbA_{1c} (%)	6.8 (1.2)	6.6 (1.1)	6.8 (1.6)	6.8 (1.0)
Total cholesterol (mg/dl)	186.9 (23.3)	175.1 (21.6)	172.7 (31.6)	173.8 (23.0)
HDL-C (mg/dl)	44.6 (8.7)	42.5 (6.7)	44.6 (10.9)	40.8 (6.2)
LDL-C (mg/dl)	111.4 (23.8)	107.8 (22.2)	93 (31.8)	110.2 (18.6)
Triglycerides (mg/dl)	143.2 (51.8)	153.7 (71.3)	133.8 (53.3)	128.1 (39.2)
Creatinine (mg/dl)	0.9 (0.1)	0.9 (0.3)	0.9 (0.2)	0.8 (0.2)
CPK (U/L)	33.1 (10.3)	34.8 (14.8)	48.5 (27.0)	38.8 (31.6)
AST (U/L)	19.8 (5.3)	19.3 (3.2)	22.0 (5.0)	22.4 (5.5)
ALT (U/L)	21.3 (7.9)	22.8 (8.9)	21.1 (5.1)	21.6 (5.3)
γGT (U/L)	25.6 (10.4)	27.9 (15.8)	23.4 (12.0)	27.2 (19.2)
Sodium (mEq/L)	138.6 (2.0)	141.5 (3.2)	139.5 (1.6)	140.9 (2.1)
Potassium (mEq/L)	4.2 (0.2)	4.6 (0.4)	4.4 (0.2)	4.4 (0.3)
Chloride (mEq/L)	100.2 (1.9)	101.9 (2.2)	100.4 (1.2)	101.5 (1.5)

Values are mean (SD).
BMI, body mass index; 24-h SBP, 24-h systolic blood pressure; 24-h DPB, 24-h systolic blood pressure; CPK, creatinine phosphokinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, gamma glutaryltransferase; HbA_{1c}, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein.
From: Barriocanal et al. 2008

Maki et al. (2008b) investigated the hemodynamic effects of rebaudioside A in healthy adults with normal and low-normal blood pressure. This randomized, double blind study evaluated the hemodynamic effects of four weeks of consumption of 1000 mg/day rebaudioside A versus placebo in 100 individuals with normal and low-normal systolic blood pressure (SBP) and diastolic blood pressure (DBP). Subjects were predominantly females (76%, rebaudioside A and 82%, placebo) with a mean age of ~41 (range 18-73) years. At baseline, mean resting, seated SBP/DBP was 110.0/73.3 mm Hg and 110.7/71.2 mm Hg for the rebaudioside A and placebo groups, respectively. Compared with placebo, rebaudioside A did not significantly alter resting, seated SBP, DBP, mean arterial pressure (MAP), heart rate (HR) or 24-h ambulatory blood pressure responses. The results of this study demonstrated that consumption of as much as 100

mg/day of rebaudioside A produced no clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure. As with the study by Barriocanal et al. (2008), this study is also well planned and conducted and utilized steviol glycoside material of high purity (in this case, 97% pure rebaudioside A).

The Maki et al. (2008b) data highlighting the blood pressure changes during baseline and week 4 and serum and hematological variables are shown in Tables 46, 47, 48 and 49 below.

A summary of studies identified in this section (Human Studies of the Effects of Steviol Glycosides on Blood Pressure) is presented in Table 50.

Table 46. 24-hour Ambulatory Blood Pressure Monitor Readings at Baseline (Week 0) and the Change from Baseline to Week 4[a,b]

Parameter	Rebaudioside A	Placebo	P-value[c]
	Mean ± SEM		
Morning			
Baseline SBP (mm Hg)	111.7 ± 1.4	114.1 ± 2.0	0.336[d]
Baseline DBP (mm Hg)	68.3 ± 1.5	69.7 ± 1.5	0.732[d]
SBP Δ(mm Hg)	2.0 ± 2.0	2.8 ± 2.0	0.426[d]
DBP Δ(mm Hg)	1.0 ± 2.1	1.6 ± 1.8	0.392[d]
Daytime			
Baseline SBP (mm Hg)	115.3 ± 1.6	117.1 ± 1.5	0.582[d]
Baseline DBP (mm Hg)	70.8 ± 1.2	70.9 ± 1.3	0.915[d]
SBP Δ(mm Hg)	1.2 ± 1.6	0.0 ± 1.7	0.833[d]
DBP Δ(mm Hg)	1.0 ± 1.8	2.4 ± 2.4	0.523[d]
Nighttime			
Baseline SBP (mm Hg)	98.1 ± 1.0	99.7 ± 1.2	0.329
Baseline DBP (mm Hg)	57.4 ± 0.9	57.6 ± 0.8	0.418
SBP Δ (mm Hg)	1.7 ± 1.2	1.1 ± 1.2	0.638[d]
DBP Δ (mm Hg)	-0.2 ± 0.9	-0.1 ± 0.8	0.742
24-h			
Baseline SBP (mm Hg)	111.0 ± 1.1	110.7 ± 1.3	0.833
Baseline DBP (mm Hg)	66.8 ± 1.0	65.8 ± 0.8	0.701
SBP Δ (mm Hg)	0.1 ± 1.0	2.2 ± 1.0	0.277[d]
DBP Δ (mm Hg)	-0.7 ± 1.4	1.6 ± 0.7	0.072

[a]Morning blood pressure = average blood pressure during the first two hours after awakening;
Daytime blood pressure = average recorded during the rest of the day; nighttime blood pressure = average recorded from the time the subject went to be until the time he/she got out of bed.
[b]Change (signified by Δ) the change from baseline (week 0) to week 4.
[c]P-values for the response variables are for the analysis of covariance with baseline value as the covariate.
[d]Indicates values were ranked prior to analysis. Median values were similar to the means shown.
Abbreviations: DBP = diastolic blood pressure, SMP = systolic blood pressure, SEM = standard error of the mean
From: Maki et al. 2008b

Table 47. Supine and Standing Blood Pressure and Heart Rate Measurements During Meal Tests at Week 0 and 4

	Week 0			Week 4		
Parameter	**Rebaudioside A**	**Placebo**	**P-value**	**Rebaudioside A**	**Placebo**	**P-value[a]**
	(*n* = 50)	**(*n* = 50)**		**(*n* = 50)**	**(*n* = 48)**	
Supine Pre-meal						
SBP (mm Hg)	111.5 ± 1.5	109.7 ± 1.3	0.367	109.5 ± 1.2	111.5 ± 1.3	0.260
DBP (mm Hg)	69.6 ± 1.1	68.9 ± 1.1	0.647	67.8 ± 1.0	69.9 ± 1.0	0.154
MAP (mm Hg)	83.6 ± 1.1	82.5 ± 1.1	0.486	81.7 ± 1.0	83.7 ± 0.9	0.140
HR (bpm)	62.9 ± 1.5	66.1 ± 1.2	0.098	63.3 ± 1.5	67.0 ± 1.3	0.071
Δ Post-meal[b,c]						
SBP (mm Hg)	0.0 ± 0.9	0.9 ± 0.7	0.674	1.5 ± 1.0	-0.8 ± 1.0	0.180
DBP (mm Hg)	-1.3 ± 0.6	-1.2 ± 0.7	0.811	0.6 ± 0.6	-1.9 ± 0.8	0.045
MAP (mm Hg)	-0.9 ± 0.6	-0.5 ± 0.6	0.955	0.9 ± 0.7	-1.5 ± 0.8	0.043
HR (bpm)	2.9 ± 1.0	4.5 ± 0.6	0.172[d]	4.4 ± 0.7	2.8 ± 0.8	0.404

