GR

ORIGINAL SUBMISSION

GRAS ASSOCIATES, LLC
20482 Jacklight Lane
Bend, OR 97702-3074
541-678-5522
mcquate@gras-associates.com

January 19, 2009

RECEIVED
JAN 21 2009
BY:

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

Attention: Dr. Robert L. Martin

Dear Dr. Martin:

On behalf of Blue California of Rancho Santa Margarita, CA, we are submitting for FDA review a GRAS notification for Rebaudioside A (97%). The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via telephone or email.

We look forward to your feedback.

Sincerely,

Robert S. McQuate, Ph.D.
CEO & Co-Founder
GRAS Associates, LLC
20482 Jacklight Lane
Bend, OR 97702-3074
541-678-5522
mcquate@gras-associates.com
www.gras-associates.com

Enclosure: GRAS Notification – Rebaudioside A (97%) (in triplicate)

000002

GRAS ASSOCIATES, LLC

COMPREHENSIVE GRAS ASSESSMENT

OF

REBAUDIOSIDE A (97%)

Food Usage Conditions for General Recognition of Safety

For

BLUE CALIFORNIA
Rancho Santa Margarita, CA

Evaluation by

Richard C. Kraska, Ph.D., DABT
Robert S. McQuate, Ph.D.
Wayne R. Bidlack, Ph.D.

January 16, 2009

TABLE OF CONTENTS

I. GRAS EXEMPTION CLAIM **4**
A. Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1) 4
B. Name and Address of Notifier 4
C. Common Name and Identity of the Notified Substance 4
D. Conditions of Intended Use in Food 5
E. Basis for the GRAS Determination 5
F. Availability of Information 5

II. INTRODUCTION **6**
A. Objective 6
B. Foreword 6
C. Summary of Regulatory History of Stevia 6
D. FDA Regulatory Framework 8

III. CHEMISTRY AND MANUFACTURE OF REBAUDIOSIDE A **9**
A. Common or Usual Name 9
B. Chemistry of Rebaudioside A 9
C. Manufacturing Processes 11
1. Scientific and Patent Literature 11
2. Blue California Manufacturing Process for Rebaudioside A 11
D. Product Specifications and Supporting Methods 12
1. JECFA Specifications 12
2. Specifications for Blue California Rebaudioside A 13
E. Stability Data 13
1. Scientific Literature 13
2. Stability of Blue California Rebaudioside A 15

IV. INTENDED DIETARY USES **17**
A. Intended Uses 17
B. Food Uses As Addressed by JECFA, Merisant and Cargill 17
C. Estimated Daily Intake 18
D. Other Information on Human Exposure to Stevia: Use as a Food Ingredient And Other Uses 21

V. SAFETY DATA FOR REBAUDIOSIDE A **22**
A. Safety Data on Steviol Glycosides: Reviews by Expert Bodies and Other Scientists 22
1. Summary of JECFA Reviews 22
2. Summary of FSANZ Review of Steviol Glycosides 24
B. Safety Data on Rebaudioside A 25
1. Mutagenicity Studies 25
2. Subchronic Studies 25
3. Reproduction and Developmental Studies 28
4. Clinical Studies on Rebaudioside A 28
5. Absorption, Distribution, Metabolosm and Execretion (ADME) Studies 29

TABLE OF CONTENTS continued

VI. DISCUSSION OF GRAS CRITERIA AND REVIEWED INFORMATION **31**
A. GRAS Criteria 31
B. Utilization of FDA Safety Assessment Methodology 32
C. Panel Discussion on the Expert Safety Reviews of Steviol Glycosides 33
D. Expert Panel Discussion of the Safety of Rebaudioside A 34
E. Discussion of Concerns Raised by UCLA Researchers and the Center for Science in the Public Interest (CSPI) 35
1. Panel's Overall Conclusions on UCLA and CSPI Concerns 36
F. Common Knowledge Elements of GRAS Determination 37

VII. CONCLUSIONS **39**

VIII. REFERENCES **40**

APPENDIX A – JECFA SPECIFICATIONS FOR STEVIOL GLYCOSIDES **44**

APPENDIX B – KEY ANALYSES FOR BLUE CALIFORNIA REBAUDIOSIDE A **49**
B-1 Eurofins Analyses of Multiple Production Batches 50
B-2 Certificates of Analysis 61
B-3 Heavy Metals & Pesticide Analyses 66
B-4 Comparative Sweetness Determination 68

APPENDIX C – STABILITY TESTING STUDIES FOR BLUE CALIFORNIA REBAUDIOSIDE A **69**
C-1 Stability Good Sweets 70
C-2 Stability Shelf Life 74

APPENDIX D – BLUE CALIFORNIA PROPOSED FOOD USES FOR REBAUDIOSIDE A **78**

APPENDIX E -- SUMMARY OF STEVIOL GLYCOSIDES SAFETY STUDIES REVIEWED BY JECFA **80**

APPENDIX F – QUALIFICATIONS OF EXPERT PANEL MEMBERS **92**
Richard C. Kraska, Ph.D., DABT 93
Robert S. McQuate, Ph.D. 97
Wayne R. Bidlack, Ph.D. 100

I. GRAS EXEMPTION CLAIM

A. Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1)[1]

Rebaudioside A, meeting the specifications for Good & Sweet® as described below, has been determined to be Generally Recognized As Safe (GRAS), in accordance with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act.* This determination was made by experts qualified by scientific training and experience; it is based on scientific procedures as described in the following sections; and the evaluation accurately reflects the conditions of the stevia-derived sweetener's intended use in foods.

Signed:

January 19, 2009

Date

Robert S. McQuate, Ph.D.
GRAS Associates, LLC
20482 Jacklight Lane
Bend, OR 97702-3074

B. Name and Address of Notifier

Blue California
30111 Tomas
Rancho Santa Margarita, CA 92688

As the notifier, Blue California ("BC") accepts responsibility for the GRAS determination that has been made for rebaudioside A (identified as Good & Sweet®) and as described in the subject notification; consequently, the rebaudioside A preparation meeting the conditions described herein is exempt from pre-market approval requirements for food ingredients.

C. Common Name and Identity of the Notified Substance

Rebaudioside A, commonly referred to as reb A or Reb A, is the common name for the notified substance; also see Section III.A.

[1] See 62 FR 18938 (17 April 1997).

D. Conditions of Intended Use in Food

The high purity (97%) rebaudioside A preparation is intended to be added as a general purpose non-nutritive sweetener into various food categories at per serving levels that reflect good manufacturing practices principles in that the quantity added to foods should not exceed the amount reasonably required to accomplish its intended technical effect.

E. Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30, the high purity rebaudioside A has been determined to be GRAS on the basis of scientific procedures as discussed in the detailed description provided below.

F. Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the US Food and Drug Administration (FDA) upon request or will be available for review and copying at reasonable times at the offices of GRAS Associates, LLC, located at 20482 Jacklight Lane, Bend, OR 97702-3074.

II. INTRODUCTION

A. Objective

At the request of Blue California, GRAS Associates, LLC (GA) has undertaken an independent safety evaluation of BC's rebaudioside A (Reb A) with a purity of 97% as found in its proprietary sweetener, Good & Sweet®. The purpose of the evaluation is to ascertain whether or not the intended food uses of the subject Reb A as a non-nutritive general purpose sweetener are generally recognized as safe, i.e., GRAS, when incorporated into various food categories.

B. Foreword

BC provided GA with background information needed to enable the GRAS assessment to be undertaken. In particular, the information provided addressed the safety/toxicity of steviol glycosides; the history of use of stevia in food; and compositional details, specifications, and method of preparation of rebaudioside A. BC was asked to provide adverse reports, as well as those that supported conclusions of safety.

Safety/toxicity studies performed with animals were noted to have value, along with available human testing. BC was also asked to supply past and present human food use information. Knowing how much steviol glycosides has been safely consumed, i.e., the so-called "dose" or use levels, is critical in extrapolating to safe exposures for rebaudioside A when consumed as a food ingredient. The composite safety/toxicity studies, in concert with exposure information, ultimately provide the specific scientific foundation for the GRAS determination.

BC supplied the product specifications and chemical properties and some consumption/ exposure information, along with other related documentation. Safety studies were identified by an independent search of the scientific and regulatory literature. A GRAS assessment based on the composite safety information, that is, based on scientific procedures was undertaken. Those references that were deemed pertinent to the objective at hand are listed in Section VIII.

C. Summary of Regulatory History of Stevia

Stevia derived-sweeteners are permitted as a food additive in South America and in several countries in Asia, including China, Japan, and Korea. As discussed more fully below, over the past few months, the subject sweeteners have received approvals in Australia, New Zealand, and Switzerland, and the US FDA has issued "no objection letters" in response to the GRAS notifications filed on behalf of rebaudioside A food uses.

In the US, steviol glycosides have been used as a dietary supplement since 1995 (Geuns, 2003). No application for dietary supplement use of purified rebaudioside A is known to have been made. At least two GRAS petitions seeking authorization for the addition of

stevioside or steviol glycosides to foods had been submitted to FDA since 1989, yet no authorizations had been issued by FDA in response to these filings, presumably because the previously available safety data---including purity considerations---for stevia, stevioside, or steviol glycosides were viewed as being inadequate. These petitions were subsequently withdrawn.

Individual GRAS notifications were submitted by Merisant and Cargill to FDA in May, 2008 for rebaudioside A, both more highly purified forms of the steviol glycosides.[2] FDA issued "no objection" letters for each of the GRAS notices on December 17, 2008.

The Food Standards Australia New Zealand (FSANZ) has completed evaluation of an application for use of steviol glycosides in foods and has recommended to the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) to amend the Australia New Zealand Food Standards Code to allow its use in food (FSANZ, 2008).

Steviol glycosides have been under a lengthy review by the Joint Expert Committee on Food Additives ("JECFA"). The original review was published in 2000 (WHO, 2000). A draft monograph was reviewed at the 51st, 63rd and 68th JECFA meetings. A temporary ADI (acceptable daily intake) of 0-2 mg/kg (on a steviol basis) was established at the 63rd meeting (WHO, 2006). In addition, food grade specifications were made final by JECFA (FAO, 2007a). At the 69th meeting, the temporary status of the ADI was removed and the ADI was raised to 0-4 mg/kg bw/day (on a steviol basis) as a result of the JECFA review of recently completed clinical studies with steviol glycosides (WHO, 2008). A final monograph on steviol glycosides is expected from JECFA.

In August 2008, Switzerland's Federal Office for Public Health cited the favorable actions of JECFA in issuing its approval for the use of stevia as a sweetener (Switzerland Office of Public Health, 2008).

The stevia-derived sweeteners are not presently permitted as an ingredient in conventional food in the EU, UK, Hong Kong, or Canada (Hawke, 2003). This likely reflects a lack of review of new data on the sweeteners rather than a continuing concern about safety.

Hong Kong maintains that stevia is not permitted as a sweetener, as cited on the government website (Hong Kong Government, 2002). The Hong Kong Government was reported to be waiting for the JECFA determination on the safety of steviol glycosides. However, no further official actions have been noted since JECFA's final resolution was reported in June 2008.

Other international bodies have investigated the safety aspects of stevia and steviol glycosides use in foods. In 1999 in the EU, the Scientific Committee on Food for the European Commission concluded that "there are no satisfactory data to support the safe use of these stevia plants and leaves," as reported in a five-page opinion dated June 17, 1999 (European Commission, 1999a). The Committee also reiterated "its earlier opinion that stevioside is not acceptable as a sweetener on the presently available data," in a

[2] GRAS notification 252 which was submitted by Merisant and GRAS notification 253 which was submitted by Cargill are listed on FDA's website at http://www.cfsan.fda.gov/~rdb/opa-grsn.html, along with the respective December 17, 2008 FDA "no objection" letters.

seven-page opinion also dated June 17, 1999 (European Commission, 1999b). Unconfirmed reports indicate that the SCF is reexamining the safety of steviol glycosides in light of JECFA's 2008 findings.

On September 24, 1998 in the UK, the Advisory Committee on Novel Foods and Processes for the Ministry of Agriculture, Fisheries and Food rejected an application for use of steviol glycosides as a sweetener in herbal teas because "the applicant had not provided all of the information necessary to enable an assessment to be made."[3]

D. FDA Regulatory Framework

Steviol glycosides or stevioside, has been used in dietary supplements in the US since 1995 (Geuns, 2003) and is widely available to consumers in the US through retail outlets and Internet purchases (Al-Achi, 2000).

In accordance with FDA regulation of foods, however, dietary supplements cannot legally be added to conventional foods. Such ingredients must undergo premarket approval by FDA as food additives or, alternatively, the ingredients to be incorporated into conventional foods must be determined to be generally recognized as safe (GRAS). The authority to make GRAS determinations is not restricted to FDA. In fact, GRAS determinations may be provided by experts who are qualified by scientific training and experience to evaluate the safety of food and food ingredients under the intended conditions of use.[4]

In 1997, FDA altered the GRAS determination process by eliminating the formal GRAS petitioning process. At that time, the petitioning process was replaced with a notification procedure.[5] While outlining the necessary content to be considered in making a GRAS determination, FDA encouraged that such determinations be provided to FDA in the form of a notification. However, notifying FDA of such determinations is strictly voluntary.

[3] See http://www.maff.gov.uk/food/novel/980924.html.
[4] See 21 CFR 170.3(i)(3).
[5] See *Federal Register* 62 April 17, 1997, 18937; or http://www.cfsan.fda.gov/~lrd/fr970417.html.

III. CHEMISTRY AND MANUFACTURE OF REBAUDIOSIDE A

A. Common or Usual Name

Rebaudioside A, also referred to as Reb A or reb A, is one of the common steviol glycosides found in nature. Rebaudioside A is also referred to by the common or usual name of rebiana.

Steviol glycosides have been referred to as stevia, stevioside, and stevia glycoside in the scientific literature. JECFA adopted the term, steviol glycosides, for the family of steviol derivatives with sweetness properties that are derived from the stevia plant. Presently, the term, stevia, is used more narrowly to describe the plant or crude extracts of the plant, while stevioside is the common name for another one of the specific glycosides that is extracted from stevia leaves.

B. Chemistry of Rebaudioside A

The following description is taken from the original JECFA monograph (WHO, 2000).

> Stevioside is a glycoside of the diterpene derivative steviol (ent-13-hydroxykaur-16-en-19-oic acid). Steviol glycosides are natural constituents of the plant *Stevia rebaudiana Bertoni*, belonging to the Compositae family. The leaves of *S. rebaudiana Bertoni* contain eight different steviol glycosides, the major constituent being stevioside (triglucosylated steviol), constituting about 5-10% in dry leaves. Other main constituents are rebaudioside A (tetraglucosylated steviol), rebaudioside C, and dulcoside A. *S. rebaudiana* is native to South America and has been used to sweeten beverages and food for several centuries. The plant has also been distributed to Southeast Asia. Stevioside has a sweetening potency 250-300 times that of sucrose and is stable to heat. In a 62-year-old sample from a herbarium, the intense sweetness of *S. rebaudiana* was conserved,indicating the stability of stevioside to drying, preservation, and storage (Soejarto et al., 1982; Hanson and De Oliveira, 1993).

The two predominant sweetener components of stevia extracts have been identified as stevioside and rebaudioside A. The chemical identities and key chemical identifiers for the two major components are shown below.

Stevioside

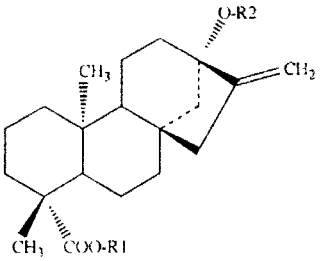
Chemical Name:	13-[2-Oβ-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Chemical formula:	$C_{38}H_{60}O_{18}$
Formula Weight:	804.88
CAS Number:	57817-89-7

Rebaudioside A

Chemical Name:	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] kaur-6-en-8-oic acid, β-D-glucopyranosyl ester
Chemical Formula:	$C_{44}H_{70}O_{23}$
Formula Weight:	967.03
CAS Number:	58543-16-1

In the most recent Chemical and Technical Assessment (FAO, 2007b), JECFA identified the sweetener components. They updated the list of common glycosides and their chemical structures which are slightly different than compounds shown in other older publications (Nanayakkara et al., 1987; Suttajit et al., 1993). They are represented in Figure 1.