From: Maki et al. 2008b

Table 48. Changes from Baseline to Treatment of Blood Pressure Measurements in the Subgroup of Subjects with Baseline Systolic Blood Pressure Below the Sex-Specific Median[a,b]

Parameters	**Rebaudioside A**	**Placebo**	**P-value[c]**
	(*n* = 28)	**(*n* = 20)**	
Resting, Seated Measurements			
Baseline SBP (mm Hg)	104.4 + 0.8	103.1 + 1.7	0.444
SBP Δ (mm Hg)	0.3 + 0.8	2.4 + 0.8	0.114
Baseline DBP (mm Hg)	67.2 + 0.9	67.2 + 1.6	0.970
DBP Δ (mm Hg)	-0.5 + 0.5	1.1 + 0.7	0.091[d]
Baseline MAP (mm Hg)	79.6 + 0.7	79.1 + 1.5	0.752
MAP Δ (mm Hg)	-0.3 + 0.6	1.5 + 0.6	0.036
24-h Measurements			
Baseline SBP (mm Hg)	108.5 + 1.2	107.1 + 1.9	0.504
SBP Δ (mm Hg)	-0.5 + 1.5	1.6 + 1.6	0.798[d]
Baseline DBP (mm Hg)	66.2 + 1.1	65.3 +1.3	0.618
DBP Δ (mm Hg)	-1.5 + 0.9	0.0 + 1.0	0.347

[a]Median baseline SBP < 108 mm Hg for women and < 117 mm Hg for male.
[b]Change (signified by Δ) in resting, seated measurements represents the change from baseline (average of weeks -1 and 0) to treatment (average of weeks 1, 2, 3, and 4), and Δ in 24-h measurements represents the change from baseline (week 0) to week 4.
[c]P-values for the response variables are for the analysis of covariance with baseline value as the covariate.
[d]Values were ranked prior to analysis. Median values were similar to the means shown.
Abbreviations: DBP = diastolic blood pressure, SBP = systolic blood pressure, SEM = standard error of the mean
From: Maki et al. 2008b

Table 49. Serum Chemistry and Hematology Parameters at Screening (Week -1) and the Changes from Screening to Week 4[a]

Parameter	Rebaudioside A (n = 50)	Placebo (n = 50)	P-Value
	Mean ± SEM		
Screening Alanine Transaminase (U/L)	22.0 ± 1.5	19.9 ± 1.1	0.264
Alanine Transaminase Δ (U/L)	−1.0 ± 0.9	0.6 ± 1.4	0.331
Screening Aspartate Transaminase (U/L)	22.6 ± 1.0	20.9 ± 0.8	0.180
Aspartate Transaminase Δ (U/L)	−0.9 ± 0.7	0.1 ± 0.7	0.288
Screening Alkaline Phosphatase (U/L)	65.2 ± 2.7	65.9 ± 2.3	0.830
Alkaline Phosphatase Δ (U/L)	0.8 ± 0.8	0.0 ± 0.9	0.476
Screening Blood Urea Nitrogen (mg/dL)	13.7 ± 0.6	14.5 ± 0.6	0.306
Blood Urea Nitrogen Δ (mg/dL)	0.1 ± 0.4	−0.8 ± 0.4	0.141
Screening Creatinine (mg/dL)	0.8 ± 0.0	0.8 ± 0.0	1.000
Creatinine Δ (mg/dL)	0.0 ± 0.0	0.0 ± 0.0	0.155
Screening Gamma Glutamyl Transferase (U/L)	20.1 ± 1.7	17.7 ± 1.5	0.278
Gamma Glutamyl Transferase Δ (U/L)	−0.7 ± 0.7	0.6 ± 0.7	0.162
Screening Red Blood Cell Count (10^6/μL)	4.4 ± 0.1	4.4 ± 0.1	0.669
Red Blood Cell Count Δ (10^6/μL)	0.0 ± 0.0	0.0 ± 0.0	0.201
Screening White Blood Cell Count (10^3/μL)	6.0 ± 0.2	6.3 ± 0.2	0.356
White Blood Cell Count Δ (10^3/μL)	0.3 ± 0.2	0.2 ± 0.2	0.884
Screening Basophil (%)[b]	0.51 ± 0.06	0.47 ± 0.07	0.655
Basophil Δ (%)	−0.09 ± 0.07	−0.05 ± 0.10	0.741
Screening Hemoglobin (g/dL)	13.3 ± 0.2	13.6 ± 0.2	0.292
Hemoglobin Δ (g/dL)	0.0 ± 0.1	−0.1 ± 0.1	0.396
Screening Hematocrit (%)	39.8 ± 0.5	40.8 ± 0.4	0.124
Hematocrit Δ (%)	0.3 ± 0.3	−0.2 ± 0.3	0.192

[a]Change from baseline to week 4 is indicated by Δ.
[b]Basophil (5) is presented because it was the only subset of white blood cells that showed a significant difference between rebaudioside A and placebo in another study (Maki *et al.* 2008).
SEM = standard error of the mean
Summary of studies identified in this section is presented in table 50.
From: Maki et al. 2008b

A summary of the studies discussed in this section is found in Table 50.

000170

0001171

Table 50. Human Studies of the Effects of Steviol Glycosides on Blood Pressure

Reference	Test Material	Subjects	Methods	Result and Discussion
Barriocanal et al., 2008	Steviol glycosides which was ≥ 92% pure (measured three times) was provided by Steviafarma Industrial S.A., Maringa, Brazil	Randomized, double-blind, placebo-controlled, longterm study in three groups of patients. 76 Subjects (30 with type 2 diabetes; 16 with type 1 diabetes; 30 without diabetes)	Group 1: 16 subjects (8 on treatment and 8 on placebo) with Type 1 diabetes (normotensive or hypertensive); Group 2: 30 subjects (15 on treatment and 15 on placebo) with Type 2 diabetes (normotensive or hypertensive); and Group 3: 30 healthy subjects (13 on treatment and 17 on placebo) with normal/low-normal blood pressure levels. The subjects in each group were randomly allocated to active treatment (the steviol glycoside stevioside: 250mg tds) or to placebo treatment and followed-up for 3 months. Blood pressures and a full panel of blood analysis were conducted at the beginning and at the end of the study.	Post-treatment systolic blood pressure, diastolic blood pressure, glucose and glycated hemoglobin (HbA_{1c}) were not significantly different from baseline measurements, except for the placebo Type 1 diabetics group where a significant difference was observed for systolic blood pressure (decreased after treatment) and glucose (increased after treatment). No side effects were observed in the two treatments for all three subject groups. This study shows that oral steviol glycosides, taken as sweetener are well tolerated and have no pharmacological effect.
Chan et al., 2000	Stevioside (Nan Kai Chemical Factory, Tien Jing, China) capsules.	106 Chinese hypertensive subjects with diastolic blood pressure between 95 and 110 mmHg and ages ranging from 28 to 75 years with 60 subjects (men 34, women 26; mean ± s.d., 54.1 ± 3.8 years) allocated to active treatment and 46 (men 19, women 27; mean ± s.d., 53.7 ± 4.1 years) to placebo treatment.	A multicenter, randomized, double-blind, placebo-controlled study Each subject was given capsules containing stevioside (250 mg) or placebo thrice daily and followed-up at monthly intervals for 1 year, for blood pressures and blood chemistry.	After 3 months, the systolic and diastolic blood pressure of the stevioside group decreased significantly (systolic: 166.0 ± 9.4–152.6 ± 6.8 mmHg; diastolic: 104.7 ± 5.2–90.3 ± 3.6 mmHg, $P < 0.05$), and the effect persisted during the whole year. Blood biochemistry parameters including lipid and glucose showed no significant changes. No significant adverse effect was observed and quality of life assessment showed no deterioration.