Figure 1. Chemical Structures of Various Steviol Glycosides Reproduced from FAO, 2007b



	Compound name	C.A.S. No.	R1	R2
1	Steviol	471-80-7	H	H
2	Steviolbioside	41093-60-1	H	β-Glc-β-Glc(2→1)
3	Stevioside	57817-89-7	β-Glc	β-Glc-β-Glc(2→1)
4	Rebaudioside A	58543-16-1	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
5	Rebaudioside B	58543-17-2	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
6	Rebaudioside C (dulcoside B)	63550-99-2	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
7	Rebaudioside D	63279-13-0	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
8	Rebaudioside E	63279-14-1	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
9	Rebaudioside F	438045-89-7	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)
10	Rubusoside	63849-39-4	β-Glc	β-Glc
11	dulcoside A	64432-06-0	β-Glc	β-Glc-α-Rha(2→1)

The structures of the components of stevia glycosides were also described in reviews by Kinghorn and Soejarto (1985), Kennelly (2002), and Geuns (2003). Non-sweet elements include the labdane diterpenes, triterpenes, sterols and flavonoid glycosides.

C. Manufacturing Processes

Various manufacturing processes yielding steviol glycosides have been described in the scientific and patent literature, and they are summarized below, along with BC's manufacturing process for Reb A.

1. Scientific and Patent Literature

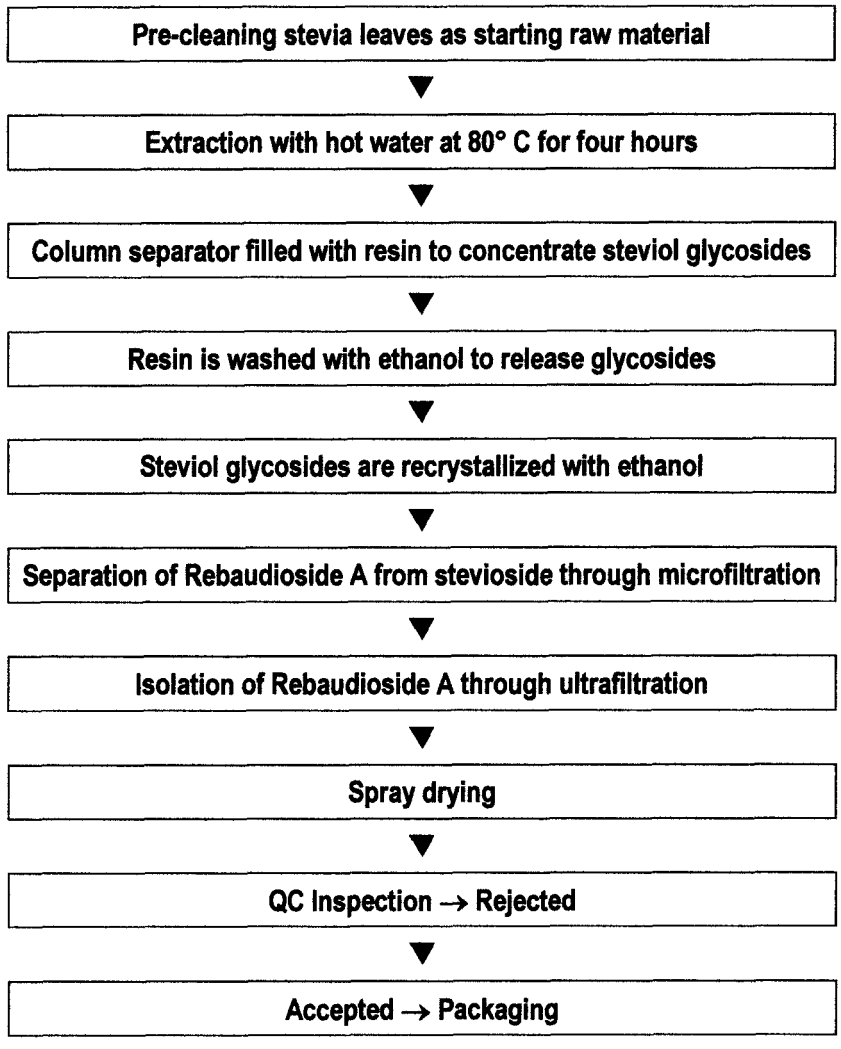
Typically, steviol glycosides are obtained by extracting leaves of *Stevia rebaudiana Bertoni* with hot water or alcohols (ethanol or methanol); the obtained extract is a dark particulate solution containing all the active principles plus leaf pigments, soluble polysaccharides, and other impurities. Some processes remove the "grease" from the leaves with solvents such as chloroform or hexane before extraction occurs (Kinghorn and Soejarto, 1985). There are dozens of extraction patents for the isolation of steviol glycosides. Kinghorn and Soejarto (1985) have categorized the extraction patents into those based on solvent, solvent plus a decolorizing agent, adsorption and column chromatography, ion exchange resin, and selective precipitation of individual glycosides. Methods using ultrafiltration, metallic ions, supercritical fluid extraction with CO_2 and extract clarification with zeolite are found within the body of newer patents.

At the 68th JECFA meeting in 2007, steviol glycosides were defined as the products obtained from the leaves of *Stevia rebaudiana Bertoni*. As cited by JECFA, the typical manufacture starts with extracting leaves with hot water and the aqueous extract is passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is washed with methanol to release the glycosides and the product is recrystallized with methanol. Ion-exchange resins may be used in the purification process. The final product is commonly spray-dried.

2. Blue California Manufacturing Process for Rebaudioside A

With the BC process, cleaned stevia leaves are extracted with a water and ethanol mixture. The ethanol grade used is high purity. The extract is concentrated and then undergoes several filtration and purification steps using membrane technology that sorts by molecular size. The filters and membranes used meet FDA requirements for food contact. The powdered product is obtained by spray drying. See flow diagram in Figure 2.

Figure 2. MANUFACTURING PROCESS FLOW DIAGRAM FOR REBAUDIOSIDE A (97%)



D. Product Specifications and Supporting Methods

1. JECFA Specifications

The composition of extracts of *Stevia rebaudiana Bertoni* depends upon the composition of the harvested leaves which are, in turn, influenced by soil, climate, and the manufacturing process itself (FAO, 2007b).

In 2007, JECFA recommended that the method of assay includes a minimum requirement of 95% of the total 7 steviol glycosides, on a dried weight basis (FAO, 2007a, see Appendix A). Stevioside and rebaudioside A are the major component

glycosides of interest because of their sweetening property. The 5 other associated glycosides found in preparations of steviol glycosides accepted by the JECFA specification for the 95% requirement are rebaudioside C, dulcoside A, rubusoside, steviolbioside and rebaudioside B. These, however, are typically found at lower levels than the stevioside or rebaudioside A.

JECFA finalized food grade specifications at the 68th JECFA meeting, which were then published in FAO JECFA Monograph 4 (FAO, 2007a). Steviol glycosides are described as a white to yellow powder, odorless to having a slight characteristic odor, and exhibiting a sweetness that is 200-300 times greater than sucrose. It is freely soluble in water and ethanol with a pH between 4.5-7.0 (1 in 100 solution). The product should not have more that 1% ash with no more than a 6% loss on drying at 105°C for 2 hours. Residual solvents (methanol)[6] should not exceed 200 mg/kg. Arsenic levels should not exceed 1 mg/kg (determined by the atomic absorption hydride technique). Lead analysis should not be more than 1 mg/kg sample. The complete listing with JECFA specifications including recommended analytical methods is attached as Appendix A.

2. Specifications for Blue California Rebaudioside A

BC has adopted product specifications for its Reb A that is contained in Good & Sweet® that meet or exceed JECFA recommendations. The specifications provided by BC as compared to JECFA specifications for the final spray dried product are given in Table 1. A report of analyses demonstrating that 5 production batches are at least 97% Reb A on a dry matter basis is attached in Appendices B-1 and B-2. Typical data on heavy metals and pesticide residues are also given in Appendix B-3, and the comparative measurement of Reb A's sweetness intensity is found in Appendix B-4.

E. Stability Data

1. Scientific Literature

Stevioside is a stable molecule over the pH range 3-9 and can be heated at 100°C for 1 hour, but rapidly decomposes at pH levels greater than 9 under these conditions (Kinghorn and Soejarto, 1985). It is speculated that steviobioside produced from stevioside by alkaline hydrolysis would be the major decomposition product obtained at pH 10 (Kinghorn and Soejarto, 1985).

Chang (1983) tested the stability of pure stevioside and rebaudioside A in carbonated phosphoric and citric acidified beverages and reported some degradation of both sweetening components after 2 months of storage at 37°C; however, there was no significant change at room temperature or below following 5 months of storage of stevioside and 3 months of storage of rebaudioside A. He also reported that exposure to 1 week of sunlight did not affect stevioside, but resulted in

[6] The BC manufacturing process utilizes a combination of water and ethanol, and not methanol, to yield its high purity Reb A.

approximately 20% loss of rebaudioside A. Heating at 60°C for 6 days resulted in 0-6% loss of rebaudioside A.

Table 1. Specifications for Steviol Glycosides & Rebaudioside A

PARAMETER	JECFA SPECIFICATION	BC SPECIFICATION
PHYSICAL SPECIFICATIONS		
APPEARANCE	WHITE TO LIGHT YELLOW POWDER	WHITE POWDER
FOREIGN MATTER	NS	ABSENT
ODOR	SLIGHT CHARACTERISTIC	SLIGHT CHARACTERISTIC
TASTE	200-300 FOLD SWEETER THAN SUGAR	400-FOLD SWEETER THAN SUGAR
CHEMICAL SPECIFICATIONS		
Rebaudioside A	NA	≥97%
Total Steviol Glycosides	>95%	NS
Moisture (loss on drying)	< 6%	≤5%
Ash	<1%	<1%
Solubility	Freely soluble in water and ethanol	Soluble in water & alcohol
pH (1% solution)	4.5-7.0	4.5-7.0
Residual Solvent	< 200 ppm methanol	NA; See footnote 6
Lead	< 1 ppm	<0.5 ppm
Arsenic	< 1 ppm	<0.5 ppm
MICROBIOLOGICAL SPECIFICATIONS		
Aerobic Plate Count	NS	< 3,000 cfu/g
Mold and Yeast	NS	< 100 CFU/g
Salmonella	NS	Negative
Total *E. coli*	NS	Negative
Fecal *E. coli*	NS	NS

Abbreviations: St = Stevioside; Reb A = Rebaudioside A; Reb B = Rebaudioside B; Reb C = Rebaudioside C; Dulc A = Dulcoside A; Rub = Rubusoside; SB = Steviolbioside; NS = not specified; NA = not applicable.

Extensive stability testing results were compiled for inclusion in both the Merisant and Cargill GRAS notifications.

Detailed stability testing was conducted by Merisant on Reb A as a powder, as a pure sweetener in solution, and on both cola-type and citrus carbonated beverages. No degradation was detected when the powder was stored at 105°C for 96 hours, and it was concluded that the powder was stable when stored for 26 weeks at 40±2°C with relative humidity of 75±5%. When considering Merisant's results of the stability investigations which include both published and unpublished testing results, it was determined that Reb A in carbonated citric acid beverages and phosphoric acid beverages showed no significant degradation during prolonged storage at refrigeration, normal ambient, or elevated ambient temperatures. Minimal loss of Reb A was detected after storage at 60°C, with considerable degradation noted after 13 hours at 100°C for carbonated beverage solutions and pure sweetener solutions (Merisant, 2008).

Cargill conducted detailed stability testing on Reb A as a powder under various storage conditions and under a range of pH and temperatures. In addition, Cargill assessed Reb A stability in several representative food matrices at room temperature and elevated temperatures. Stability profiles were created for table top sweetener applications, mock beverages including cola, lemon-lime, and root beer, yogurt, thermally processed beverages, and white cake. The stability testing revealed some degradation products that had not been detected in bulk Reb A. However, it was noted that these degradation products were structurally related to the steviol glycosides that are extracted from the leaves of *Stevia rebaudiana Bertoni*. The degradation products all share the same steviol aglycone backbone structure as found in stevioside and rebaudioside A, but they differ by virtue of the glucose moities present.

Photostability studies were also conducted on the dry powder and mock beverages to ascertain Reb A behavior under defined conditions of fluorescent and near UV light exposure. Reb A was determined to be photostable under the defined conditions of analysis.

From the stability testing reported, it was concluded that Reb A is stable in various food matrices following several days or weeks of storage. The extent and rate of degradation is dependent on pH, temperature, and time. When placed in beverages, Reb A is more stable in the pH range 4 to 6 and at temperatures from 5°C to 25°C (Cargill, 2008).

2. Stability of Blue California Rebaudioside A

BC has conducted various studies of short term stability on rebaudioside A at elevated temperatures at 100°C at various pH levels. No appreciable degradation was seen over 3 hours at pH 2, 4, 6 and 8.

BC continues to investigate longer term stability studies including those that will estimate product shelf life. A preliminary report indicates that the product is stable for at least three months under expected storage conditions. Preliminary reports of these stability studies are included in Appendix C.

The stability test results conducted by Merisant and Cargill also have application to BC's Reb A in light of the comparable purities of all three Reb A sources which fall in the narrow range of 95-97% Reb A.

IV. INTENDED DIETARY USES

A. Intended Uses

BC intends to market its 97% pure Reb A in Good'N Sweet as a table top sweetener and for incorporation into various food categories as a general purpose sweetener which will include those food categories listed in Appendix D. Rebaudioside A will function as a non-nutritive sweetener as defined in 21 CFR 170.3(o)(19). The use levels will vary by food category but the actual levels are self-limiting due to organoleptic factors and consumer taste considerations. However, the amounts of Reb A to be added to foods will not exceed the amounts reasonably required to accomplish its intended technical effect in foods as required by FDA regulation.[7]

B. Food Uses As Addressed by JECFA, Merisant, and Cargill

JECFA reviewed various estimates of possible consumption of steviol glycosides (WHO, 2006) as part of its safety deliberations. Estimated maximum use levels in various foods as evaluated by the Committee are summarized in Table 2a.

Table 2a. Food Uses of Steviol Glycosides Reported to JECFA With Calculated Steviol Equivalents

FOOD TYPE	MAXIMUM USE LEVEL REPORTED[a] (mg STEVIOL GLYCOSIDES /kg OF FOOD)	MAXIMUM USE LEVEL CALCULATED FOR REBAUDIOSIDE A[b] MG REBAUDIOSIDE A / KG OF FOOD	MAXIMUM USE LEVEL CALCULATED FOR REBAUDIOSIDE A[b] MG STEVIOL EQUIVALENTS/ KG OF FOOD
Desserts	500	250	83
Cold confectionery	500	250	83
Pickles	1000	500	167
Sweet corn	200	100	33
Biscuits	300	150	50
Beverages	500	250	83
Yogurt	500	250	83
Sauces	1000	500	167
Delicacies	1000	500	167
Bread	160	80	27

[a] Reproduced from WHO, 2006. [b] Calculated by Expert Panel assuming twice the sweetness intensity for rebaudioside A and three-fold difference in molecular weight between rebaudioside A and steviol.

Merisant listed expected levels of use for various food applications in their GRAS Notification. Their consumer estimates were largely based on food consumption survey

[7] See 21 CFR 182.1(b)(1).

data from 2003-2004 NHANES, a resource that reflects food intake over a two-day time period. Statistically weighted values were utilized to provide reliable quantitative findings that are representative of food consumption of actual "users" within the US population. The 2-day food surveys are known to overestimate actual consumption levels when compared to longer term food surveys, such as those based on 14-day surveys. On a per user basis, the mean daily consumption of Reb A was calculated to be 2.0 mg/kg bw/day, and that for the 90th percentile consumer was found to be 4.7 mg/kg bw/day. Specific food categories and use levels are given in Table 2b.

Cargill utilized a different approach in estimating dietary intake figures for Reb A when incorporated as a general sweetener in a broad cross-section of processed foods (Cargill, 2008). Cargill reasoned that Reb A uses and use levels would be rather comparable to aspartame uses in the US with a few minor exceptions. They performed a side-by-side consumption analysis for Reb A versus aspartame, using post-market surveillance consumption data and published data for consumption of aspartame and other high intensity sweeteners (Renwick, 2008). Their findings are considered further in Section IV.C and are tabulated in Table 3b.

Table 2b. Proposed Uses and Levels of Rebaudioside A by Merisant (2008)

FOOD GROUP	REB A (PPM)
Tabletop sweeteners	30,000[a]
Sweetened ready-to-drink teas	90-450
Fruit juice drinks	150-500
Diet soft drinks	150-500
Energy drinks	150
Flavored water	150
Cereals (oatmeal, cold cereal, cereal bars)	150

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

C. Estimated Daily Intake

BC intends to incorporate its Reb A into a broad selection of foods as noted in Appendix D, but BC has not provided specific consumption estimates (i.e., numbers of servings or firm use levels) for the individual food categories Instead, the very conservative consumer intake estimates provided by JECFA as shown above in Table 2a were utilized to gauge the potential human exposures of steviol glycosides and Reb A in foods as reported in the US and in other countries. Since Reb A is about twice as sweet as the mixed glycosides, these levels can be adjusted downward accordingly.