Table 50. Human Studies of the Effects of Steviol Glycosides on Blood Pressure

Reference	Test Material	Subjects	Methods	Result and Discussion
Ferri et al., 2006	Standard crude stevioside was obtained from dried leaves, which were collected from a farm in north-eastern of Parana state (southern Brazil), resulting in a mixture of crude stevioside and rebaudioside A.	Previously untreated patients with mild hypertension	A single center, randomized, double-blind, placebo-controlled study on drug naive patients with mild hypertension, who received capsules containing either placebo for 24 weeks or crude stevioside 3.75 mg/kg/day (7 weeks), 7.5 mg/kg/day (11 weeks) and 15.0 mg/kg/day (6 weeks). Each group consisted of 7 patients (6 men and 1 woman), who were prescribed with capsules twice a daily (b.i.d.), i.e. before lunch and before dinner. After the placebo phase and after each administration of crude stevioside, body mass index, electrocardiogram and laboratory tests were performed. Blood pressure was measured biweekly and the blood chemistry including metabolic and hormonal parameters was analyzed at the end of each stevioside administration step.	No major adverse clinical effects were observed during the trial. Systolic and diastolic blood pressure decreased ($p < 0.05$) during the treatment with crude stevioside, but a similar effect was also observed in the placebo group. Therefore, crude stevioside up to 15.0 mg/kg/day did not show an antihypertensive effect. Moreover, the results suggest that oral crude stevioside is safe and supports the well-established tolerability during long term use as a sweetener in Brazil.
Geuns et al., 2007	Commercial stevioside (Stevita, Maringa, Brazil) that was purified by repeated crystallization from methanol to a purity level of more than 97%. Steviolbioside (around 2.7%) and rebaudioside A (around 0.3%) were the main impurities.	10 healthy subjects (5 female and 5 male)	Stevioside (250-mg capsules) thrice daily for 3 days. Control study (a glass of drinking water without stevioside capsule) was conducted on the same subjects 3 weeks after the stevioside test. Urine, blood and feces were analyzed and stevioside was incubated with stomach juice to study the metabolism and fate of oral stevioside. In addition, blood pressure and plasma glucose and insulin concentrations were measured.	Between the control and stevioside, there are no significant differences for blood pressure and blood biochemical parameters, for urinary volume and urinary excretion of electrolytes, and for mean blood glucose and insulin. Thus, oral stevioside is not directly effective as a hypotensive or hypoglycemic agent in healthy subjects at the level administered in this study. Stevioside is not up taken by the gastrointestinal tract, because stomach juice does not degrade stevioside. All the stevioside reaching the colon is degraded by microorganisms into steviol, the only metabolite found in feces.

000172

000173

Table 50. Human Studies of the Effects of Steviol Glycosides on Blood Pressure

Reference	Test Material	Subjects	Methods	Result and Discussion
Hsieh et al., 2003	Stevioside (Nan Kai Chemical Factory, Tien Jing, China) capsules.	Male and female Chinese with mild essential hypertension. Aged 20-75 years	Multicenter, randomized, double-blind, placebo-controlled trial Patients took capsules containing 500 mg stevioside powder or placebo 3 times daily for 2 years. Blood pressure was measured at monthly clinic visits. Patients were also encouraged to monitor blood pressure at home. Left ventricular mass index (LVMI) was determined by 2-dimensional echocardiography at baseline and after 1 and 2 years of treatment. Quality of life (QOL) was assessed using the Medical Outcomes Study 36-Item Short-Form Health Survey. Electrocardiographic, laboratory, and QOL parameters were assessed at the beginning of treatment, and at 6 months, 1 year, and 2 years.	82 (42 men and 40 women) in the stevioside group and 86 (44 women and 42 men) in the placebo group completed the trial. After 2 years, the stevioside group had significant decreases in mean [SD] SBP and DBP compared with baseline (SBP, from 150 [7.3] to 140 [6.8] mm Hg; DBP, from 95 [4.2] to 89 [3.2] mm Hg; $P < 0.05$) and compared with placebo ($P < 0.05$). These effects were noted beginning approximately 1 week after the start of treatment and persisted throughout the study. There were no significant changes in body mass index or blood biochemistry, and the results of laboratory tests were similar in the 2 groups throughout the study. No significant difference in the incidence of adverse effects was noted between groups, and QOL scores were significantly improved overall with stevioside compared with placebo ($P < 0.001$). Neither group had a significant change in mean LVMI.
Maki et al. 2008b	Rebaudioside A	50 M+F; age 18-74 yrs; (rebaudioside A) 50 M+F; age 18-74 yrs; (placebo) all subjects have normal and low-normal blood pressure	250 mg rebaudioside A 4x/d (total of 1000 mg/d); rebaudioside A purity 97%) 250 mg microcrystalline cellulose 4x/d (total of 1000 mg/d) 4 weeks	Subjects were predominantly females (76%, rebaudioside A and 82%, placebo) with a mean age of ~41 (range 18-73) years. At baseline, mean resting, seated SBP/DBP was 110.0/73.3 mm Hg and 110.7/71.2 mm Hg for the rebaudioside A and placebo groups, respectively. Compared with Placebo, rebaudioside A did not significantly altered resting, seated SBP, DBP, mean arterial pressure (MAP), heart rate (HR) or 24-h ambulatory blood pressure responses. Consumption of as much as 1000 mg/day of rebaudioside A produced no clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure.

V. References

Abudula R, Matchkov VV, Jeppesen PB, Nilsson H, Aalkjær C, Hermansen K. 2008. Rebaudioside A directly stimulates insulin secretion from pancreatic beta cells: a glucose-dependent action via inhibition of ATP-sensitive K(+)-channels. *Diabetes Obes Metab.* [Epub ahead of print]

Abudula R, Jeppesen PB, Rolfsen SE, Xiao J, and Hermansen K. 2004. Rebaudioside A potently stimulates insulin secretion from isolated mouse islets: studies on the dose-, glucose-, and calcium-dependency. *Metabolism.* 53:1378-1381.

Ahmed MS and Dobberstein RH. 1982. III. High-performance liquid chromatographic separation and quantitation of rebaudiosides B, D, and E, dulcoside A, and steviolbioside. *J Chromatog.* 245:373-376.

Alvarez M. 2006. Stevioside: Toxicological Aspects. http://cookingwithstevia.com/web/reports12.shtml.

Alves LM and Ruddat M. 1979. The presence of gibberellin A20 in stevia rebaudiana and its significance for the biological activity of steviol. *Plant and Cell Physiol.* 20:123-130.

Aritajat S, Kaweewat K, Manosroi J, and Manosroi A. 2000. Dominant lethal test in rats treated with some plant extracts. *Southeast Asian J Trop Med Public Health.* 31 (Suppl 1):171-3.

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

Barriocanal LA, Palacios M, Benitez G, Benitez S, Jimenez JT, Jimenez N, Rojas V. 2008. Apparent lack of pharmacological effect of steviol glycosides used as sweeteners in humans. A pilot study of repeated exposures in some normotensive and hypotensive individuals and in Type 1 and Type 2 diabetics. *Regul Toxicol Pharmacol.* 51(1):37-41.