In concert with the JECFA intake estimates, further consideration was given to anticipated human exposures as projected independently and with different approaches by both Merisant and Cargill in compiling their GRAS dossiers (Merisant, 2008 and Cargill, 2008). As noted below, the multiple approaches tended to converge to yield estimated daily intakes (EDIs) in the range of 1.3 – 4.7 mg/kg bw/day that, when compared to the acceptable daily intake (ADI), constitutes an integral component in the subject GRAS evaluation.

The Committee evaluated information on exposure to steviol glycosides as submitted by Japan and China. Additional information was available from a report on *Stevia rebaudiana Bertoni* plants and leaves that was prepared for the European Commission by the Scientific Committee on Food.

JECFA used the GEMS/Food database to prepare international estimates of exposure to steviol glycosides (as steviol). JECFA assumed that **steviol glycosides would replace all dietary sugars**, at the lowest reported relative sweetness ratio for steviol glycosides and sucrose which is 200:1. The intakes ranged from 1.3 mg/kg bw/day with the African diet to 3.5 mg/kg bw/day with the European diet.

JECFA also estimated the per capita exposure derived from disappearance (poundage) data supplied by Japan and China. The Committee evaluated exposures to steviol glycosides by assuming full replacement of all dietary sugars in the diets for Japan and the US. Table 3a summarizes the exposures to steviol glycosides (as steviol) as evaluated or derived by the Committee.

Table 3a. Summary of Estimates of Exposure to Steviol Glycosides (as Steviol)

ESTIMATE	EXPOSURE (mg/kg BW/DAY)
GEMS/Food (International)[a]	1.3–3.5 (for a 60 kg person)
Japan, Per Capita	0.04
Japan, Replacement Estimate[b]	3
US, Replacement Estimate[b]	5

[a] WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme.
[b] These estimates were prepared in parallel to those for the international estimates; it was assumed that all dietary sugars in diets in Japan and the US would be replaced by steviol glycosides on a sweetness equivalent basis, at a ratio of 200:1.

JECFA concluded that the replacement estimates were highly conservative---that is, the calculated dietary exposure overestimates likely consumption---and that true dietary intakes of steviol glycosides (as steviol) would probably be **20 – 30% of these values** or 1.0 - 1.5 mg/kg bw/day on a steviol basis, **or 3.0 – 4.5 mg/kg bw/day for Reb A based on the molecular weight adjustment.** Furthermore, by adjusting for the 400-fold increased sweetness of Reb A relative to sucrose (see Appendix B-4) compared to the mixed steviol glycosides sweetness factor of 200-fold relative to sucrose assumed by JECFA, **the estimated dietary intake of Reb A would likely be about 1.5 – nearly 2.3 mg/kg bw/day.**

FSANZ (2008) similarly estimated steviol glycoside dietary intake for adult consumers in New Zealand, assuming a full sugar replacement scenario which resulted in estimated exposures of 0.3 - 1.0 mg/kg bw/day on a steviol basis, or **0.5 – 1.5 mg/kg bw/day for Reb A when making both the molecular weight and sweetness equivalency calculations**.

Merisant also calculated a dietary estimate for rebaudioside A of 2.0 mg/kg bw/day for the average consumer of the foods listed in Table 2b and 4.7 mg/kg bw/day for a 90th percentile consumer.

In another recent review conducted on behalf of Cargill and included in their GRAS notification, the intake of Reb A when used as a complete sugar replacement was estimated at 1.3 – 3.4 mg/kg bw/day when calculated as Reb A (Renwick, 2008). The estimated daily intake assessments have been compiled in Table 3b, and we can see that **total daily consumption of Reb A for defined food categories and as a general purpose sweetener is expected to be 5 mg/kg bw/day or less**, for a total daily dietary exposure of 300 mg Reb A or less for an adult.

Table 3b. Summary of Estimated Daily Intake Assessments for Rebaudioside A

Scenarios	EDI			
	As Steviol (mg/kg bw/day)	As Reb A[a] (mg/kg bw/day)	As Reb A[b] (mg/kg bw/day)	Total Daily Intake[c] (mg/day)
JECFA				
100% Reb A replacement of sugars	5.0	15.0	7.5	450
20-30% Reb A replacement of sugars	1.0 - 1.5	3.0 - 4.5	1.5 - 2.3	90 - 140
FSANZ				
100% Reb A replacement of sugars	0.3 - 1.0	0.9 - 3.0	0.5 - 1.5	30 - 90
MERISANT				
		2.0 - 4.7		120 - 282
CARGILL				
		1.3 - 3.4		78 - 204

[a] Values for JECFA and FSANZ estimates reflect molecular weight conversions from steviol to rebaudioside A.
[b] Values for JECFA and FSANZ estimates reflect the application of the correction factor for the increased sweetness of rebaudioside A (see Appendix B-4).
[c] Total daily intake figures were calculated for a 60 kg adult.

D. Other Information on Human Exposure to Stevia: Use as a Food Ingredient and Other Uses

There are no reported uses of purified Reb A as a sweetener or dietary supplement. The predominant use of steviol glycosides as a food ingredient has occurred in Brazil and Japan.[8] It is reported that 40% of the artificial sweetener market in Japan is stevia based and that steviol glycosides are commonly used in processed foods in Japan (Lester, 1999).

Steviol glycoside usage as a dietary supplement is presently permitted in the US, Canada, Australia and New Zealand. It has wide use in China and Japan in food and in dietary supplements. In the US, stevia is available in packets containing 60 - 90 mg steviol glycoside for home supplement uses, such as in beverages or other foods. It is estimated that sales of stevia in the US reached $45 million in 2005 (The Food Institute Report, 2006). No estimates are available on the daily consumption levels of steviol glycosides consumed in the US *via* dietary supplements.

During the second quarter in 2008, as a result of selected firms obtaining independent GRAS determinations for the steviol glycoside-derived sweeteners, such materials have begun to be incorporated into foods in the US. In light of FDA's review of the Merisant and Cargill GRAS notifications and issuance of "no objection" letters, the use of steviol glycoside-derived sweeteners such as rebaudioside A is anticipated to grow substantially in the US, and international uses are also expected to increase with the favorable JECFA determination at its 2008 meeting.

In South America, stevia is commonly used as a treatment for Type II diabetes (Hawke, 2003). However, elevated doses in the range of 1 gram per person per day or more were reported to be necessary to achieve this therapeutic effect (Gregersen et al., 2004).

[8] See Raintree Nutrition Tropical Plant Database (www.rain-tree.com/stevia.html).

V. SAFETY DATA FOR REBAUDIOSIDE A

A. Safety Data on Steviol Glycosides: Reviews by Expert Bodies and Other Scientists

The biological, toxicological, and clinical data on stevia and steviol glycosides have been assessed by a number of reviewers (Brusick, 2008a; Carakostas et al., 2008; Geuns, 2003; Huxtable, 2002) and most notably through the extended evaluation by JECFA (WHO, 2000, 2006, 2007, 2008) and a review by Food Standards Australia New Zealand (FSANZ, 2008) for use in food. The JECFA reviews, as well as the other reviews completed before 2008, primarily focused on mixtures of steviol glycosides typically and were not specific for purified rebaudioside A.

Some of the earliest studies on steviol glycosides were of limited value regarding safety assessments since the actual compositions of materials investigated and their questionable purities undermined drawing firm toxicological conclusions. For example, it had been reported that there was a decrease in fertility with crude stevia preparations and the mutagenic activity of the principle metabolite, steviol, was called into question. FDA was unwilling to authorize the use of stevia based on questions raised about safety by studies with materials of lesser purity and by studies with unusual protocols in *in vivo* and in *in vitro* systems usually employing high doses or high concentrations of test materials. These concerns included renal toxicity, effects on glucose metabolism, and inhibition of mitochondrial enzymes. However, over the last 15 years, the safety of steviol glycosides and rebaudioside A in particular were rather thoroughly studied with comprehensive and modern toxicology protocols using scientifically accepted dosing regimens of purified test substances. The results of these investigations are discussed below.

In addition, JECFA encouraged the further elucidation of clinical effects on blood pressure and glucose metabolism on hypertensive and diabetic individuals, respectively, in normal human subjects. By 2006, sufficient favorable data were generated for JECFA to generate a temporary ADI which was finalized in 2008. More details on the JECFA reviews are discussed in Section V.A.1. The key toxicology and clinical data on steviol glycosides (primarily stevioside) and the principle metabolite steviol reviewed by JECFA and other reviewers are summarized in Appendix E.

1. Summary of JECFA Reviews

In 1999, the 51st meeting of JECFA (WHO, 2000) expressed the following reservations about the safety data available at that time for steviol glycosides:

> The Committee noted several shortcomings in the information available on stevioside. In some studies, the material tested (stevioside or steviol) was poorly specified or of variable quality, and no information was available on other constituents or contaminants. Furthermore, no studies of human metabolism of stevioside and steviol were available. In addition, data on long-term toxicity and carcinogenicity were available for stevioside in only one species. The mutagenic potential of steviol has been tested sufficiently only *in vitro*.

Additional data were subsequently provided on the metabolism of steviol glycosides. These data helped understand that the common steviol glycosides are converted to

steviol by intestinal bacteria and then rapidly converted to glucuronides that are excreted. The committee now had a molecular basis to become comfortable with studies on test materials which consisted of variable composition but were relatively high purity mixtures of the common steviol glycosides. The committee came to the conclusion that steviol glycosides are not mutagenic and that steviol is mutagenic in *in vitro* studies but not *in vivo*. The committee became convinced that purified steviol glycosides did not impair reproductive performance as did crude preparations of stevia and that there was sufficient chronic studies in rats with adequate no observed effect levels (NOEL) that could support a reasonable acceptable daily intake (ADI) in the range of doses that would be encountered by the use of steviol glycosides as a sugar substitute. The mutagenic, reproductive and chronic studies relied upon by JECFA are summarized in Appendix E. However, JECFA wanted more clinical data to rule out pharmacological effects at the expected doses. The following excerpt was taken from the report of the 63rd meeting (WHO, 2006):

> The Committee noted that most of the data requested at its fifty-first meeting, e.g., data on the metabolism of stevioside in humans, and on the activity of steviol in suitable studies of genotoxicity *in vivo*, had been made available. The Committee concluded that stevioside and rebaudioside A are not genotoxic *in vitro* or *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives *in vitro* is not expressed *in vivo*.
>
> The NOEL for stevioside was 970 mg/kg bw/day in a long-term study (Toyoda et al., 1997) evaluated by the Committee at its fifty-first meeting. The Committee noted that stevioside has shown some evidence of pharmacological effects in patients with hypertension or with type-2 diabetes at doses corresponding to about 12.5–25 mg/kg bw/day (equivalent to 5–10 mg/kg bw/day expressed as steviol). The evidence available at present was inadequate to assess whether these pharmacological effects would also occur at lower levels of dietary exposure, which could lead to adverse effects in some individuals (e.g., those with hypotension or diabetes).
>
> The Committee therefore decided to allocate a temporary ADI, pending submission of further data on the pharmacological effects of steviol glycosides in humans. A temporary ADI of 0–2 mg/kg bw was established for steviol glycosides, expressed as steviol, on the basis of the NOEL for stevioside of 970 mg/kg bw/day (or 383 mg/kg bw/day, expressed as steviol) in the 2-year study in rats and a safety factor of 200. This safety factor incorporates a factor of 100 for inter- and intra-species differences and an additional factor of 2 because of the need for further information. The Committee noted that this temporary ADI only applies to products complying with the specifications.
>
> The Committee required additional information, to be provided by 2007, on the pharmacological effects of steviol glycosides in humans. These studies should involve repeated exposure to dietary and therapeutic doses, in normotensive and hypotensive individuals and in insulin-dependent and insulin-independent diabetics.

At the 68th meeting in 2007, JECFA concluded that sufficient progress had been made on the clinical studies and extended the temporary ADI until 2008 (WHO, 2007). Furthermore, sufficient data had been received to revise and finalize food additive specifications for steviol glycosides (FAO, 2007a). The Chemical and Technical Assessment report written after the 2007 meeting, explained the Committee's thinking which resulted in flexibility in the identity specifications (FAO, 2007b).

> In response to the call for data on "stevioside" for the 63rd meeting of the Committee, 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 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 additive could be produced with high purity (at least 95%) and that all the steviol glycosides hydrolyze upon ingestion to steviol, on which the temporary ADI is based, the 68th JECFA decided it was unnecessary to maintain a limit for the sum of stevioside and rebaudioside content. The Committee recognized that the newly revised specifications would cover a range of compositions that could include, on the dried basis, product that was at least 95% stevioside or at least 95% rebaudioside A.

At the 69th meeting in 2008, JECFA issued a final evaluation (WHO, 2008) based on their satisfaction with the completed clinical studies and actually raised the ADI. A final toxicology monograph is expected in the near future. The summary of the meeting is as follows:

> ADI of 0–4 mg/kg bw expressed as steviol, based on a NOEL of 970 mg/kg bw per day from a long-term experimental study with stevioside (383 mg/kg bw per day expressed as steviol) and a safety factor of 100. The results of the new studies presented to the Committee showed no adverse effects of steviol glycosides when taken at doses of about 4 mg/kg bw per day, expressed as steviol, for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal or low-normal blood pressure for 4 weeks.
>
> Some estimates of high-percentile dietary exposure to steviol glycosides exceeded the ADI, particularly when assuming complete replacement of caloric sweeteners with steviol glycosides. The Committee recognized that these estimates were highly conservative and that actual intakes were likely to be within the ADI.

2. Summary of FSANZ Review of Steviol Glycosides

Food Standards Australia New Zealand (FSANZ) completed a review of the safety of steviol glycosides for use as a sweetener in foods in 2008. The risk assessments undertaken by FSANZ concluded that steviol glycosides are well-tolerated and unlikely to have adverse effects on blood pressure, blood glucose or other parameters in normal, hypotensive or diabetic subjects at doses up to 11 mg/kg bw/day. The FSANZ review discussed the adequacy of the existing database and several new studies, including the clinical studies reviewed by JECFA in the summer of 2007, most notably the work of Barriocanal et al., which was later published in 2008.

Prior to publishing their final report which occurred after the JECFA meeting of 2008, FSANZ, in their draft document, also indicated that the new data in humans provides a basis for revising the uncertainty factors that were used by JECFA to derive the temporary ADI for steviol glycosides in 2005. In particular, the evidence surrounding the pharmacological effects of steviol glycosides on blood pressure and blood glucose has been strengthened so that the additional 2-fold safety factor for uncertainty related to effects in normotensive or diabetic individuals is no longer required. Therefore, FSANZ established an ADI of 4 mg/kg bw/day for steviol

glycosides as steviol equivalents, derived by applying a 100-fold safety factor to the NOEL of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol) in a 2-year rat study (FSANZ, 2008).

B. Safety Data on Rebaudioside A

Only limited studies were available on rebaudioside A during the JECFA deliberations. Several toxicology studies have been recently reported on purified rebaudioside A, although it is uncertain whether or not these studies were considered by JECFA during its 2008 deliberations. These studies include additional mutagenicity data, comparative pharmacokinetic studies with stevioside in rats and humans, several subchronic studies in rats and one in dogs and additional reproduction and developmental studies in rats, as well as additional clinical studies.

1. Mutagenicity Studies

Rebaudioside A was evaluated for genotoxicity with a set of *in vitro* and *in vivo* assays covering mutation, chromosome damage and DNA strand breakage with consistent and uniformly negative results (Pezzuto et al, 1985; Nakajima 2000a; Nakajima 2000b; Sekihashi et al, 2002) as reviewed by Brusick (2008b).

An unpublished chromosome aberration assay of rebaudioside A in cultured mammalian cells was submitted for JECFA review (Nakajima, 2000a). The JECFA review of this study indicated that no increase in chromosome aberrations was found. In their GRAS Notification, Merisant submitted three unpublished studies on rebaudioside A including a bacterial mutagenicity study, a mouse lymphoma study, and a mouse micronucleus study. All three studies indicated lack of mutagenic or genotoxic activity.

Table 4 summarizes the key mutagenicity testing results for Reb A. For a more comprehensive summary of mutagenicity studies on steviol glycosides, see Appendix E.