Bondarev NI, Sukhanova MA, Reshetnyak OV, and Nosov AM. 2003. Steviol glycoside content in different organs of stevia rebaudiana and its dynamics during ontogeny. *Biologia Plantarum.* 47:261-264.

Boonkaewwan C, Toskulkao C, and Vongsakul M. 2006. Anti-inflammatory and immunomodulatory activities of stevioside and its metabolite steviol on THP-1 cells. *J Agric Food Chem.* 54:785-789.

Bovanova L, Brandsteterova E, and Baxa S. 1998. HPLC determination of stevioside in plant material and food samples. *Z Lebensm Unters Forsch A.* 207:352-355.

Brandle JE, Starratt AN, and Gijzen M. 1998. Stevia rebaudiana: Its biological, chemical and agricultural properties. *Can J Plant Sci.* 78:527-536.

Carakostas M.C., Curry L.L., Boileau A.C., Brusick D.J. 2008. Overview: The history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. *Food Chem. Toxicol.* 46: S1-S10

Cardoso VN, Barbosa MF, Muramoto E, Mesquita CH, and Almeida MA. 1996. Pharmacokinetic studies of 131I-stevioside and its metabolites. *Nucl Med Biol.* 23:97-100.

Chan P, Xu DY, Liu JC, Chen YJ, Tomlinson B, Huang WP, and Cheng JT. 1998. The effect of stevioside on blood pressure and plasma catecholamines in spontaneously hypertensive rats. *Life Sci.* 63:1679-1684.

Chan P, Tomlinson B, Chen YJ, Liu JC, Hsieh MH, and Cheng JT. 2000. A double-blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in human hypertension. *Br J Clin Pharmacol.* 50:215-220.

Chang J-C, Wu MC, Liu I-M, and Cheng J-T. 2005. Increase of insulin sensitivity by stevioside in fructose-rick chow-fed rats. *Horm Metab Res.* 37:610-616.

Charles River. 2006. Clinical laboratory parameters for Crl:CD(SD) rats. March 2006.

Chen TH, Chen, SC, Chan P, Chu YL, Yang HY and Cheng JT. 2005. Mechanism of the hypoglycemic effect of stevioside, a glycoside of *Stevia rebaudiana*. *Planta Med.* 71:108-113.

Chen J, Jeppensen PB, Abudula R, Dyrskog SEU, Colombo M, and Hermansen K. 2006. Stevioside does not cause increased basal insulin secretion or beta-cell sensitization as does the sulphonylurea, glibenclamide: Studies *in vitro*. *Life Sci.* 78:1748-1753.

Chen J, Jeppesen PB, Nordentoft I, Hermansen K. 2007. Stevioside improves pancreatic beta-cell function during glucotoxicity via regulation of acetyl-CoA carboxylase. *Am J Physiol Endocrinol Metab.* 292: E1906-16.

Chung MS, Suh HJ, Yoo W, Choi SH Cho YJ, Cho YH, and Kim CJ. 2005. Daily intake assessment of saccharin, stevioside, D-sorbitol and aspartame from various processed foods in Korea. *Food Addit Contam.* 22(11):1087-1097.

Cioni PL, Morelli I, Andolfi L, Macchia M, and Ceccarini L. 2006. Qualitative and quantitative analysis of essential oils of five lines Stevia rebaudiana Bert. genotypes cultivated in Pisa (Italy). *J Essent Oil Res.* 18:76-79.

Compadre CM, Hussain RA, Nanayakkara NP, Pezzuto JM, and Kinghorn AD. 1988. Mass spectral analysis of some derivatives and *in vitro* metabolites of steviol, the aglycone of the natural sweeteners, stevioside, rebaudioside A, and rubusoside. *Biomed Environ Mass Spectrom.* 15:211-222.

000175

Curi R, Alvarez M, Bazotte RB, Botion LM, Godoy JL, and Bracht A. 1986. Effect of Stevia rebaudiana on glucose tolerance in normal adult humans. *Braz J Med Biol Res.* 19:771-774.

Curry LL, Roberts A, Brown N. 2008. Rebaudioside A: Two-generation reproductive toxicity study in rats. *Food Chem Toxicol.* 46: S21-S30.

Curry LL and Roberts A. 2008. Subchronic toxicity of rebaudioside A. *Food Chem Toxicol.* 46: S11-S20.

Dyrskog SE, Jeppesen PB, Colombo M, Abudula R, and Hermansen K. 2005a. Preventive effects of a soy-based diet supplemented with stevioside on the development of the metabolic syndrome and type 2 diabetes in Zucker diabetic fatty rats. *Metabolism.* 54:1181-1188.

Dyrskog SE, Jeppesen PB, Chen J, Christensen LP, Hermansen K. 2005b. The diterpene glycoside, rebaudioside A, does not improve glycemic control or affect blood pressure after eight weeks treatment in the Goto-Kakizaki rat. *Rev Diabet Stud.* 2:84-91.

Ferreira EB, Assis Rocha NF, da Costa MA, do Prado WA, Araujo Funari FL, and Bazotte RB. 2006a. Comparative effects of stevia rebaudiana leaves and stevioside on glycaemia and hepatic gluconeogenesis. *Planta Med.* 72:691-696.

Ferreira EB, Assis Rocha NF, da Costa MA, do Prado WA, Araujo Funari FL, and Bazotte RB. 2006b. Supporting information to: Comparative effects of stevia rebaudiana leaves and stevioside on glycaemia and hepatic gluconeogenesis. *Planta Med.* 72:1-2.

Ferri LA, Alves-Do-Prado W, Yamada SS, Gazola S, Batista MR, and Bazotte RB. 2006. Investigation of the antihypertensive effect of oral crude stevioside in patients with mild essential hypertension. *Phytother Res.* Sep;20(9):732-6.

Food and Drug Administration. 2006. Estimating Exposure To Direct Food Additives And Chemical Contaminants in the Diet.

Food Standards Australia New Zealand (FSANZ). 2008. Final assessment report application A540. Steviol glycosides as intense sweeteners. August 6, 2008

Gardana C, Simonetti P, Canzi E, Zanchi R, and Pietta P. 2003. Metabolism of stevioside and rebaudioside A from Stevia rebaudiana extracts by human microflora. *J Agric Food Chem.* 51:6618-6622.

Geuns JMC. 2000. Safety of *Stevia* and stevioside. *Recent Res. Devel. Phytochem.* 4:75-88.

Geuns JMC. 2003. Stevioside. *Phytochem.* 64:913-921.

Geuns JM, Bruggeman V, and Buyse JG. 2003a. Effect of stevioside and steviol on the developing broiler embryos. *J Agric Food Chem.* 51:5162-5167.

Geuns JM, Augustijns P, Mols R, Buyse JG, and Driessen B. 2003b. Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. *Food Chem Toxicol.* 41:1599-1607.

Geuns JM, Buyse J, Vankeirsbilck A, Temme EH, Compernolle F, and Toppet S. 2006. Identification of steviol glucuronide in human urine. *J Agric Food Chem.* 54:2794-2798.

Geuns JM. 2007. Letter to the Editor. Comments to the paper by Nunes et al. (2007), Analysis of genotoxic potentiality of stevioside by comet assay, *Food and Chemical Toxicology* 45 (2007) 662-666. *Food Chem Toxicol.* 45:2601-2.

Geuns JM, Buyse J, Vankeirsbilck A, Temme EH. 2007. Metabolism of stevioside by healthy subjects. *Exp Biol Med.* 232(1):164-73.

Gregersen S, Jeppesen PB, Holst JJ, and Hermansen K. 2004. Antihyperglycemic effects of stevioside in type 2 diabetic subjects. *Metabolism.* 53:73-76.