2. Subchronic Studies

Two repeated dose studies were conducted by the oral route in Wistar rats (Curry and Roberts, 2008). In a 4-week study, were administered rebaudioside A (97% purity) was administered at dietary concentrations of 0, 25,000, 50,000, 75,000 and 100,000 ppm. The NOAEL, including an evaluation of testes histopathology, was determined to be 100,000 ppm. In the 13-week study, Wistar rats were administered rebaudioside A at dietary concentrations of 0, 12,500, 25,000 and 50,000 ppm. Reductions in body weight gain attributable to initial taste aversion and lower caloric density of the diet were observed in high-dose male and females groups. Inconsistent reductions in serum bile acids and cholesterol 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. Significant changes in other clinical

Table 4. Mutagenicity Studies on Rebaudioside A

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCEN-TRATION / DOSE	RESULT	REFERENCE
Forward mutation	*S. typhimurium* TM677	Stevioside	NS	10 mg/plate	Negative[a]	Pezzuto et al. (1985)
Chromosomal aberration	*CHL/IU Chinese hamster lung fibroblasts*	Rebaudio-side A	NS	1.2—55 mg/mL	Negativea	Nakajima (2000a)
DNA damage (comet assay)	*Male BDF1 mouse stomach, colon, liver*	Stevia extract	Stevioside, 52; rebaudioside A, 22	250—2000 mg/kg	Negativec	Sekihashi et al. (2002)
Micronucleus formation	*BDF1 mouse bone marrow*	Rebaudio-side A	NS	500-2000 mg/kg bw per day for 2 days	Negatived	Nakajima (2000b)
Comet Assay	*Wistar rats (Blood, liver and brain cells examined)*	Stevioside	88.62%	Wistar rats treated with 4 mg/ml stevioside solution via oral administrati on for 45 days.	Positive Stevioside generated DNA lesions in the blood, liver (36 x higher than control), brain (2.5 x higher than control) and spleen (3.4 x higher than control).	Nunes et al., 2007
Bacterial Mutagenicity	5 Salmonella strains with and without exogenous metabolic activation system	Rebaudio side A	99.5%	1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.	No mutagenic responses at dose	Wagner and Van Dyke, 2006
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence and presence of exogenous metabolic activation system	Rebaudio side A	99.5%	Cloning concentrati ons of 500, 1000, 2000, 3000, 4000 and 5000 µg/mL	No mutagenic or clastogenic response	Clarke, 2006
Mouse Micronucleus	Micronucleus study consisted of seven groups, each containing 5 male and 5 female ICR mice.	Rebaudio side A	99.5%	500, 1000 and 2000 mg/kg;	No increase in micronuclei formation	Krsmanovic and Huston, 2006

pathology results, organ weights and functional observational battery test results were not observed. Macroscopic and microscopic examinations of all organs, including testes 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 4,161 and 4,645 mg/kg body weight/day in male and female rats, respectively.

Rebaudioside A (99.5% purity) was administered in the diet at target exposure levels of 500, 1000, and 2000 mg/kg bw/day to Sprague-Dawley rats for 90 days (Nikiforov and Eaton, 2008). There were no treatment-related effects on the general condition and behavior of the animals as determined by clinical observations, functional observational battery, and locomotor activity assessments. Evaluation of clinical pathology parameters revealed no toxicologically relevant, treatment-related effects on hematology, serum chemistry, or urinalysis. Macroscopic and microscopic findings revealed no treatment-related effects on any organ evaluated. Lower mean body weight gains were noted in males in the 2000 mg/kg/day group throughout the study, which was considered by the authors to be test article related; however, given the small magnitude of the difference as compared to controls, this effect was not considered to be adverse.

A 90-day dietary toxicity study was conducted in Crl:CD(SD) rats with Reb A (99.5% purity) doses of 500, 1000 and 2000 mg/kg bw/day (Eapen, 2007). Each group consisted of 20/animals/sex. There were no treatment related effects on clinical observations, food consumption, and functional observational or locomotor activity parameters. No treatment related macroscopic, organ weight or microscopic findings were reported. Significantly lower body weight gains were noted in the 2000 mg/kg bw/day group in males but not females. The body weight in males was 9.1% lower than the control group at the end of the dosing period (study week 13). The investigators did not consider this result to be adverse due to the small magnitude of difference from the control group value and were most likely due to the large proportion of the diet represented by the test material. The assigned NOAEL was ≥2000 mg/kg bw/day.

A 6-month dietary toxicity study in Beagle dogs was conducted to evaluate the potential toxic effects of Reb A (97.5% purity) at dosage levels of 0, 500, 1000 or 2000 mg/kg bw/day (Eapen, 2008). All groups consisted of 4 males and 4 females. During the course of the study, there were no unscheduled deaths. No treatment-related clinical observations were noted. Home cage, open field observations and functional observations and measurements were unaffected by the administration of rebaudioside A. There were no differences in hematology findings, serum chemistry findings, or urinalysis findings between groups. In addition, no treatment related gross necropsy observations, alterations in final body weight, alterations in organ weights, or histological changes were noted. Based on the results of this study, the authors concluded that no systemic toxicity of rebaudioside A was observed at dosage levels up to 2000 mg/kg bw/day and the assigned NOAEL was ≥2000 mg/kg bw/day.

3. Reproduction and Developmental Studies

Rebaudioside A (97% purity) was administered via the diet to male and female Han Wistar rats at 0, 7,500, 12,500, and 25,000 ppm for two generations (Curry, et al., 2008). Rebaudioside A treatment was not associated with any signs of clinical toxicity or adverse effects on body weight, body weight gain, or food consumption. No treatment-related effects of rebaudioside A were observed in either the F0 or F1 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 F1 and F2 offspring, their pre-weaning reflex development, overall body weight gains, and the timing of sexual maturation, were not adversely affected by rebaudioside A treatment. The NOAEL for reproductive effects was 25,000 ppm and the NOAEL for the survival, development, and general condition of the offspring also was considered to be 25,000 ppm or 2,048 to 2,273 mg/kg body weight/day.

The results of the published studies are supported by the results of two unpublished studies with Reb A (Sloter, 2008a and b). In a two-generation dietary reproduction study, four groups of male and female Crl:CD(SD) rats (30/sex/group) were offered either basal diet or the test article, rebaudioside A (purity 95.7%), continuously in the diet for at least 70 consecutive days prior to mating (Sloter 2008a). Rebaudioside A doses were 0, 500, 1000 and 2000 mg/kg/day for the F0 and F1 generations. F0 animals were approximately 7 weeks of age at the initiation of test diet exposure. The test diet was offered to the offspring selected to become the F1 generation following weaning (beginning on postnatal day [PND] 21). The F0 and F1 males continued to receive rebaudioside A throughout mating, continuing through the day of euthanasia. The F0 and F1 females continued to receive rebaudioside A throughout mating, gestation and lactation until day of euthanasia. The authors concluded that there were no effects on reproduction in males or females (estrus cycles, mating, fertility, conception or copulation indices, number of days between pairing and coitus, gestation length, and spermatogenic endpoints). A dose level ≥2000 mg/kg bw/day (highest dose administered) was assigned to be the NOAEL for parental systemic and reproductive toxicity.

Reb A was tested by gavage in an embryo/fetal development study in rats (Sloter, 2008b). Intrauterine growth and survival were unaffected by the test article, and there were no test article-related fetal malformations or developmental variations at any dosage level. In the absence of maternal or developmental toxicity a dose level ≥2000 mg/kg bw/day (highest dose administered) was considered to be the NOAEL for maternal and embryo/fetal developmental toxicity when Reb A was administered by oral gavage to pregnant rats.

4. Clinical Studies on Rebaudioside A

A randomized, double-blind trial evaluated the hemodynamic effects of four weeks' consumption of 1000 mg/day rebaudioside A (97% purity) versus placebo in 100 individuals with normal and low-normal systolic blood pressure (SBP) and diastolic blood pressure (DBP) (Maki et al., 2008a). Subjects were predominantly female (76%, rebaudioside A and 82%, placebo) with a mean age of ~41 (range 18 to 73)

years. At baseline, mean resting, seated SBP/DBP was 110.0/70.3 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-hour ambulatory blood pressure responses. The authors concluded that these results indicate that 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.

Another trial evaluated the effects of 16 weeks of consumption of 1000 mg rebaudioside A (97% purity, n = 60), a steviol glycoside with potential use as a sweetener, compared to placebo (n = 62) in men and women (33-75 years of age) with type 2 diabetes mellitus (Maki, et al., 2008b). Mean ± standard error changes in glycosylated hemoglobin levels did not differ significantly between the rebaudioside A (0.11 ± 0.06%) and placebo (0.09 ± 0.05%; $p = 0.355$) groups. Changes from baseline for rebaudioside A and placebo, respectively, in fasting glucose (7.5 ± 3.7 mg/dL and 11.2 ± 4.5 mg/dL), insulin (1.0 ± 0.64 µU/mL and 3.3 ± 1.5 µU/mL), and Cpeptide (0.13 ± 0.09 ng/mL and 0.42 ± 0.14 ng/mL) did not differ significantly ($p > 0.05$ for all). Assessments of changes in blood pressure, body weight, and fasting lipids indicated no differences by treatment. Rebaudioside A was well-tolerated, and records of hypoglycemic episodes showed no excess versus placebo. The authors suggest that these result that chronic use of 1000 mg rebaudioside A does not alter glucose homeostasis or blood pressure in individuals with type 2 diabetes mellitus.

5. Absorption, Distribution, Metabolism and Excretion (ADME) Studies

Three recently completed studies have shed light on the absorption and fate of rebaudioside A in rats and humans.

The toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol were examined in rats for comparative purposes to determine whether toxicological studies conducted previously with stevioside would be applicable to the structurally-related glycoside, rebaudioside A (Roberts and Renwick, 2008). Single, oral doses of the radiolabelled compounds were extensively and rapidly 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 metabolite profiles; the predominant radioactive component in all samples was steviol, with lower amounts of steviol glucuronide(s) and low levels 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 hours. Urinary excretion accounted for less than 2% of the administered dose for all compounds in both intact and bile duct-cannulated rats, and the majority of the absorbed dose was excreted *via* the bile. After administration of the compounds to 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 de-conjugation in the lower intestine. The authors concluded that the overall data on toxicokinetics and metabolism indicate that

rebaudioside A and stevioside are handled in an almost identical manner in the rat after oral dosing.

This randomized, double-blind, cross-over study assessed the comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A and stevioside in healthy adult male subjects (Wheeler et al., 2008). Steviol glucuronide appeared in the plasma of all subjects after administration of rebaudioside A or stevioside, with median Tmax values of 12.0 and 8.00 hours post-dose, 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 Cmax value (1472 ng/ml) than administration of stevioside (1886 ng/mL). The geometric mean AUC0-t value for steviol glucuronide after administration of rebaudioside A (30788 ng*hr/mL) was approximately 10% lower than after administration of stevioside (34090 ng*hr/mL). Steviol glucuronide was excreted primarily in the urine of the subjects during the 72 hour collection period, accounting for 59% and 62% of the rebaudioside A and stevioside doses, respectively. No steviol glucuronide was detected in feces. Pharmacokinetic analysis indicated that both rebaudioside A and stevioside were hydrolyzed to steviol in the gastrointestinal tract prior to absorption. The majority of circulatory steviol was in the form of steviol glucuronide indicating rapid first-pass conjugation prior to urinary excretion. Only a small amount of steviol was detected in urine (rebaudioside A: 0.04%; stevioside: 0.02%). The authors concluded 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. No safety concerns were noted as determined by reporting of adverse events, laboratory assessments of safety or vital signs.

Another pharmacokinetic study was done as a toxicokinetic (TK) phase of a dietary study to determine the potential of rebaudioside A toxicity in rats at levels up to 2000 mg/kg bw/day (Sloter, 2008a). Reb A and total steviol were detected in peripheral blood of rats during daily administration of 2000 mg/kg bw/day of Reb A at extremely low levels, with mean plasma concentrations of approximately 0.6 and 12 ug/mL, respectively. Estimates of absorbed dose for Reb A and total steviol were approximately 0.02% and 0.06%, respectively, based on the amounts measured in urine collected over 24 hours in comparison to daily administered dietary dose to rats. Mean fecal Reb A and measured hydrolysis products expressed as *Total Reb A Equivalents* compared to daily administered dose results in an estimate of percent of dose recovered ≈ 84%.

VI. DISCUSSION OF GRAS CRITERIA AND REVIEWED INFORMATION

A. GRAS Criteria

FDA defines "safe" or "safety" as it applies to food ingredients as:

> "...reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use. It is impossible in the present state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any substance."[9]

Amplification is provided in that the determination of safety is to include probable consumption of the substance in question, the cumulative effect of the substance and appropriate safety factors. It is FDA's operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that:

> "...General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food."
>
> "General recognition of safety through experience based on common use in food prior to January 1, 1958, shall be based solely on food use of the substance prior to January 1, 1958, and shall ordinarily be based upon generally available data and information."[10]

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following component elements:[11]

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and
- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles, textbooks, or compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

---

[9] See 21 CFR 170.3(i).
[10] See 21 CFR 170.30(a).
[11] See Footnote 1.

The apparent imprecision of the terms "appreciable", "at the time" and "reasonable certainty" demonstrates that the FDA recognizes the impossibility of providing absolute safety, in this or any other area (Lu 1988; Renwick 1990).

As noted below, the safety assessment to ascertain GRAS status for rebaudioside A with the defined food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

B. Utilization of FDA Safety Assessment Methodology

Safety assessment methodology has been defined by advances in the science of risk assessment. Risk assessment, simply defined, consists of an estimate of exposure to a chemical or food ingredient coupled with an assessment of assigning a safe dose or level of exposure. Exposure estimates are based on knowledge of how the chemical and ingredient will be used. Assigning a safe dose can be a highly scientific mathematical approach or a judgment approach or a blend of these two approaches. The approach is usually dictated by the quantity, quality and rigor of the safety data available. For example, assessment of carcinogenic risk is usually a highly mathematical approach relying on specialized safety data. GRAS assessments based largely on history of use are more a function of judgment stemming from information about use as opposed to analysis of safety data.

For ingredients where there is insufficient history of use, FDA has traditionally used an approach that relies on simple mathematics using safety data and some measure of scientific judgment (Kokoski et al., 1990). FDA primarily relies on the review of laboratory animal data. More recently, FDA has begun to partially rely on human clinical information when available. FDA toxicologists first determine that the study does not demonstrate any indication of a carcinogenic effect. The next step is to carefully review the findings at each dose level and assign the dose level without effects as the NOEL or "no observed effect level" or without adverse effects as the NOAEL or "no observed adverse effect level." The NOEL or NOAEL expressed as a weight of ingredient per kilogram of body weight of test animal is divided by an appropriate safety factor to obtain an acceptable daily intake (ADI). The ADI is then compared to an estimated daily intake (EDI) for humans expressed in the same units for sake of comparison. If the ADI comfortably exceeds the EDI, the ingredient is considered to be safe under intended conditions of use. If the ADI and EDI are nearly equivalent, or even if the EDI slightly exceeds the ADI, scientific judgment based on a variety of factors can be used to consider the ingredient to be safe under intended conditions of use (Frankos and Rodricks, 2001; Kokoski et al., 1990).

Detailed guidelines are given by FDA on design and conduct of the study, including number of animals per dose group and tissues and fluids to be examined (FDA, 2006). FDA also requires that the studies are conducted according to Good Laboratory Practice regulations. FDA sets data requirements based on concern levels which are largely determined by the combination of level of use in food and chemical structure, if the ingredient is structurally similar to a chemical with toxicity of concern (FDA, 2006). These criteria are fairly conservative; except in the most trivial exposure situations, most new ingredients require a set of chronic and developmental toxicity studies as well as a full battery of short term studies for mutagenicity and genotoxicity. In these cases, FDA uses a 100-fold safety

factor to calculate the ADI from the NOEL or NOAEL. If only subchronic studies are available, FDA uses an additional uncertainty factor of ten, which converts to a safety factor of 1000 (Frankos and Rodricks, 2001; Kokoski et al.; 1990, Lu, 1988).

Safety assessments eventually rely on scientific judgment. Several additional considerations, including the assessment of available clinical data, need to be considered in setting an ADI. These are more fully discussed in FDA guidelines and JECFA reviews (FDA, 1993, 2006; WHO (JECFA) 1987).

C. Panel Discussion on the Expert Safety Reviews of Steviol Glycosides

Steviol glycosides are unique compounds in that they have viable uses as a non-nutritive sweetener in foods.[12] The series of reviews by JECFA indicate the progression of knowledge on the toxicology of these compounds. Many early toxicology studies were conducted on crude extracts of stevia and there were also several studies with *in vivo* and *in vitro* models which explored the biological activity of stevia extracts at high doses or high concentrations. Several concerns were noted, including impairment of fertility, renal effects, interference with glucose metabolism and inhibition of mitochondrial enzymes. As more studies were done on purified glycosides, the toxicology profile of steviol glycosides eventually proved out to be rather unremarkable. A number of subchronic, reproductive and chronic studies have been conducted in laboratory animals. The studies were, in general, adequately designed with appropriate dosing regimens and adequate numbers of animals to maximize the probability of detection of important effects. Notably the reproductive studies with purified steviol glycosides refuted the concern of effects on fertility that were initially reported with stevia leaves or crude extracts. All other concerns failed to manifest themselves at the doses employed in long-term rat studies.

As discussed in Section V, JECFA reasoned that there were adequate chronic studies in rats, particularly the study by Toyoda et al., (1997) on which to base an ADI with an adequate margin of safety. The committee was satisfied that the lack of carcinogenic response in these well-conducted studies justified their conclusion that the *in vitro* mutagenic activity of steviol did not present a risk of carcinogenic effects *in vivo* and, therefore, all common steviol glycosides which share the same basic metabolic and excretory pathway and that the use of high purity preparations of various steviol glycosides is safe to use as a sugar substitute. The additional clinical data subsequently presented allowed JECFA to establish a permanent ADI of 0-4 mg/kg bw/day (based on steviol equivalents) or 0 - 12 mg/kg bw for rebaudioside A over and above the temporary ADI of 0 - 2 mg/kg bw/day (based on steviol equivalents).

The Panel agrees with this reasoning. It should be noted that in a recent study, DNA damage was seen in a variety of organs in a comet assay in rats maintained on drinking water containing 4 mg/mL steviol glycosides for up to 45 days (Nunes et al., 2007). Several experts in the field have questioned the methodology used in this study (Geuns, 2007; Williams, 2007; Brusick, 2008b). The Panel has reviewed the cited publications and agrees and discounts the importance of the Nunes study.

[12] It has also been reported that steviol glycosides can impart pharmacological properties, which can be utilized in the treatment of certain disease conditions, such as hypertension and Type 2 diabetes when administered at elevated levels.

Regarding clinical effects noted in humans, in order to corroborate the observations in these studies that these effects of steviol glycosides only occur in patients with either elevated blood glucose or blood pressure (or both), JECFA called for studies in individuals that are neither hypertensive nor diabetic (WHO, 2006). As reviewed by FSANZ, new data presented to JECFA demonstrate the lack of pharmacological effects of steviol glycosides at 11 mg/kg bw/day in normal individuals or approximately slightly more than 4 mg/kg bw on the basis of steviol equivalents (Barriocanal et al., 2008). JECFA may also have had preliminary results associated with the recently published clinical studies on rebaudioside A (Maki et al., 2008a, b). The Panel has reviewed the clinical studies and concludes that there will no effects on blood pressure and glucose metabolism in humans at the doses of rebaudioside A expected from use in food as a non-nutritive sweetener.

Part of JECFA's review included anticipated dietary patterns and the use concentrations expected in various foods in order to calculate an estimated daily intake or EDI (WHO, 2003, 2006). For US consumption, based on the assumption of 100% substitution of steviol glycosides for sugar, an EDI of 5 mg/kg bw/day steviol was calculated. JECFA concluded that the replacement estimates were highly conservative and that this calculated intake of steviol glycosides (as steviol) would more likely be 20–30% of these values. Except for the scenario developed by JECFA with 100% replacement of sugars by steviol glycosides, and as discussed in Section IV.C and summarized in Table 3b, the highest dietary estimate for use in foods for Reb A is 4.7 mg/kg bw/day. The Panel embraces the JECFA ADI of 4 mg/kg bw/day based on steviol equivalents which corresponds to 12 mg/kg bw/day for Reb A and notes that the estimates as contained in Table 3b of anticipated dietary intake are below the ADI.

D. Expert Panel Discussion of the Safety of Rebaudioside A

Eleven papers describing the results of a comprehensive research program on Reb A were published in July, 2008. These studies formed the basis of the Cargill GRAS notification (GRN 253). Several other studies were sponsored by Merisant and similarly these were then submitted with their GRAS notification (GRN 252). Previously, only a limited number of toxicology studies specifically on rebaudioside A were conducted. As in the previous section, JECFA, as a world renowned expert body for the evaluation of food ingredient safety, had concluded even before these new studies were completed that seven common steviol glycosides are safe for use as sweetener preparations when present in any combination as long as the combined purity of 95% or more was established.

The presumed strategy of the most recent research on rebaudioside A was to conduct a limited number of well-designed and executed toxicology studies on the specific compound and to demonstrate in rats and in humans that it is handled pharmacokinetically similarly to stevioside, which is the steviol glycoside on which most previous pharmacokinetic research was conducted. This was done to justify using the JECFA generated ADI without having to conduct a chronic study in rats with rebaudioside A. In addition, the Merisant group upgraded the mutagenicity and genotoxicity data available on rebaudioside A with three assays that FDA generally considers to be most predictive for carcinogenicity potential. The Cargill group conducted two clinical studies to assure that rebaudioside A does not have potentially problematic pharmacological effects on blood glucose and blood pressure as was demonstrated for stevioside.

The most recent research on rebaudioside A was summarized by Carakostas et al. (2008) and Brusick (2008a). These reviews summarized the findings of the Cargill research program as follows:

- Steviol glycosides, rebaudioside A, and stevioside are not genotoxic *in vitro*.
- Steviol glycosides, rebaudioside A, and stevioside have not been shown to be genotoxic *in vivo* in well-conducted assays.
- A report indicating that stevioside produces DNA breakage *in vivo* appears to be flawed (Nunes, et al., 2007) and was improperly interpreted as a positive response.
- Steviol genotoxicity in mammalian cells is limited to *in vitro* tests that may be affected by excessive concentrations of the compound.
- The primary evidence for steviol genotoxicity is derived from very specific bacterial tests or purified plasmid DNA that lack DNA repair capabilities.
- Stevioside is not a carcinogen or cancer promoter in well-conducted rodent chronic bioassays.
- The pharmacokinetic similarity between rebaudioside A and stevioside justifies the use of the ADI established by JECFA that was determined on studies employing stevioside as the main component as the ADI for rebaudioside A.
- The dietary levels expected from consumption of rebaudioside A as a total replacement of sugar (Renwick, 2008) are less than the ADI and, therefore, there is no safety concern for consumers

The Panel concurs that both the JECFA and Renwick (2008) consumption estimates very conservatively represent a potential high user of rebaudioside A if this non-nutritive sweetener becomes widely available in food. As part of this GRAS evaluation, the Panel adopts the JECFA EDI for application to BC's rebaudioside A that is contained in Good & Sweet®.

In consideration of the aggregate safety information available, the Panel has concluded that JECFA has conducted an expert evaluation and agrees that, at the present time, the ADI for steviol glycosides of adequate purity as defined by JECFA specifications has been properly determined to be 4 mg/kg bw/person as steviol equivalents, which is equivalent to 12 mg/kg bw/day for rebaudioside A on a weight basis. The Panel agrees that unwanted pharmacological effects are not likely to occur at this level and that high consumers of rebaudioside A are not likely to exceed this level. Therefore, the Panel adopts the JECFA-derived ADI as a safe exposure for rebaudioside A and that food uses meeting the specifications within the limits determined by this esteemed international body of food safety experts can be considered to be generally recognized as safe (GRAS) within the meaning of the Food, Drug, and Cosmetic Act.

E. Discussion of Concerns Raised by UCLA Researchers and the Center for Science in the Public Interest (CSPI)

In August of 2008, two UCLA researchers published a criticism of the GRAS Assessment by Cargill (Kobylewski and Eckhert, 2008). They were recruited for this task by CSPI, long known as a "public watchdog" on food ingredient safety. The basic deficiencies contained within the toxicology review generated by the UCLA group can be summarized as follows:

- There are insufficient mutagenicity and genotoxicity data on rebaudioside A compared to comparable data available for stevioside to confirm that rebaudioside A is not likely to have carcinogenic properties.
- The metabolism of rebaudioside A is too different from stevioside to rely on the rat chronic studies on stevioside to set an ADI for rebaudioside A.
- The carcinogenic potential of both stevioside and rebaudioside A should be examined in a second rodent species. They suggest that a mouse study is needed according to FDA Redbook guidelines.[13]

1. Panel's Overall Conclusions on UCLA and CSPI Concerns

The Panel has reviewed the UCLA paper, as well as the Reb A studies submitted by Merisant and Cargill as part of their GRAS notifications to FDA. CSPI has challenged the safety determination for Reb A based to a great extent on the UCLA toxicology review. The Panel recognizes that one can always avoid making food ingredient safety decisions by asking for more data, and CSPI has adopted this position.

Based on the review of the UCLA evaluation and the composite safety information on steviol glycosides and Reb A and for the reasons summarized below, the Panel disagrees with the conclusions of the UCLA study.

The pharmacokinetic work shows that stevioside and rebaudioside A are not absorbed *per se* but are converted to steviol in the GI tract. This occurs more slowly for rebaudioside A due to the fact that it has a disaccharide side chain instead of a monosaccharide side chain present in stevioside. In both humans and rats, the steviol is rapidly converted to the glucuronide. The glucuronide is not further metabolized but is efficiently excreted. In the rat, elimination occurs in the bile to the large intestine. In humans, elimination of the glucuronide occurs both in bile and urine. The UCLA group indicates that this is a profound difference and suggests that this makes the rat a poor model for the extrapolation of an ADI. The Panel disagrees with this concern. It is more important that the glucuronide is not expected to be toxic and is not further metabolized and is efficiently eliminated. The route of elimination is different, but elimination is elimination. A mouse carcinogenicity study is not indicated because the rat is pharmacokinetically a model sufficiently similar to the human. Moreover, there are no data to indicate that the mouse is a better model than the rat. In addition, the Merisant mouse lymphoma study and mouse micronucleus study, as well as the mouse micronucleus study conducted by others, do not indicate that the mouse is especially sensitive to rebaudioside A, other steviol glycosides or steviol if formed in the mouse in a way that would manifest an undiscovered carcinogenic pathway.

Consequently, the Panel rejects the concerns of the two UCLA authors.

The Panel further notes that JECFA is composed of dozens of scientists that are experts on food ingredient safety that have established ADIs for food ingredients over the last 40 years.

[13] See Toxicological Principles for the Safety Assessment of Food Ingredients Redbook 2000 (http://www.cfsan.fda.gov/~redbook/red-toca.html) and Guidance for Industry Summary Table of Recommended Toxicological Testing for Additives Used in Food (http://www.cfsan.fda.gov/~dms/opatxgui.html).

Both Merisant and Cargill took rather rigorous scientific approaches to demonstrate the safety of rebaudioside A. The studies were equally well conducted. The safety profiles compiled by Merisant and Cargill differ somewhat, yet the results are complementary and are mutually reinforcing of rebaudioside A safety.[14]

The Cargill studies provided significant insight into the pharmacokinetics of rebaudioside A while demonstrating clinical safety of rebaudioside A regarding lack of effects on blood pressure and glucose metabolism that could result from doses expected from use in food. The Merisant notification augmented genotoxicity data in three systems recognized by FDA as good predictors of carcinogenic potential. Two of these assays were conducted in mouse systems. Merisant added a subchronic study in dogs and a teratology study in rats. Both Cargill and Merisant relied on the JECFA ADI for steviol glycosides as determined largely by published chronic studies in rats. Both groups justified the use of the ADI on pharmacokinetic arguments showing the similarity of stevioside and rebaudioside A metabolism and excretion.

The Panel endorses the conclusion of JECFA and the Cargill and Merisant Expert Panels in that there are a sufficient number of good quality health and safety studies to support the determination that the intended use of purified preparations of steviol glycosides, including Reb A, when added to food at levels up to full replacement of sugar on a sweetness equivalency basis, meets FDA's definition of safe.

F. Common Knowledge Elements of GRAS Determination

The first common knowledge element for a GRAS determination is that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing published, peer-reviewed scientific journals. The majority of studies reviewed as part of this safety assessment have been accepted for publication in the scientific literature as reported in Section V. Most of the literature relied upon by JECFA has also been published, most importantly the chronic rat studies on steviol glycosides. JECFA did make limited use of unpublished studies, and they were summarized in the two JECFA monographs. Moreover, JECFA publicly releases the results of their safety reviews, and their meeting summaries and monographs are readily available on their website. Thus, these studies become generally available to the scientific community. JECFA only reviewed a limited number of studies conducted specifically on rebaudioside A. The collection of supporting data on rebaudioside A has recently been enhanced by the 2008 studies cited earlier. The newest clinical studies that address JECFA's concern on unwanted pharmacological effects with steviol glycosides (Barriocanal et al., 2008) and with rebaudioside A (Maki et al., 2008 a, b) are now published in the peer-reviewed scientific literature.

To be sure, the Panel recognizes that the safety of steviol glycoside in human foods has been the subject of interest for many years. In addition to the reported substantial history of consumption of stevia, especially in South America and Asia, many scientific studies have been conducted and published. Some of the studies have raised concerns about the safety, and the Panel has given careful attention to such concerns. The overriding

[14] We note that the UCLA group did not review the Merisant studies.

evidence has diminished the Panel's concerns based on better study designs, better execution, or simply updated investigations that better reflect state-of-the art toxicological principles and findings.

The remaining common knowledge element for a GRAS determination is that there must be a basis to conclude that there is consensus among qualified scientists about the safety of the substance with its intended use. The JECFA opinion largely meets the common knowledge test on its own. The Panel is cognizant of the scientific rigor and broad base of scientific expertise that resides with the prestigious JECFA. JECFA is composed of expert scientists from various regulatory agencies around the world, as well as other scientists chosen because of their specific expertise on various classes of food ingredients. In addition, FDA participated in the JECFA deliberations.

The JECFA conclusion has been reviewed and validated by other respected regulatory agencies including FSANZ and the Switzerland Office of Public Health (FSANZ, 2008 and Switzerland Office of Public Health, 2008). A number of other well-respected scientists have indicated that steviol glycosides are safe for human consumption at doses in the range of the JECFA ADI (Xili et al., 1992; Toyoda et al., 1997; Geuns, 2003; Williams, 2007).

The common knowledge element has been recently embellished by the many respected scientists that participated in the Cargill-sponsored new research conducted on rebaudioside A, most notably Brusick and Renwick. An assertion of "general recognition of safety" was made by Carakostas et al. (2008). In summary, there are many diverse groups of scientists from all corners of the globe that together provide strong fulfillment of the consensus requirement. Of particular significance from the perspective of establishing consensus for the safety of high purity steviol glycosides is the mid-December 2008 "no objection" determinations by FDA for the GRAS notifications for rebaudioside A as submitted by Merisant and Cargill.

While the scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, the Panel believes that a wide consensus does exist in the scientific community to support the GRAS conclusion on rebaudioside A as outlined in this evaluation. The broader scientific community has concluded that past concerns expressed by others over the years (Huxtable, 2002) and earlier safety issues noted by FDA have been resolved by newer data on more purified test materials and the rigid specifications for purity published by JECFA for steviol glycosides, including rebaudioside A. Indeed, scientists from FDA are members of JECFA and have not objected to the safety decision on steviol glycosides. There is also a wider consensus that the body of new research on rebaudioside A is sufficient as opposed to the small group of scientists that argue that more studies need to be done before the sweetener is made available in the US.

VI. CONCLUSIONS[15]

Blue California's rebaudioside A, which is incorporated in its Good & Sweet® formulation, and having a purity of 97% as expressed on a dry weight basis, is Generally Recognized As Safe when consumed as a non-nutritive sweetener when: (1) it is produced in accordance with FDA Good Manufacturing Practices requirements; (2) it meets or exceeds the JECFA purity specifications for steviol glycosides; and (3) it is consumed within the designated JECFA ADI of 12 mg/kg bw/day on a rebaudioside A basis. In order to remain within the designated ADI, it is important to observe good manufacturing practices principles in that the quantity of a substance added to food shall not exceed the amount reasonably required to accomplish its intended technical effect.

This declaration has been made in accordance with FDA's standard for food ingredient safety, i.e., reasonable certainty of no harm under the intended conditions of use.