Hagiwara A, Fukushima S, Kitaori M, Shibata M, and Ito N. 1984. Effects of three sweeteners on rat urinary bladder carcinogenesis initiatiated by N-butyl-N-(4-hydroxybutyl)-nitrosamine. *Gann.* 75:763-786.

Hashimoto Y, Moriyasu M, Nakamura S, Ishiguro S, and Komuro M. 1978. High-performance liquid chromatographic determination of stevia components on a hydrophilic packed column. *J Chromatogr.* 161:403-405.

Hirokado M, Nakajima I, Nakajima K, Mizoiri S, and Endo F. 1980. An analytical method for stevia components in natural sweetener preparation. *J Food Hyg Soc Jpn.* 21:451-456.

Hsieh MH, Chan P, Sue YM, Liu JC, Liang TH, Huang TY, Tomlinson B, Chow MS, Kao PF, and Chen YJ. 2003. Efficacy and tolerability of oral stevioside in patients with mild essential hypertension: a two-year, randomized, placebo-controlled study. *Clin Ther.* 25:2797-2808.

Hsu YH, Liu JC, Kao PF, Lee CN, Chen YJ, Hsieh MH, and Chan P. 2002. Antihypertensive effect of stevioside in different strains of hypertensive rats. *Zhonghua Yi Xue Za Zhi (Taipei).* 65:1-6.

Hübler MO, Bracht A and Kelmer-Bratch AM. 1994. Influence of stevioside on hepatic glycogen levels in fasted rats. *Research Communications in Chemical Pathology and Pharmacology.* 84(1):111-118.

Hutapea AM, Toskulkao C, Wilairat P, and Buddhasukh D. 1999. High-performance liquid chromatographic separation oand quantitation of stevioside and its metabolites. *J Liq Chromatogr Rel Technol.* 22:1161-1170.

Hutapea AM, Toskulkao C, Buddhasukh D, Wilairat P, and Glinsukon T. 1997. Digestion of steviosdie, a natural sweetener, by various digestive enzymes. *J Clin Biochem Nutr.* 23:177-186.

Ishidate M, Jr., Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada M, and Matsuoka A. 1984. Primary mutagenicity screening of food additives currently used in Japan. *Food Chem Toxicol.* 22:623-636.

Ishii EL, Schwab AJ, and Bracht A. 1987. Inhibition of monosaccharide transport in the intact rat liver by stevioside. *Biochem Pharmacol.* 36:1417-1433.

Ishii-Iwamoto E and Bracht A. 1995. Stevioside is not metabolized in the isolated perfused rat liver. *Research Comm Molecular Pathol Pharmacol.* 87:167-175.

JECFA (Joint FAO/WHO Expert Committee on Food Additives). 2007. (Sixty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives).

JECFA (Joint FAO/WHO Expert Committee on Food Additives). 2005. 64th Meeting, Rome, Italy [ftp://ftp.fao.org/es/esn/jecfa/jecfa64_summary.pdf]

JECFA (Joint FAO/WHO Expert Committee on Food Additives). 1999. 53rd Meeting, ftp://ftp.fao.org/es/esn/jecfa/2002-09-10_Explanatory_note_Heavy_Metals.pdf

Jeppesen PB, Dyrskog SE, Agger A, Gregersen S, Colombo M, Xiao J, Hermansen K. 2006. Can stevioside in combination with a soy-based dietary supplement be a new useful treatment of type 2 diabetes? An in vivo study in the diabetic goto-kakizaki rat. *Rev Diabet Stud.* 3:189-99.

Jeppesen PB, Gregersen S, Rolfsen SE, Jepsen M, Colombo M, Agger A, Xiao J, Kruhoffer M, Orntoft T, and Hermansen K. 2003. Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. *Metabolism.* 52:372-378.

Jeppesen PB, Gregsen S, Alstrupp KK and Hermansen K. 2002. Stevioside induces antihyperglycaemic, insulinotropic and glucagonostatic effects *in vivo*: studies in the diabetic Goto-Kakizaki (GK) rats. *Phytomedicine.* 9:9-14.

Jeppesen PB, Gregersen S, Poulsen CR, and Hermansen K. 2000. Stevioside acts directly on pancreatic β cells to secrete insulin: actions idependent of cyclic adenosine monophosphate and adenosine triphosphate-sensitive K^+-channel activity. *Metabolism.* 49(2):208-214.

Jeppesen, P. B., Gregersen, S., Hermansen. K. 1996. Stevioside and steviol stimulate insulin secretion from isolated mouse islets. *Diabetologia.* Aug;39(Suppl 1)A125: 472 (Abstract)

Jutabha P, Toskulkao C, and Chatsudthipong V. 2000. Effect of stevioside on PAH transport by isolated perfused rabbit renal proximal tubule. *Can J Physiol Pharmacol.* 78:737-744.

Kemmelmeier FS., Yammamoto NS., Ishii EL., Kelmer-Bracht AM., Bracht A. 1984. Acao dos produtos naturais da stevia rebaudiana sobre a glicolise em reticulociters e eritrocitos. *Rev Unimar*, 6: 83-97.

Kerr WE, Mello M.L.S., and Gonadio E. 1983. Mutagenicty tests on the stevioside from stevia rebaudiana (Bert.) Bertoni. *Rev Brasil Genet.* VI:173-176.

Kitada Y, Sasaki M, Yamazoe Y, and Nakazawa H. 1989. Simultaneous determination of stevioside, rebaudioside A and C and dulcoside A in foods by high-performance liquid chromatography. *J Chromatog.* 474:447-451.

Klongpanichpak S, Temcharoen P, Toskulkao C, Apibal S, and Glinsukon T. 1997. Lack of mutagenicity of stevioside and steviol in Salmonella typhimurium TA 98 and TA 100. *J Med Assoc Thai.* 80 Suppl 1:S121-8.

Kobayashi M, Horidawa S, Degrandi IH, Ueno J, and Mitsuhashi H. 1977. Dulcosides A and B, new diterpene glycosides from stevia rebaudiana. *Phytochem.* 16:1405-1408.

Kolb N, Herrera JL, Ferreyra DJ, and Uliana RF. 2001. Analysis of sweet diterpene glycosides from stevia rebaudiana: Improved HPLC method. *J Agric Food Chem.* 49:4538-4541.

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

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

Kuznesof PM. 2007. Steviol Glycosides, Chemical and Technical Assessment (Original prepared by Harriet Wallin for the 63rd JECFA; Revised by Paul M. Kuznesof, Ph.D. for the 68th JECFA). Available online at http://www.fao.org/ag/agn/agns/files/jecfa68/CTA%20Steviol%20glycosides%20-%20Final%202007.pdf accessed on July 21, 2008

Lailerd N, Saengsirisuwan V, Sloniger JA, Toskulkao C and Henriksen EJ. 2004. Effects of Stevioside on glucose transport activity in insulin-sensitive and insulintesistant rat skeletal muscle. *Metabolism.* 53(1):101-107.

Lee CN, Wong KL, Liu JC, Chen YJ, Cheng JT, and Chan P. 2001. Inhibitory effect of stevioside on calcium influx to produce antihypertension. *Planta Med.* 67:796-799.

Liu J-C, Kao P-K, Hsu Y-H, Hou C-C, Lien G-S, Hsieh M-H, Chen Y-J, and Cheng J-T. 2003. Mechanism of the antihypertensive effect of stevioside in anesthetized dogs. *Pharmacol.* 67:14-20.