Richard C. Kraska, Ph.D., DABT	January 16, 2009
Robert S. McQuate, Ph.D.	January 16, 2009
Wayne R. Bidlack, Ph.D.	January 16, 2009

[15] The credentials for the individuals serving on the Expert Panel can be found in Appendix F, where the educational and professional backgrounds for Richard C. Kraska, Ph.D., DABT, Robert S. McQuate, Ph.D. and Dr. Wayne R. Bidlack are summarized. Each has extensive technical background in the evaluation of food ingredient safety. Drs. Kraska and McQuate each worked on GRAS and food additive safety issues within FDA's GRAS Review Branch earlier in their careers and subsequently continued working within this area in the private sector. Dr. Bidlack is Professor of Food Science and former Dean of the College of Agriculture at California State Polytechnic University, Pomona. He has worked extensively in food safety matters over the years and frequently serves as a consultant to the food industry. Dr. Kraska served as Chair of the Panel.

VII. REFERENCES

Al-Achi, A. and Greenwood, R., 2000. Stevia: a plant for Sweetness? US Pharmacist 25, 12.

Barriocanal, L.A., Palacios, M., Benitez, G., Benitez, S., Jimenez, J.T., 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, 37-41.

Brusick, D.J., 2008a. Postscript. Food Chem.Toxicol. doi:10.1016/j.fct.2008.05.010.

Brusick, D.J., 2008b. A critical review of the genetic toxicity of steviol and steviol glycosides. Food Chem. Toxicol. doi:10.1016/j.fct.2008.05.002.

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. doi:10.1016/j.fct.2008.05.003.

Cargill GRAS Notification for Rebaudioside A, 2008, Submitted to the US Food and Drug Administration, Washington, DC and identified as GRAS Notification 253; see FDA website at http://www.cfsan.fda.gov/~rdb/opa-grsn.html.

Chang, S.S., and Cook, J.M., 1983. Stability studies of stevioside and rebaudioside A in carbonated beverages. J. Agric. Food Chem. 31, 409-414.

Clarke, J.J., 2006. *In Vitro* Mammalian Cell Gene Mutation Test (L5178Y/TK+/- Mouse Lymphoma Assay) [with Rebaudioside A]. BioReliance, Rockville, MD. Unpublished Report (Study Number AB21TG.704.BTL).

Curry, L.L., Roberts, A., 2008. Subchronic toxicity of rebaudioside A. Food Chem. Toxicol. doi:10.1016/j.fct.2008.04.042.

Curry, L.L., Roberts, A., Brown, N., 2008. Rebaudioside A: two-generation reproductive toxicity study in rats. Food Chem. Toxicol. doi:10.1016/j.fct.2008.05.005.

Eapen, A.K., 2007. A 90-Day Oral (Dietary) Toxicity Study of Rebaudioside A in Rats. WIL Research Laboratories, LLC. Unpublished Report (Study Number WIL-568002).

Eapen, A.K. 2008. A 6-Month Oral (Dietary) Toxicity Study of Chrysanta® 99-P in Beagle Dogs. WIL Research Laboratories, LLC. Unpublished Report (Study Number WIL-568011).Krsmanovic, L., Huston, T., 2006. Rebaudioside A: Mammalian Erythrocyte Micronucleus Test. BioReliance, Rockville, MD. 2007. Unpublished Report (Study Number AB21TG.123.BTL).

European Commission, 1999a. Opinion on Stevia Rebaudiana Bertoni plants and leaves. Scientific Committee on Food (CS/NF/STEV/3 Final, 17 June 1999).

European Commission, 1999b. Opinion on stevioside as a sweetener. Scientific Committee on Food (CS/ADD/EDUL/167Final, 17 June 1999).

FAO (Food and Agriculture Organization), 2007a. Steviol Glycosides. FAO JECFA Monographs 4.

FAO (Food and Agriculture Organization), 2007b. Chemical and Technical Assessment: Steviol Glycosides. Revised by Paul M. Kuznesof, PhD for the 68th JECFA Meeting.

FDA, (Food and Drug Administration), 1993. Clinical Evaluation of Foods and Food Additives In: Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food Redbook II Draft Guidance. US Food and Drug Administration, Bureau of Foods, Washington, DC. Available: http://www.cfsan.fda.gov/~acrobat/red-via.pdf.

FDA, (Food and Drug Administration), 2006. Toxicological Principles for the Safety of Food Ingredients. Redbook, US Food and Drug Administration, Bureau of Foods, Washington, DC. Available: http://www.cfsan.fda.gov/~redbook/red-toca.html.

Frankos, V. and Rodricks, J., 2001. In: Gad, S. (Ed.), Regulatory Toxicology, Food Additives and Nutrition Supplements. Taylor and Francis, New York, 133–166.

FSANZ, (Food Standards Australia New Zealand), 2008. Final Assessment Report, Application A540, Steviol Glycosides As Intense Sweeteners.

Geuns, J.M.C., 2003. Molecules of interest stevioside. Phytochemistry 64, 913-921.

Geuns, J.M.C., 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.

Gregersen, S., Jeppensen, P.B., Holst, J.J.,Hermansen, K., 2004. Antihyperglycemic effects of stevioside in type 2 diabetic subjects. Metabolism, 53, 73–76.

Hawke, J., 2003. The Bittersweet Story of the Stevia Herb. *Nexus Magazine* vol.10 no. 2. Available at: http://www.nexusmagazine.com/articles/stevia.html.

Hanson, J.R. and De Oliveira, B.H., 1993. Stevioside and related sweetditerpenoid glycosides. Nat. Prod. Rep.,10, 301-309.

Hong Kong Government, 2002. Updated July 2002. [Online]. Risk in Brief, issue no. 10, "Stevioside in Foods", Hong Kong Government, Risk Assessment Section at: http://www.info.gov.hk/fehd/textmode/safefood/report/stevioside/stevioside.html.

Huxtable, R.J., 2002. Pharmacology and toxicology of stevioside, rabaudioside A, and steviol. In: Kinghorn, A.D. (Ed.) Stevia: The Genus of Stevia, Taylor and Francis Inc., New York.

Kennelly, E.J., 2002. Sweet and non-sweet constituents of Stevia rebaudiana (Bertoni). In: Kinghorn, A.D. (Ed.), Stevia, The Genus Stevia. Medicinal and Aromatic Plants – Industrial Profiles, Vol. 19. Taylor and Francis Inc., London and New York, pp. 68-85.

Kinghorn, A.D. and Soejarto, D.D., 1985. Current status of stevioside as a sweetening agent for human use. In: H. Wagner, H. Hikino, and N.R. Farnsworth (Eds.) Economic and Medicinal Plant Research, Vol.1. Academic Press, New York, pp. 1-52.

Kobylewski, S., and Eckhert, C.D., 2008. Toxicology of Rebaudioside A: A Review. University of California at Los Angeles.

Kokoski, C.J., Henry, S.H., Lin, C.S.,Ekelman, K.B., 1990. In: Branen, A.L., Davidson, P.M., Salimen, S. (Eds.), Methods Used in Safety Evaluation. Food Additives, Marcel Dekker, New York, pp. 579-616.

Krsmanovic, L. and Huston, T., 2006. Rebaudioside A: Mammalian Erythrocyte Micronucleus Test. BioReliance, Rockville, MD. Unpublished Report (Study Number AB21TG.123.BTL).

Lester, T.,1999. *Stevia rebaudiana*. The Australian New Crops Newsletter, Issue 11, January 1999. Available: www.newcrops.uq.edu.au/newslett/ncn11161.html.

Lu, F.C., 1988. Acceptable daily intake: inception, evolution and application. Regul. Toxicol. Pharmacol. 8, 45-60.

Maki, K.C., Curry, L.L., Carakostas, M.C., Tarka, S.M., Reeves, M.S., Farmer, M.V., McKenney, J.M., Toth, P.D., Schwartz, S.L., Lubin, B.C., Dicklin, M.R., Boileau, A.C., Bisognano, J.D., 2008a. The hemodynamic effects of rebaudioside A in healthy adults with normal and low-normal blood pressure. Food Chem. Toxicol. doi:10.1016/j.fct.2008.04.040.

Maki, K.C., Curry, L.L., Reeves, M.S., Toth, P.D., McKenney, J.M., Farmer, M.V., Schwartz, S.L., Lubin, B.C., Boileau, A.C., Dicklin, M.R., Carakostas, M.C., Tarka, S.M., 2008b. Chronic consumption of rebaudioside A, a steviol glycoside, in men and women with type 2 diabetes mellitus. Food Chem. Toxicol. doi:10.1016/j.fct.2008.05.007.

Merisant GRAS Notification for Rebaudioside A, 2008, Submitted to the US Food and Drug Administration, Washington, DC under the name Whole Earth Sweetener Company LLC and identified as GRAS Notification 252; see FDA website at http://www.cfsan.fda.gov/~rdb/opa-grsn.html.

Nakajima, (initials unknown), 2000a. Chromosome aberration assay of rebaudioside A in cultured mammalian cells. Test number 5001 (079–085). Ministry of Health and Welfare, Japan.

Nakajima, (initials unknown), 2000b. Micronucleus test of rebaudioside A in mice. Test number 5002 (079-086). Unpublished report of a study conducted at the Biosafety Research Center, Japan. Submitted to WHO by Ministry of Health and Welfare, Japan.

Nanayakkara, N.P., Klocke, J.A., Compadre, C.M., Hussain, R.A., Pezzuto, J.M.,Kinghorn, A.D., 1987. Characterization and feeding deterrent effects on the aphid, Schizaphis graminum, of some derivatives of the sweet compounds, stevioside and rebaudioside A. J. Nat. Prod. 50, 434-441.

Nikiforov, A.I. and Eaton, A.K., 2008. A 90-day (oral) toxicity study of rebaudioside A in Sprague-Dawley rats. Int. J. Toxicol. 27, 65-80.

Nunes, A.P., Ferreira-Machado, S.C., Nunes, R.M., Dantas, F.J., De Mattos, J.C., Caldiera de Araujo, A., 2007. Analysis of genotoxic potentiality of stevioside by comet assay. Food Chem. Toxicol. 45, 662-666.

Pezzuto, J.M., Compadre, C.M., Swanson, S.M., Nanayakkara, D., Kinghorn, A.D., 1985. Metabolically activated steviol, the aglycone of stevioside, is mutagenic. Proc. Natl. Acad. Sci. USA 82, 2478–2482.

Renwick, A.G., 1990. Acceptable daily intake and the regulation of intense sweeteners. Food Addit. Contam. 7, 463-75.

Renwick, A.G., 2008. The use of a sweetener substitution method to predict dietary exposures for the intense sweetener rebaudioside A. Food Chem. Toxicol. doi:10.1016/j.fct.2008.05.009.

Roberts, A., Renwick, A.G., 2008. Comparative toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol in rats. Food Chem. Toxicol. doi:10.1016/j.fct.2008.05.006.

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

Sloter, E.D. 2008a. A Dietary Two-Generation Reproductive Toxicity Study of Chrysanta® 99-P in Rats. WIL Research Laboratories, LLC. Unpublished Report (Study Number WIL-568006).

Sloter, E.D., 2008b. Oral (Gavage) Study of Chrysanta® 99-P on Embryo/Fetal Development in Rats. WIL Research Laboratories, LLC. Unpublished Report (Study Number WIL-568004).

Soejarto, D.D., Kinghorn, A.D.,Farnsworth, N.R., 1982. Potential sweetening agents of plant origin. III. Organoleptic evaluation of stevia leaf herbarium samples for sweetness. J. Nat. Prod., 45, 590-599.

Suttajit, M., Vinitketkaumnuen, U., Meevatee U.,Buddhasukh, D.,1993. Mutagenicity and human chromosomal effect of stevioside, a sweetener from Stevia rebaudiana Bertoni. Environ. Health Perspect.101, 53–56.

Switzerland Office of Public Health, 2008, http.www.bag.admin.ch/themen/lebensmittel/04861/04972/index.html?lang=fr.

The Food Institute Report, 2006. FDA News May 15, 2006. From Newsday, May 2, 2006.

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

Wagner, V.O. and Van Dyke, M.R., 2006. Bacterial Reverse Mutation Assay of Rebaudioside A. BioReliance, Rockville, MD. Study Number AB21TG.503.BTL.

Williams, G. M., 2007. Letter to the Editor, Food Chem. Toxicol. 45 (2007) 2597-2598.

Wheeler, A., Boileau, A.C., Winkler, P.C., Compton, J.C., Prakash, I., Jiang, X., Mandarino, D.A., 2008. Pharmacokinetics of rebaudioside A and stevioside after single oral doses in healthy men. Food Chem. Toxicol. doi:10.1016/j.fct.2008.04.041.

WHO, 1987. Setting the ADI. In principles for the safety assessment of food additives and contaminants in foods, pp. 75-85. Environmental Health Criteria 70. IPCS International programme on chemical safety in cooperation with the joint FAO/WHO expert committee on food additives (JECFA). World Health Organization, Geneva.

WHO, 2000. Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additive Series; 42. Safety evaluation of certain food additives. Stevioside.

WHO, 2003. GEMS/Food regional diets (regional per capita consumption of raw and semi processed agricultural commodities). Geneva: Global Environment Monitoring System 144 steviol glycosides K2 Food Contamination Monitoring and Assessment Programme and Food Safety Department, World Health Organization.

WHO, 2006. Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additive Series; 54. Safety evaluation of certain food additives, Steviol Glycosides, pp.117-144.

WHO, 2007. Joint FAO/WHO Expert Committee on Food Additves. 68th Meeting, Summary and Conclusions, Steviol Glycosides. Issued July 12, 2007.

WHO, 2008. Joint FAO/WHO Expert Committee on Food Additves. 69th Meeting, Summary and Conclusions, Steviol Glycosides. Issued July 4, 2008

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, 957-965.

APPENDIX A

JECFA SPECIFICATIONS FOR STEVIOL GLYCOSIDES

STEVIOL GLYCOSIDES

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

SYNONYMS INS no. 960

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

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

Chemical name

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

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

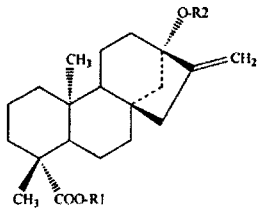
C.A.S. number

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

Chemical formula

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

Structural formula The seven named steviol glycosides:



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

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

Formula weight

Stevioside: 804.88
Rebaudioside A 967 03

Assay

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

DESCRIPTION

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

FUNCTIONAL USES

Sweetener

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Freely soluble in water and in ethanol

Stevioside and rebaudioside A

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

pH (Vol 4)

Between 4 5 and 7.0 (1 in 100 solution)

PURITY

Total ash (Vol. 4)

Not more than 1%

Loss on drying (Vol. 4)

Not more than 6% (105°, 2h)

Residual solvents (Vol. 4)

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

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

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

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

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

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

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

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

Chromatography Conditions
Column: Supelcosil LC-NH2 or equivalent (length: 15-30 cm; inner diameter: 3.9-4.6 mm)
Mobile phase: A 80.20 mixture of acetonitrile and water (see above)
Flow rate: Adjust so that the retention time of rebaudioside A is about 21 min.
Injection volume: 5-10 µl
Detector: UV at 210 nm
Column temperature: 40°

Procedure
Equilibrate the instrument by pumping mobile phase through it until a drift-free baseline is obtained. Record the chromatograms of the sample solution and of the standard solutions.

The retention times relative to rebaudioside A (1.00) are:

0.45-0.48 for stevioside	0.12-0.16 for rubusoside
0 25-0.30 for dulcoside A	0.35-0.41 for steviolbioside
0.63-0.69 for rebaudioside C	0.73-0 79 for rebaudioside B

Measure the peak areas for the seven steviol glycosides from the sample solution (the minor components might not be detected). Measure the peak area for stevioside for the standard solution.

Calculate the percentage of each of the seven steviol glycosides, *X*, in the sample from the formula:

$$\%X = [W_S/W] \times [f_X A_X/A_S] \times 100$$

where

W_S is the amount (mg) of stevioside in the standard solution

W is the amount (mg) of sample in the sample solution

A_S is the peak area for stevioside from the standard solution

A_X is the peak area of *X* for the sample solution

f_X is the ratio of the formula weight of *X* to the formula weight of stevioside: 1.00 (stevioside), 0.98 (dulcoside A), 1.20 (rebaudioside A), 1.18 (rebaudioside C), 0.80 (rubusoside), 0.80 (steviolbioside), and 1 00 (rebaudioside B).

Calculate the percentage of total steviol glycosides (sum the seven percentages).