Liu J and Li SFY. 1995. Separation and determination of stevia sweeteners by capillary electrophoresis and high performance liquid chromatography. *J Liq Chromatogr.* 18:1703-1713.

Makapugay HC, Nanayakkara NPD, and Kinghorn AD. 1984. Improved high-performance liquid chromatographic separation of the stevia rebaudiana sweet diterpene glycosides using linear gradient elution. *J Chromatogr.* 283:390-395.

000179

Maki KC, Curry LL, Reeves MS, Toth PD, McKenney JM, Farmer MV, Schwartz SL, Lubin BC, Boileau AC, Dicklin MR, Carakostas MC, Tarka SM. 2008a. Chronic consumption of rebaudioside A, a steviol glycoside, in men and women with type 2 diabetes mellitus. *Food Chem Toxicol.* 46:S47-S53.

Maki KC, Curry LL, Carakostas MC, Tarka SM, Reeves MS, Farmer MV, McKenney JM, Toth PD, Schwartz SL, Lubin BC, Dicklin MR, Boileau AC, Bisognano JD. 2008b. The hemodynamic effects of rebaudioside A in healthy adults with normal and low-normal blood pressure. *Food Chem Toxicol.* 46: S40-S46.

Malaisse WJ, Vanonderbergen A, Louchami K, Jijakli H, and Malaisse-Lagae F. 1998. Effects of artificial sweeteners on insulin release and cationic fluxes in rat pancreatic islets. *Cell Signal.* 10:727-733.

Matsui M, Matsui K, Kawasaki Y, Oda Y, Noguchi T, Kitagawa Y, Sawada M, Hayashi M, Nohmi T, Yoshihira K, Ishidate M Jr, and Sofuni T. 1996. Evaluation of the genotoxicity of stevioside and steviol using six *in vitro* and one *in vivo* mutagenicity assays. *Mutagenesis.* 11(6):573-9.

Mauri P, Catalano G, Gardana C, and Pietta P. 1996. Analysis of stevia glycosides by capillary electrophoresis. *Electrophoresis.* 17:367-371.

McGarvey BD, Attygalle AB, Starratt AN, Xiang B, Schroeder FC, Brandle JE, and Meinwald J. 2003. New non-glycosidic diterpenes from the leaves of stevia rebaudiana. *J Nat Prod.* 66:1395-1398.

McNeil. 2008. 90-day oral (diet) toxicity study with stevia glycosides in Sprague-Dawley rats. RCC NA Study Number RCCMIS-008.

Medon PJ, Pezzuto, JM, Hovanec-Brown JM, Nanayakkara, NPD, Soejarto, DD, Kamath SK, Kinghorn AD. 1982. Safety assessment of some stevia rebaudiana sweet principles. *Fed Proc*. 41:Absract No 7857.

Melis MS and Sainati AR. 1991a. Effect of calcium and verapamil on renal function of rats during treatment with stevioside. *J Ethnopharmacol.* 33:257-262.

Melis MS and Sainati AR. 1991b. Participation of prostaglandins in the effect of stevioside on rat renal function and arterial pressure. *Braz J Med Biol Res.* 24:1269-76.

Melis MS. 1992a. Stevioside effect on renal function of normal and hypertensive rats. *J Ethnopharmacol.* 36:213-217.

Melis MS. 1992b. Renal excretion of stevioside in rats. *J Nat Prod.* 55:688-90.

Mélis MS. 1992c. Influence of calcium on the blood pressure and renal effects of stevioside. *Braz J Med Biol Res.* 1992; 25:943-9.

Melis MS. 1995. Chronic administration of aqueous extract of Stevia rebaudiana in rats: renal effects. *J Ethnopharmacol.* 28; 47:129-34.

Melis MS. 1996. A crude extract of Stevia rebaudiana increases the renal plasma flow of normal and hypertensive rats. *Braz J Med Biol Res.* 29:669-75.

Melis MS. 1999. Effect of crude extract of Stevia rebaudiana on renal water and electrolytes excretion. *Phytomedicine.* 6:247-250.

Metivier J and Viana AM. 1979. Determination of microgram quantities of stevioside from leaves of stevia rebaudiana Bert. by two-dimensional thin layer chromatography. *J Exp Bot.* 30(4):805-810.

Mizukami H, Shiiba K, and Ohashi H. 1982. Enzymatic determination of stevioside in stevia rebaudiana. *Phytochem.* 21:1927-1930.

Mori N, Sakanoue M, Takeuchi M, Shimpo K, and Tanabe T. 1981. Effect of stevioside on fertility in rats. *J Food Hyg Soc Jpn.* 22(5):409-414.

Nakayama K, Kashara D, and Yamamoto F. 1986. Absorption, distribution, metabolism and excretion of stevioside in rats. *J Food Hyg Soc Jpn.* 27:1-8.

Nepovím A, Drahosova H, Valícek P, and Vanek T. 1998. The effect of cultivation conditions on the content of stevioside in stevia rebaudiana Bertoni. *Pharm Pharmacol Lett.* 8(1):19-21.

Nikiforov AI and Eapen AK. 2008. A 90-day oral (dietary) toxicity study of rebaudioside A in Sprague-Dawley rats. *Int J Toxicol.* 27(1):65-80.

Nikolova-Damyanova B, Bankova V, and Popov S. 1994. Separation and quantitation of stevioside and rebaudioside A in plant extracts by normal-phase high performance liquid chromatography and thin-layer chromatograph: A comparison. *Phytochem analysis.* 5:81-85.

Nishiyama P, Alvarez M, and Viera LGE. 1992. Quantitative analysis of stevioside in the leaves of stevia rebaudiana by near infrared reflectance spectroscopy. *J Sci Food Agric.* 59:281.

Nunes AP, Ferreira-Machado SC, Nunes RM, Dantas FJ, De Mattos JC, Caldeira-de-Araújo A. 2007. Analysis of genotoxic potentiality of stevioside by comet assay. *Food Chem Toxicol.* 45:662-6.

Oh H-Y, Han E-S, Choi D-W, Kim J-W, Eom M-O, Kang I-H, Kang H-J, Ha K-W. 1999. *In vitro* and *In vivo* evaluation of genotoxicity of stevioside and steviol, natural sweetener. J *Pharmaceut Soc Korea.* 43(5): 614-622.

Oliveira-Filho RM, Uehara OA, Minetti CA, and Valle LB. 1989. Chronic administration of aqueous extract of Stevia rebaudiana Bertoni in rats: endocrine effects. *Gen Pharmacol.* 20:187-191.

Oshima Y, Saito J, and Hikino H. 1986. Sterebins A, B, C and , bisnorditerpenes of stevia rebaudiana leaves. *Tetrahedron.* 42:6443-6446.

Oshima Y, Saito J, and Hikino H. 1988. Sterebins E, F, G, and H, diterpenoids of stevia rebaudiana leaves. *Phytochemistry.* 27:624-626.

Pasquel A, Meireles MAA, Marques MOM, and Petenate AJ. 2000. Extraction of stevia glycosides with CO_2 + water, CO_2 + ethanol, and CO_2 + water + ethanol. *Braz J Chem Eng.* 17(3). http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0104-66322000000300003&lng=pt&nrm=iso&tlng=en.

Pezzuto JM, Compadre CM, Swanson SM, Nanayakkara D, and Kinghorn AD. 1985. Metabolically activated steviol, the aglycone of stevioside, is mutagenic. *Proc Natl Acad Sci U S A.* 82:2478-2482.

Pezzuto JM, Nanayakkara NP, Compadre CM, Swanson SM, Kinghorn AD, Guenthner TM, Sparnins VL, Lam LK. 1986. Characterization of bacterial mutagenicity mediated by 13-hydroxy-ent-kaurenoic acid (steviol) and several structurally related derivatives and evaluation of potential to induce glutathione S-transferase in mice. *Mutat Res.* 169(3):93-103.

Planas GM and Kuc J. 1968. Contraceptive properties of stevia rebaudiana. *Science.* 162:1007.

Procinska E, Bridges BA, and Hanson JR. 1991. Interpretation of results with the 8-azaguanine resistance system in Salmonella typhimurium: no evidence for direct acting mutagenesis by 15-oxosteviol, a possible metabolite of steviol. *Mutagenesis.* 6:165-167.

Rajbhandari A and Roberts MF. 1983. The flavonoids of stevia rebaudiana. *J Nat Prod.* 46:194-195.

Raskovic A, Jakovljevic V, Mikov M, Gavrilovic M. 2004a. Joint effect of commercial preparations of Stevia rebaudiana Bertoni and sodium monoketocholate on glycemia in mice. *Eur J Drug Metab Pharmacokinet.* 29:83-6.

Raskovic A, Gavrilovic M, Jakovljevic V, Sabo J. 2004b. Glucose concentration in the blood of intact and alloxan-treated mice after pretreatment with commercial preparations of stevia rebaudiana (Bertoni). *European Journal of Drug Metabolism and Pharmacokinetics.* 29(2):87-90.

Renwick AG. 2008. The use of a sweetener substitution method to predict dietary exposures for the intense sweetener rebaudioside A. Food Chem Toxicol. 46:S61-9.

Renwick AG and Tarka SM. 2008. Microbial hydrolysis of steviol glycosides. *Food Chem Toxicol.* 46: S70-S74.

Roberts A and Renwick AG. 2008. Comparative toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol in rats. *Food Chem Toxicol.* 46: S31-S39.

Sakamoto I, Yamasaki K, and Tanaka O. 1977. Application of ^{13}C NMR spectroscopy to chemistry of natural glycosides: rebaudioside-C, a new sweet diterpene glycoside of stevia rebaudiana. *Chem Pharm Bull.* 25:844-846.

Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K, Taniguchi K, and Tsuda S. 2002. The comet assay with 8 mouse organs: Results with 39 currently used food additives. *Mutat Res.* 519:103-119.

Scientific Committee on Food (SCF). 1999. *Opinion on stevioside as a sweetener.* http://www.dbcomputers.com.py/www/stevia/ftp/Yerba%20Dulce/Comis%20Euro%2099.pdf.

Sekihashi K, Saitoh H, and Sasaki Y. 2002. Genotoxicity studies of stevia extract and steviol by the comet assay. *J Toxicol Sci.* 27 Suppl 1:1-8. [Japanese]

Shibata H, Sonoke S, Ochiai H, Nishihashi H, and Yamada M. 1991. Glucosylation of steviol and steviol-glucosides in extracts from stevia rebaudiana bertoni. *Plant Physiol.* 95:152-156.

Shibata H, Sawa Y, Oka T, Sonoke S, Kim KK, and Yoshioka M. 1995. Steviol and steviol-glycoside: glucosyltransferase activities in stevia rebaudiana Bertoni--purification and partial characterization. *Arch Biochem Biophys.* 321:390-396.

Suanarunsawat T and Chaiyabutr N. 1997. The effect of stevioside on glucose metabolism in rat. *J Physiol Pharmacol.* 75:976-982.

Suttajit M, Vinitketkaumnuen U, Meevatee U, and Buddhasukh D. 1993. Mutagenicity and human chromosomal effect of stevioside, a sweetener from Stevia rebaudiana Bertoni. *Environ Health Perspect.* 101(Suppl 3):53-6.

Tamura Y, Nakamura S, Fukui H, and Tabata M. 1984. Comparison of stevia plants grown from seeds, cuttings and stem-tip cultures for growth and sweet diterpene glucosides. *Plant Cell Reports.* 3:180-182.

Temcharoen P, Suwannatrai M, Klongpanichpak S, Apibal S, Glinsukon T, and Toskulkao C. 2000. Evaluation of the effect of steviol on chromosomal damage using micronucleus test in three laboratory animal species. *J Med Assoc Thai.* 83(Suppl 1):S101-8.

Terai T, Ren H, Mori G, Yamaguchi Y, Hayashi T. 2002. Mutagenicity of steviol and its oxidative derivatives in Salmonella typhimurium TM677. *Chem Pharm Bull* (Tokyo). 50(7):1007-10.

Tomita T, Sato N, Arai T, Shiraishi H, Sato M, Takeuchi M, and Kamio Y. 1997. Bactericidal activity of a fermented hot-water extract from Stevia rebaudiana Bertoni towards

enterohemorrhagic Escherichia coli O157:H7 and other food-borne pathogenic bacteria. *Microbiol Immunol.* 41:1005-1009.

Toskulkao C, Sutheerawattananon M, and Piyachaturawat P. 1995. Inhibitory effect of steviol, a metabolite of stevioside, on glucose absorption in everted hamster intestine *in vitro*. *Toxicol Lett.* 80:153-159.

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

Toskulkao C, Suwannatrai M, Temcharoen P, Chaturat L, Suttajit M, Sahaphong S, and Glinsukon T. 2001. Chronic oral toxicity and carcinogenicity study of steviol, a metabolite of stevioside, in hamsters. *J Toxicol Pub Health.* 17:263-270.

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

United States Agency for International Development (USAID). 2004. *Stevia from Paraguay.* http://pdf.usaid.gov/pdf_docs/PNADB003.pdf.

Usami M, Sakemi K, Kawashima K, Tsuda M, and Ohno Y. 1995. Teratogenicity study of stevioside in rats. *Eisei Shikenjo Hokoku.* 113:31-35.

Wasuntarawat C, Temcharoen P, Toskulkao C, Mungkornkarn P, Suttajit M, and Glinsukon T. 1998. Developmental toxicity of steviol, a metabolite of stevioside, in the hamster. *Drug Chem Toxicol.* 21:207-222.

Wheeler A, Boileau AC, Winkler PC, Compton JC, Prakash I, Jiang X, Mandarino DA. 2008. Pharmacokinetics of rebaudioside A and stevioside after single oral doses in healthy men. *Food Chem Toxicol.* 46: S54-S60.

Wingard RE, Jr., Brown JP, Enderlin FE, Dale JA, Hale RL, and Seitz CT. 1980. Intestinal degradation and absorption of the glycosidic sweeteners stevioside and rebaudioside A. *Experientia.* 36:519-520.

World Health Organization (WHO). 1999. *Safety evaluation of certain food additives. WHO food additive series: 42.* http://www.inchem.org/documents/jecfa/jecmono/v042je07.htm.

World Health Organization (WHO). 2000. *Safety evaluation of certain food additives. WHO food additives series 42. Fifty-first report of the Joint FAO/WHO Expert Committee on Food Additives.* Geneva.

World Health Organization (WHO). 2004. *Compendium of food additive specifications. Joint FAO/WHO Expert Committee on Food Additives. 63rd Meeting.* Geneva. http://www.fao.org/docrep/007/y5777e/y5777e00.htm.

World Health Organization (WHO). 2005. *Evaluation of certain food additives. WHO technical report series 928. Sixty-third report of the Joint FAO/WHO Expert Committee on Food Additives*. Geneva. http://whqlibdoc.who.int/trs/WHO_TRS_928.pdf.

World Health Organization (WHO). 2006. *Safety evaluation of certain food addtives. WHO food additive series 54*. Geneva. http://whqlibdoc.who.int/publications/2006/9241660546_eng.pdf.