APPENDIX B

KEY ANALYSES FOR BLUE CALIFORNIA REBAUDIOSIDE A

B-1 Eurofins Analyses of Multiple Production Batches

B-2 Certificates of Analysis

B-3 Heavy Metals & Pesticide Analyses

B-4 Comparative Sweetness Determination

Eurofins Scientific, Inc.
1365 Redwood Way
Petaluma, Ca 94951

Summary Report

Method Verification of the Determination of Steviol Glycosides/Rebaudioside A by High Performance Liquid Chromatography (HPLC) and Purity Analysis of Five Production Samples

Prepared by: _____
Jules Skamarack, Operations Manager
Eurofins Scientific, Inc.

Approved by: _____
Cecilia McCollum, Executive Vice President
Blue California.

Date Issued: December 2008

I. Study Identification

1. Study Title:

Method Verification of the Determination of Steviol Glycosides/Rebaudioside A by High Performance Liquid Chromatography (HPLC), and Purity Analysis of Six Production Samples

2. Study Objective:

The objective of this study is to verify the assay for rebaudioside A in the Blue California supplied Good & Sweet Rebaudioside A powder.

3. Study Coordinator/Performing Laboratory:
Jules Skamarack, Eurofins Scientific, Inc.

4. Study Monitors:
Cecilia McCollum, Executive Vice President
Blue California.

5. Method References:
High Performance Liquid Chromatographic Determination of Individual Sweet Diterpenoid Glycosides of *Stevia rebaudiana*, W.A.Court, Agriculture & Food Canada Pest Management Research Centre, P.O. Box 186, Ontario, N4B 2W9

Steviol glycosides, Prepared at the 69th JEFCA (2008) published in FAO JECFA Monographs 5 (2008) superseding specification prepared in the 68th JEFCA (2007), published in FAO JECFA Monographs 5 (2008). An ADI of 0-4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

II. Study Description

1. Scope:
This method is applicable to the determination of rebaudioside A, stevioside and Stevia glycosides in raw materials and *Stevia rebaudiana* plant extracts.

2. Test Materials:
Stevia rebaudiana Leaf extracts

(1) Eurofins sample **5444**, Good & Sweet Rebaudioside A , Powder, Lot # 33308092926, for method verification

(2) Eurofins sample **5949**, Good & Sweet Rebaudioside A , Powder, Lot # 33308093026

(3) Eurofins sample **5950**, Good & Sweet Rebaudioside A , Powder, Lot # 33308100328

(4) Eurofins sample **5951**, Good & Sweet Rebaudioside A , Powder, Lot #33308100928

(5) Eurofins sample # **5952**, Good & Sweet Rebaudioside A , Powder, Lot #33308101529

(6) Eurofins sample # **5953**, Good & Sweet Rebaudioside A , Powder, Lot #33308101730

3. Test Reagents:

(1) Acetonitrile, HPLC Grade
Fisher P/N A998-4, VWR P/N JT9017-3

(2) Stevioside ChromaDex., Lot # 19351-0364 (98.4%) C.A.S # 57817-89-1

(3) Rebaudioside A, Lot # ALN6700 (98.4%) from Wako Pure Chemical Industries, Ltd. Japan. C.A.S # 58543-16-1

(4) Rebaudioside A, ChromaDex, Lot # 18226-201 (97.88%). C.A.S number 58543-16-1

(5) *Stevia rebaudiana* Leaf Voucher Specimen, ChromaDex Lot # 30120-095, Positive Control Sample.

(6) Phosphoric Acid, Fischer Chemical Company P/N A260

(7) Steviolbioside ChromaDex, Lot # 19349-2871-16 (100.00%) C.A.S. # 41093-60-1

(8) Rebaudioside B ChromaDex, Lot # 18227-101 (100.00%) C.A.S. # 58543-17-2

(9) Rebaudioside C ChromaDex, Lot # 18228-1857 (96.9%) C.A.S. # 63550-99-2

(10) Stevia plant extracts positive control (internal), number 04-1172

4. Mobile Phase Preparation:

A. 80% HPLC grade acetonitrile: 20% Milli-Q water (pH adjusted to 3.0 with phosphoric acid) filtered through 0.5 μm filter (V/V).

5. Reference Standards:

Separate Standards (stevioside and rebaudioside A)

A. Stock standards.

1. Adjust standard concentration for purity and moisture levels (WAKO, ChromaDex). Corrections are made based on suppliers C of A.

2. On a microbalance, accurately weigh 20.0 ± 1 mg of stevioside ChromaDex standard and 20.0 ± 1 mg of rebaudioside A WAKO standard; quantitatively transfer to a 10-ml volumetric flask with mobile phase.
Dissolve using heat if necessary. Cool to room temperature and dilute to volume with mobile phase. Concentration is approximately 2 mg/ml stevioside and rebaudioside A. Adjust concentrations for vendor purity.

3. On a microbalance, accurately weigh 10.0 ± 1 mg of rebaudioside A Chromadex standard and quantitatively transfer to a 5-mL volumetric flask with mobile phase. Dissolve using heat if necessary. Cool to room temperature and dilute to volume with mobile phase. Concentration is approximately 2 mg/ml rebaudioside A. Adjust concentrations for vendor purity. This standard is used for a retention time confirmation and accuracy determinations.

B. Calibration standards (WAKO rebaudioside A, ChromaDex stevioside (mixed standard was used for this portion of the study). The range of quantitation will roughly be between 0.5 mg and 1.5 mg in solution. Per ICH guidelines a 5 point curve is utilized initially for determination of linearity. A three point curve was used for routine quantitation that covers the range defined by the method and listed above. The sample test concentration will be at approximately 1 mg/ml rebaudioside A, based on the expected test sample concentration. To accommodate this, dilute the stock standard volumetrically to include 1 mg/ml standard as the midpoint of calibration.

C. The accuracy stock standards were prepared as follows to confirm accuracy at the high mid and low points of the calibration curve. Rebaudioside A (reb A), ChromaDex standard Lot 18226-584 was utilized as the second source to determine accuracy of the WAKO primary standard. Three separate standards were used for each Day 1, Day 2 and Day 3 analyses. Preparation and stock concentrations are listed here:

Amount weighted (mg)	Final volume (mls)	Concentration (mg/ml)
10.109	5	1.955626
10.057	5	1.945567
10.027	5	1.939763

D. System suitability standards, retention time confirmation (rebaudioside A ChromaDex accuracy standard) and mid point calibration standards (WAKO rebaudioside A and Chromadex Stevioside) for system suitability were utilized. See results section for concentrations.

6. Single Lab Verification Study Results:

A. Primary method: See provided method.

B. Rebaudioside A standard stability (midpoint standard at 0.9617 mg/ml)

1. Room Temperature Results Rebaudioside A:

Day	Day 1	Day 2	Day 3	Day 4	Day 5
Area	2271	2274	2287	2283	2295
% Recovery	100	100.1	100.7	100.5	101.1

Day 1 is an average of 8 injections of the calibration standard. RSD of 8 injections = 0.230 and passes the criteria for calibration using a single point (see methodology)

2. Performance Characteristics:

a. **All results were in the acceptable range of 99-101%. Standards appear to be stable for the duration of 5 days; however, the trend of increasing standard concentration indicates that a fresh standard should be made for each 3 to 4 consecutive days for analysis. The high level of solvent (acetonitrile) may be indicative of evaporation over time causing a concentration effect.**

C. Linearity:

1. A five point calibration curve for both stevioside and rebaudioside were developed. The stock standard was then diluted using mobile phase to create a 5 point calibration curve for validation with concentrations for **stevioside** as follows (adjusted for standard purity and moisture):

Stock (mls)	Final volume (mls)	Concentration (mg/ml)
1	1	1.9816776
2	3	1.3211184
1	2	0.9908388
1	3	0.6605592
1	4	0.4954194

Linearity Results Stevioside:

Correlation Coefficient	**Specification**	**Result**
0.99993	**>/= 0.999**	**PASS**

Concentrations for **rebaudioside A** are as follows (adjusted for standard purity and moisture):

Stock (mls)	Final volume (mls)	Concentration (mg/ml)
1	1	1.9727472
2	3	1.3151648
1	2	0.9863736
1	3	0.6575824
1	4	0.4931868

Linearity Results Rebaudioside A:

Correlation Coefficient	**Specification**	**Result**
0.99994	**>/= 0.999**	**PASS**

a. The relative standard deviation (RSD) for the response factor ((amount/area) mg/mL/mAU) was determined between calibration levels. The RSD expressed as a percent is to achieve a specification of <5%. The %RSDs achieved between calibration levels was acceptable at 0.6003 for stevioside and 0.5736 for rebaudioside A .
b. Correlation coefficients for both compounds met the criteria.

D. Selectivity: For purposes of this study, selectivity is specificity
1. Perform selectivity procedures:
a. Analyze an acetonitrile blank.
b. Analyze positive control sample
c. Forced degradation studies: Create a mixed stock standard solution, subject 10.0-mL aliquots to the following conditions:
1. Photolytic degradation: standard fluorescent light, ambient temperature for 4, 8 and 24 hours
2. Thermal degradation:
a. 90°C for 4, 8 and 24 hours
b. 20-25°C for 4, 8 and 24 hours
c. -15 to-20°C for 4, 8 and 24 hours

2. Results:
a. Two blanks have been provided in the study report, the acetonitrile blank (ACN) and the preparation solvent blank (prepsolv). Both chromatograms were free of interfering peaks. No additional peaks were present in the blank chromatograms.

b. Positive control samples were tested. All compounds of interest were detected in the positive controls. Additionally the "unknown" peak found in the purity samples was also identified in each positive control. The internal positive control (04-1172) also serves as a confirmation of identification for dulcoside A for which a standard was not obtained. Dulcoside A retention time was originally confirmed with a standard in this control (04-1172) and has been monitored for approximately 4 years. In addition to the control samples a mixed standard chromatogram showing a profile of all purchased standards is included here as well as individual standard chromatograms.

c. Exposure to standard fluorescent light had little or no effect (showed no degradation) on the stevioside and rebaudioside A concentrations for the 24 hour period. See the following Table:

Time	Stevioside		Reb A	
--	Area	% recovery	Area	% Recovery
= 0 hours	2635	100	2259	100
= 4 hours	2609	99.0	2235	98.9
= 8 hours	2606	98.9	2235	98.9
= 24 hours	2609	99.0	2238	99.1

All results were in the acceptable range of 99-101%. Standards are stable for the duration of 24 hours.

d. Results for the thermal degradation follow with interpretation below. The solutions were stored in crimped vials, and brought to ambient conditions prior to analysis. The solutions were observed to be clear (free of solids) without volume change. No new additional peaks were detected during any of these experiments:

000056

Time/Condition	Stevioside		Rebaudioside A	
--	Area	% recovery	Area	% Recovery
= 0 hours	2635	100	2259	100
RT = 4 hours	2608	99.0	2235	98.9
RT = 8 hours	2612	99.1	2240	99.2

RT = 24 hours	2606	98.9	2236	99.0
90C = 4 hours	2607	98.9	2235	98.9
90C = 8 hours	2811	106.7	2409	106.6
90C = 24 hours	2708	102.8	2320	102.7
FRZ = 4 hours	2614	99.2	2241	99.2
FRZ = 8 hours	2614	99.2	2240	99.2
FRZ = 24 hours	2611	99.1	2238	99.1

RT = storage at room temperature
90C = Storage at 90 degrees Celsius
FRZ = freezer storage at – 5 to – 25 degrees Celsius.

The results for thermal degradation had no effect (showed no degradation) on the stevioside and rebaudioside A concentrations for the room temperature, 90 degrees C and freezer storage studies.

Two samples (90C 4 hours and 90C 8 hours) showed higher results for both stevioside and rebaudioside A for this portion of the study. This is likely a result of concentration (evaporation of the volatile solution, acetonitrile, during storage at this high temperature, creating excessive pressure that would not be seen at room temperature or freezing storage temperatures) and container specific, not an affect to the compound itself. Stevioside and rebaudioside A had nearly identical recoveries from the same vial. This was shown at both the 8 hour and 24 hour vial. The 24 hour vial showed less of an affect than the 8 hour. This may indicate that the 8 hour vial was not crimped as effectively as the 24 hour vial. Higher temperatures should be avoided with diluted samples and standards.

d. **Positive controls showed complete separation between stevioside, rebaudioside A, rebaudioside B, rebaudioside C, dulcoside A and steviolbioside. Standards were used to mark the retention times of the steviolbioside, rebaudioside B, rebaudioside C, stevioside and rebaudioside A. Two positive controls were utilized one, a ChromaDex dried leaf material and one internal. The ChromaDex leaf material is a complex full plant matrix. The second control that was used is a plant extract where previous work was done to confirm the retention of the major steviol glycosides including dulcoside A. The unknown compound found at approximately 12 minutes in the test samples is also present in both control samples, ChromaDex and internal.**
Note: chromatograms are included in hard copy.

E. System Suitability:

1. Minimum of five injections of an approximately 1.0 mg/ml standard solution were injected during each analysis sequence for each of three days.

2. Acceptance criteria: The system is considered suitable if the retention times of the standard peaks do not deviate more than 1 minute during an analytical run and the RSD of the peak retention times are less than 2%. Results follow:

	Day 1	Day 2	Day 3
Retention time (Rt) Range (minutes)	15.786-16.465	15.673-15.720	15.348-15.579
Rt % RSD	1.664	0.131	0.554
Rebaudioside A Peak Area RSD	0.300	0.161	0.898
Number of Data Points	8	7	7

000057

Rebaudioside A Retention Time Range meets the criteria of deviation of less than 1 minute.
Rebaudioside A Retention time % RSD = PASS.

Rebaudioside A Peak Area RSD, all PASS with results of less than 1.5 percent as per the method.

3. An Extended Performance report was generated using Agilent Chem Station software to include resolution, tailing and theoretical plate counts, comparing stevioside to rebaudioside A (Reb A). Results were determined at the beginning and end of the Day 1 runs. Results are as follows;

Beginning of run;
USP Resolution Stevioside/ Reb A = 1.156
USP Tailing Stevioside = 1.048
USP Tailing Reb A = 1.051
USP Plate Count Tangent Method, 11843/11267

End of run;
USP Resolution Stevioside/ Reb A = 1.156
USP Tailing Stevioside = 1.055
USP Tailing Reb A = 1.054
USP Plate Count Tangent Method, 12175/11784

4. The retention time and identity for Rebaudioside A was confirmed using the ChromaDex rebaudioside A standard. Chromatograms are located in the accuracy portion of the package.

F. Accuracy:

Accuracy was determined by applying the analytical procedure to an analyte of known purity. For this purpose a Chromadex Rebaudioside A standard of known purity was used. Per ICH recommendations, a minimum of 9 determinations each was performed on three concentration levels covering the range of the method (e.g., 3 concentrations/3 replicates).

1. Accuracy stock standards:

The ChromaDex rebaudioside A standard was diluted separately on three days Stock concentrations used on each day are listed here:

Stock	Concentration (mg/ml)
Day 1	1.955626
Day 2	1.945567
Day 3	1.939763

The accuracy stock standards were diluted 1:2 and 1:4 to complete the mid and low level standards. Concentrations are listed in the associated table below with the results for the accuracy tests:

Accuracy Continued:

2. Standard concentrations with accuracy results:

ChromaDex Lot Number	Concentration (mg/ml)	Stevioside Result, Percent (%w/w)	Day Tested
18226-584	1.956	101	Day1
18226-584	1.946	97.9	Day2
18226-584	1.94	101.1	Day3
18226-584	0.9778	101.3	Day1
18226-584	0.9728	98.4	Day2
18226-584	0.9699	101.9	Day3
18226-584	0.4889	100.4	Day1
18226-584	0.4864	99.2	Day2
18226-584	0.4849	101.9	Day3

000058

RSD between levels = 1.49

3. Accuracy Acceptance criteria:
   a. Recoveries must be 98 -102%
   b. RSD between levels must be ≤ 5%.

All results meet the criteria for % recovery. The average percent recovery over all 9 determinations is 100.3%. The RSD calculated on all nine data points is 1.49%.

G. Repeatability:

1. For the sample, perform 5 sample preparations. Repeat over 2 separate days, for a total of 10 per matrix. Results follow:

Lot # 33308092926 Eurofins Sample # 08-5444	Amount (mg)	Final Volume	Concentration (mg/mL)	Reb A Result (% w/w) as is
Day1-1	40.22	40	1.0055	93.9
Day1-2	42.04	40	1.0510	94.2
Day1-3	40.94	40	1.0235	93.5
Day1-4	41.37	40	1.03425	93.4
Day1-5	41.01	40	1.02525	93.9
Day2-1	40.9	40	1.0225	91.7
Day2-2	40.94	40	1.0235	91.4
Day2-3	40.55	40	1.01375	92.3
Day2-4	41.27	40	1.03175	91.4
Day2-5	40.9	40	1.0225	91.8

Repeatability Continued:

% RSD Day 1 = 0.33912
Average Value Day 1 = 93.8 (as is)

% RSD day 2 = 0.40354
Average Value Day 2= 91.7(as is)

% RSD Days 1 and 2 = 1.23567
Average Value Day 1 and 2 = 92.8(as is)

2. Acceptance criteria:
   a. Determined response factors.
      1. RSD of each set of 3 performed on same day must be ≤ 5%.
      2. RSD of each set of 10 performed on both days must be ≤ 5%.