Xili L, Chengjiany B, Eryi X, Reiming S, Yuengming W, Haodong S, Zhiyian H. 1992. Chronic oral toxicity and carcinogenicity study of stevioside in rats. *Food Chem Toxicol.* 30:957-65.

Yamada A, Ohgaki S, Noda T, and Shimizu M. 1985. Chronic toxicity study of dietary stevia extracts in F344 rats. *J Food Hyg Soc Jpn.* 26:169-183.

Yoda SK, Marques MOM, Petenate AJ, and Meireles MAA. 2003. Supercritical fluid extraction from stevia rebaudiana Bertoni using CO_2 and CO_2 + water: Extraction kinetics and identification of extracted components. *J Food Eng.* 57:125-134.

Yodyingyuad V and Bunyawong S. 1991. Effect of stevioside on growth and reproduction. *Hum Reprod.* 6:158-165.

000185

THE GENERALLY RECOGNIZED AS SAFE STATUS OF McNEIL STEVIA

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of food and food ingredients, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized as Safe (GRAS) status of a stevia extract, hereinafter referred to as McNeil Stevia, that is proposed for use in table-top sweetener products by McNeil Nutritionals, LLC. This data and information is summarized in the GRAS determination document, *Generally Recognized As Safe Determination for the Use of McNeil Stevia*, produced by Spherix, Inc., for McNeil Nutritionals, LLC, and that is appended herewith. This GRAS determination was made based on scientific procedures for GRAS determinations described under 21 CFR §170.30(b) and in accordance with the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s).

Based upon our review of the information and data available, we find that McNeil Stevia is safe and is GRAS, under the intended conditions of use. McNeil Stevia has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information. The conclusion of the Panel is based on the following:

- McNeil Stevia is a well-characterized and high-purity substance that complies with established food grade specifications for steviol glycosides set by JECFA (JECFA 2007). This allows for seven steviol glycosides (rebaudioside A, stevioside, rebaudioside B, steviolbioside, rebaudioside C, dulcoside A and rubusoside) to be present, the sum of which accounts for a minimum of 95% of the dried substance.
- McNeil Stevia is comprised largely of rebaudioside A and stevioside that, like all steviol glycosides, are completely metabolized to steviol by the microbial flora of the cecum. Steviol present in the digestive tract is readily absorbed to the bloodstream and rapidly glucuronidated in both humans and rats, with excretion occurring via the bile (and into feces) in rats and via the urine in humans. Evidence indicates that no stevia glycosides are absorbed intact.
- Based on the similarity between rats and humans in gastrointestinal microflora metabolism of stevioside and rebaudioside A to steviol, and metabolism of absorbed

Spherix Incorporated
6430 Rockledge Drive, #503, Westmoreland Building, Bethesda, MD 20817
Tel.: +1-301-897-2540 • Fax: +1-301-897-2567

steviol to the primary metabolite, steviol glucuronide, rat studies are an appropriate model to assess safety of steviol glycoside ingestion in humans.

- Additionally, both studies of stevia extracts containing primarily rebaudioside A and studies of stevia extracts containing predominantly stevioside are appropriate for inclusion in the safety assessment of stevia extracts, since both are acted upon by gastrointestinal microflora to yield steviol, and no glycated steviol is absorbed.
- Steviol glycosides have very low acute toxicity in animals and there is no evidence of health risk, including carcinogenicity, developmental, or reproductive effects. Weight of the evidence indicates that steviol glycosides are not genotoxic.
- A 90-day rodent study using two suppliers of McNeil Stevia at up to 4% in the diet was also conducted and showed no adverse treatment related effects. The NOAEL was determined to be the highest dose tested (up to 3300 mg/kg/day).
- Some animal and human studies of steviol glycosides have reported hypotensive and hypoglycemic effects. Critical new published studies resolve questions about untoward effects after long term repeated exposure to steviol glycosides. Studies have shown that steviol glycosides are well tolerated in humans and unlikely to have adverse effects on blood pressure in normal, hypotensive or hypertensive individuals or on glucose homeostasis in normal and type 2 diabetic individuals at doses well exceeding maximum expected intakes.
- Safety of ingestion of steviol glycosides in humans has been corroborated in clinical trials; measures of tolerance, body weight, clinical chemistry, hematology and urinalysis did not show any evidence of untoward effects.
- Intake as a table-top sweetener is estimated to be 17 and 35 mg steviol equivalents/day at the mean and 9^{0th} percentile of intake for ages $\geq$ 19 years (equivalent to 0.28 and 0.58 mg steviol equivalents/kg/day at the mean and 9^{0th} percentile of intake) (NHANES 2003-2004) and 7 and 18 mg steviol equivalents/day at the mean and 9^{0th} percentile of intake for all ages, eaters only (equivalent to 0.14 and 0.33 mg steviol equivalents/kg/day at the mean and 9^{0th} percentile of intake) (MRCA 1992-1993).

- An ADI of 4 mg steviol equivalents/kg bw/day, derived by applying a 100-fold safety factor to the NOAEL of 970 mg stevioside/kg bw/day (equivalent to 383 mg/kg bw/kg steviol equivalents) in a 2-year rat study, is appropriate. This ADI includes steviol glycoside mixtures with different ratios of stevioside/rebaudioside. The ADI of 4 mg/kg bw/day steviol equivalents is greater than the proposed EDI of up to 0.58 mg/kg bw/day steviol equivalents for the use of McNeil Stevia as a table-top sweetener.

Spherix Incorporated
6430 Rockledge Drive, #503, Westmoreland Building, Bethesda, MD 20817
Tel.: +1-301-897-2540 • Fax: +1-301-897-2567

GENERALLY RECOGNIZED AS
SAFE DETERMINATION OF MCNEIL STEVIA

Therefore, based on our independent and collective critical evaluation of the available information on McNeil Stevia, we, members of the Expert Panel, conclude that the intended use of McNeil Stevia, produced in accordance with current Good Manufacturing Practice and meeting the specifications referenced in the GRAS determination document, is safe and is Generally Recognized as Safe (GRAS) based on scientific procedures and corroborated by history of safe exposure. Because the intended use of McNeil Stevia is safe and GRAS, it is excluded from the definition of a food additive, and thus may be marketed for these uses without the need to promulgate a specific food additive regulation under 21 CFR.

It is our opinion that other experts qualified by training and/or experience to evaluate the safety of food and food ingredients would concur with these conclusions.

Roger A. Clemens, DrPH, CNS, FACN, FIFT
GRAS Expert Panel Member
School of Pharmacy
University of Southern California

Signature: _____
Date: 8/21/08

A. Wallace Hayes, Ph.D., DABT, FATS, ERT
GRAS Expert Panel Member
Harvard School of Public Health

Signature: _____
Date: 8/21/08

John A. Thomas, Ph.D., DATS, FACT
GRAS Expert Panel Member
Department of Pharmacology & Toxicology
School of Medicine
Indiana University

Signature _____
Date: 8/21/08

William J. Waddell, M.D.
GRAS Expert Panel Member
Department of Pharmacology & Toxicology
School of Medicine
University of Louisville

Signatu
Date: 8/21/08

Claire L. Kruger, Ph.D., DABT
Scientific Advisor to the Panel
Spherix Incorporated

Signature: _____
Date: 8/21/08

Spherix Incorporated
6430 Rockledge Drive, #503, Westmoreland Building, Bethesda, MD 20817
Tel.: +1-301-897-2540 • Fax: +1-301-897-2567

SUBMISSION END