All results meet acceptance criteria.

H. Ruggedness (intermediate precision):

1. For the sample, second analyst performs
   a. Five preparations
   b. Different instrument of exact specifications as primary study, results follow:

Lot # 33308092926 Eurofins Sample # 08-5444	Amount (mg)	Final Volume	Concentration (mg/mL)	Reb A Result (% w/w) as is
Day3-1	40.03	40	1.0008	94.0
Day3-2	39.96	40	0.9990	92.7
Day3-3	40.8	40	1.0200	92.1
Day3-4	39.81	40	0.9953	91.6
Day3-5	40.9	40	1.0225	93.2

% RSD day 3 = 0.93648
Average Value Day 3 = 92.7 (as is)

% RSD Days 1, 2 and 3 = 1.04667
Average Value Day 1, 2 and 3 = 92.7 (as is)

2. Acceptance criteria:
   a. Determined response factors.
      1. RSD of each set of 5 performed on same day must be ≤ 5%.
      2. RSD of each set of 10 performed on all days must be ≤ 5%.
   b. Determine average result for each matrix for each analyst.
      1. RSD of average results must be ≤ 5% between analysts.

All results meet acceptance criteria.

7. Purity Analysis of Five Production Samples:

A. Five additional samples were analyzed for purity. Each sample was tested for rebaudioside A, identified steviol glycosides and one unknown compound. Steviol glycosides were quantified using the molecular weight conversions from rebaudioside A as noted in the method. One unknown was quantified as rebaudioside A assuming a 1:1 relationship. The results for the five samples are reported below. Each sample was tested 5 times. Average results as well as relative standard deviation percent (% RSD) are also reported for each sample.

Sample 5949	Run 1	Run 2	Run 3	Run 4	Run 5		
Compound	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside C	0 734	0 749	0.732	0 738	0.73	0 737	1.023
Stevioside	0 806	0 8	0.82	0 806	0 812	0 809	0 935
Rebaudioside B	1.15	1 16	1 15	1.17	1.16	1 158	0 723
Rebaudioside A	96 8	97 5	97 1	97 3	97 1	97.2	0 268
Unknown	0 447	0 459	0 446	0 44	0 432	0 445	2.234
Total	99.937	100 668	100.248	100.454	100.234	100.3082	0.272

Sample 5950	Run 1	Run 2	Run 3	Run 4	Run 5		
Compound	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside A	97 4	97 5	97 1	97 2	98.1	97 460	0.401
Rebaudioside B	1 17	1.11	1 14	1 13	1.13	1.136	1 929
Stevioside	0.828	0 766	0 783	0 769	0 788	0.787	3 154
Rebaudioside C	0 747	0.705	0 73	0 719	0 734	0 727	2.181
Unknown	0.46	0 434	0 448	0 433	0 434	0 442	2.699
Total	100 605	100 515	100 201	100.251	101 186	100 552	0 392

	Run 1	Run 2	Run 3	Run 4	Run 5		
Compound	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside A	97 5	97	97 5	96.6	97 4	97 2	0.405
Rebaudioside B	1 16	1 1	1.13	1 16	1 19	1 148	2.980
Stevioside	0.809	0.774	0.779	0 788	0 819	0 7938	2 448
Rebaudioside C	0.739	0.698	0 718	0 739	0 745	0 7278	2.688
Unknown	0.446	0 413	0.442	0.456	0 431	0.4376	3.750
Total	100.654	99 985	100 569	99.743	100 585	100.3072	0.413

Sample 5952	Run 1	Run 2	Run 3	Run 4	Run 5		
Compound	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside A	97 5	95.3	97 3	97.2	97 0	96 9	0 919
Rebaudioside B	1 14	1 17	1 19	1 10	1 11	1.142	3 357
Stevioside	0.802	0 815	0 807	0.752	0 763	0 7878	3.594
Rebaudioside C	0.719	0.751	0.763	0 700	0.697	0 726	4 109
Unknown	0 424	0.452	0 458	0 434	0 411	0.4358	4 462
Total	100.585	98.488	100.518	100.186	99.981	99 9516	0.855

Sample 5953	Run 1	Run 2	Run 3	Run 4	Run 5		
Compound	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside A	97 3	98 3	97 1	97.7	97.5	97.6	0 472
Rebaudioside B	1 12	1 18	1.14	1 12	1 11	1 134	2.463
Stevioside	0 776	0.814	0 785	0 777	0.764	0.7832	2 398
Rebaudioside C	0 721	0 759	0 728	0 717	0 699	0 7248	3.023
Unknown	0 438	0.456	0 44	0.431	0 411	0 4352	3 753
Total	100 355	101 509	100.193	100.745	100 484	100.6572	0 514

8. Conclusions:

The results generated meet and exceed the acceptance criteria as established in the method verification proposal. All analyses were performed on Agilent 1100 series HPLCs with Agilent Chem Station software. The primary objective of the study has been to show that the method as designed can accurately determine the concentration of rebaudioside A in "Good & Sweet Rebaudioside A, Powder". The results show that the method is precise and accurate.

In addition to the rebaudioside A low levels of three additional steviol glycosides were identified using reference standards. Quantitation of these three compounds was accomplished using relative response factors to the rebaudioside A as described in the method. A fourth compound that does not match standards was quantified as rebaudioside A. Even though not identified, this unknown peak is present in both the internal control sample as well as the ChromaDex voucher leaf specimen used as controls.

Limit of detection and limit of quantitation were beyond the scope of this project due to the concentrated nature of the samples. However quantitation of the impurities can be performed at the low levels that are found in these samples. The ICH visual inspection method (ICH Validation of Analytical Procedures: Methodology, section 6.1) for determining limit of detection and limit of quantitation was utilized. Limit of detection and limit of quantitation for these compounds are roughly estimated at 0.05% and 0.5 percent respectively. In the future additional work can be performed to statistically determine these limits if requested.

Five lots of "Good & Sweet Rebaudioside A, Powder" were tested by this method. The results show that the method can accurately determine the concentration of rebaudioside A in this material as well as the 4 additional related peaks. The results have shown accurate and precise determination of rebaudioside A as well as identification of three additional peaks and quantitation of these peaks with one additional unknown peak.

9. Moisture Correction for Rebaudioside A:
Discrepancies between the result listed on the chromatogram for the rebaudioside A and that reported in section 7 table of this report are explained as follows. The results on the sample chromatogram for rebaudioside A have not been adjusted for moisture. All of the results in section 7 table have been adjusted for the moisture correction and reported on the dry weight basis.

The equation for moisture correction is as follows;
Rebaudioside A dry weight basis = rebaudioside A result as is / (100- % moisture / 100).

Results for the measured percent moisture are listed here;

Sample #	Measured Moisture (%)
5949	2.0
5950	2.0
5951	2.0
5952	1.9
5953	1.9

Results for the additional peaks were moisture in the Agilent Chem Station software during reprocessing. Results for these compounds expressed on the chromatogram are corrected for moisture and reported on a dry weight basis.

Blue California

CERTIFICATE OF ANALYSIS

Product: Good & Sweet ™ Rebaudioside A (***Stevia Rebaudiana, Leaves***)

Lot No:	**33308100328**	**Original Manufacturer:**	**Blue California Co.**
Date of Manufacturing:	**10-03-08**	**Expiration/Re-test date:**	**10-03-10**
QC acceptance date:	**10-27-08**	**Country of Origin:**	**China**

This product has NOT been treated by Irradiation or ETO

ATTRIBUTES	SPECIFICATION	METHODS	RESULTS
APPEARANCE	WHITE POWDER	VISUAL	PASS
FOREIGN MATTER	ABSENT	VISUAL	PASS
ODOR	CHARACTERISTIC	OLFACTORY	PASS
TASTE	400 FOLD SWEETER THAN SUGAR	GUSTATORY	
REBAUDIOSIDE A	**≥ 97%**	**HPLC**	**97.46% (on dry basis)**
LOSS ON DRYING	≤ 5%	USP 29	2.0%
HEAVY METALS	< 10 ppm	USP 29	PASS
LEAD	< 0.5 ppm	USP 29	0.10 ppm
ARSENIC	< 0.5 ppm	USP 29	PASS
ASH	< 1%	USP 29	0.19%
SOLUBILITY	SOLUBLE IN WATER AND ALCOHOL	USP 29	PASS
pH (1 % solution)	4.5 - 7.0	USP 29	6.0
BULK DENSITY	≥ 0.15 g/ml	USP 29	0.23 g/ml
TAP DENSITY	≥ 0.30 g/ml	USP 29	0.35 g/ml
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP 29	96.7%
TOTAL PLATE COUNT	< 3,000 cfu/gm	AOAC	200 cfu/gm
TOTAL COLIFORM	< 100 cfu/gm	AOAC	PASS
YEAST AND MOLDS	< 100 cfu/gm	AOAC	PASS
E. COLI:	NEGATIVE	AOAC	PASS
SALMONELLA	NEGATIVE	AOAC	PASS
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: Dr.Z.B.Yu (QA/QC Manager) Issued date: 11-12-2008

* THIS DOCUMENT CONTAINS CONFIDENTIAL INFORMATION THAT IS INTENDED ONLY FOR THE USE OF THE PARTY TO WHOM IT IS ADDRESSED. ANY DISCLOSURE, COPYING, DISTRIBUTION

Blue California

CERTIFICATE OF ANALYSIS

Product: Good & Sweet ™ Rebaudioside A (*Stevia Rebaudiana, Leaves*)

Lot No:	**33308093026**	**Original Manufacturer:**	**Blue California Co.**
Date of Manufacturing:	**09-30-08**	**Expiration/Re-test date:**	**09-30-10**
QC acceptance date:	**10-27-08**	**Country of Origin:**	**China**

This product has NOT been treated by Irradiation or ETO

ATTRIBUTES	SPECIFICATION	METHODS	RESULTS
APPEARANCE	WHITE POWDER	VISUAL	PASS
FOREIGN MATTER	ABSENT	VISUAL	PASS
ODOR	CHARACTERISTIC	OLFACTORY	PASS
TASTE	400 FOLD SWEETER THAN SUGAR	GUSTATORY	
REBAUDIOSIDE A	**≥ 97%**	**HPLC**	**97.2% (on dry basis)**
LOSS ON DRYING	≤ 5%	USP 29	2.10%
HEAVY METALS	< 10 ppm	USP 29	PASS
LEAD	< 0.5 ppm	USP 29	0.10 ppm
ARSENIC	< 0.5 ppm	USP 29	PASS
ASH	< 1%	USP 29	0.20%
SOLUBILITY	SOLUBLE IN WATER AND ALCOHOL	USP 29	PASS
pH (1 % solution)	4.5 - 7.0	USP 29	6.0
BULK DENSITY	≥ 0.15 g/ml	USP 29	0.22 g/ml
TAP DENSITY	≥ 0.30 g/ml	USP 29	0.35 g/ml
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP 29	96.6%
TOTAL PLATE COUNT	< 3,000 cfu/gm	AOAC	200 cfu/gm
TOTAL COLIFORM	< 100 cfu/gm	AOAC	PASS
YEAST AND MOLDS	< 100 cfu/gm	AOAC	PASS
E. COLI:	NEGATIVE	AOAC	PASS
SALMONELLA	NEGATIVE	AOAC	PASS
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: Dr.Z.B.Yu (QA/QC Manager) Issued date: 11-12-2008

* THIS DOCUMENT CONTAINS CONFIDENTIAL INFORMATION THAT IS INTENDED ONLY FOR THE USE OF THE PARTY TO WHOM IT IS ADDRESSED ANY DISCLOSURE, COPYING, DISTRIBUTION

Blue California

CERTIFICATE OF ANALYSIS

Product: Good & Sweet ™ Rebaudioside A (*Stevia Rebaudiana, Leaves*)

Lot No:	**33308101529**	**Original Manufacturer:**	**Blue California Co.**
Date of Manufacturing:	**10-15-08**	**Expiration/Re-test date:**	**10-15-10**
QC acceptance date:	**10-30-08**	**Country of Origin:**	**China**

This product has NOT been treated by Irradiation or ETO

ATTRIBUTES	SPECIFICATION	METHODS	RESULTS
APPEARANCE	WHITE POWDER	VISUAL	PASS
FOREIGN MATTER	ABSENT	VISUAL	PASS
ODOR	CHARACTERISTIC	OLFACTORY	PASS
TASTE	400 FOLD SWEETER THAN SUGAR	GUSTATORY	
REBAUDIOSIDE A	**≥ 97%**	**HPLC**	**97% (on dry basis)**
LOSS ON DRYING	≤ 5%	USP 29	2.90%
HEAVY METALS	< 10 ppm	USP 29	PASS
LEAD	< 0.5 ppm	USP 29	0.10 ppm
ARSENIC	< 0.5 ppm	USP 29	PASS
ASH	< 1%	USP 29	0.25%
SOLUBILITY	SOLUBLE IN WATER AND ALCOHOL	USP 29	PASS
pH (1 % solution)	4.5 - 7.0	USP 29	5.90
BULK DENSITY	≥ 0.15 g/ml	USP 29	0.23 g/ml
TAP DENSITY	≥ 0.30 g/ml	USP 29	0.35 g/ml
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP 29	96.2%
TOTAL PLATE COUNT	< 3,000 cfu/gm	AOAC	200 cfu/gm
TOTAL COLIFORM	< 100 cfu/gm	AOAC	PASS
YEAST AND MOLDS	< 100 cfu/gm	AOAC	PASS
E. COLI:	NEGATIVE	AOAC	PASS
SALMONELLA	NEGATIVE	AOAC	PASS
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: Dr.Z.B.Yu (QA/QC Manager) Issued date: 11-12-2008

* THIS DOCUMENT CONTAINS CONFIDENTIAL INFORMATION THAT IS INTENDED ONLY FOR THE USE OF THE PARTY TO WHOM IT IS ADDRESSED. ANY DISCLOSURE, COPYING, DISTRIBUTION

Blue California

CERTIFICATE OF ANALYSIS

Product: Good & Sweet ™ Rebaudioside A (***Stevia Rebaudiana, Leaves***)

Lot No:	**33308100928**	**Original Manufacturer:**	**Blue California Co.**
Date of Manufacturing:	**10-09-08**	**Expiration/Re-test date:**	**10-09-10**
QC acceptance date:	**10-27-08**	**Country of Origin:**	**China**

This product has NOT been treated by Irradiation or ETO

ATTRIBUTES	SPECIFICATION	METHODS	RESULTS
APPEARANCE	WHITE POWDER	VISUAL	PASS
FOREIGN MATTER	ABSENT	VISUAL	PASS
ODOR	CHARACTERISTIC	OLFACTORY	PASS
TASTE	400 FOLD SWEETER THAN SUGAR	GUSTATORY	
REBAUDIOSIDE A	**≥ 97%**	**HPLC**	**97.2% (on dry basis)**
LOSS ON DRYING	≤ 5%	USP 29	2.20%
HEAVY METALS	< 10 ppm	USP 29	PASS
LEAD	< 0.5 ppm	USP 29	0.10 ppm
ARSENIC	< 0.5 ppm	USP 29	PASS
ASH	< 1%	USP 29	0.22%
SOLUBILITY	SOLUBLE IN WATER AND ALCOHOL	USP 29	PASS
pH (1 % solution)	4.5 - 7.0	USP 29	6.0
BULK DENSITY	≥ 0.15 g/ml	USP 29	0.23 g/ml
TAP DENSITY	≥ 0.30 g/ml	USP 29	0.35 g/ml
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP 29	96.7%
TOTAL PLATE COUNT	< 3,000 cfu/gm	AOAC	300 cfu/gm
TOTAL COLIFORM	< 100 cfu/gm	AOAC	PASS
YEAST AND MOLDS	< 100 cfu/gm	AOAC	PASS
E. COLI:	NEGATIVE	AOAC	PASS
SALMONELLA	NEGATIVE	AOAC	PASS
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: Dr.Z.B.Yu (QA/QC Manager) Issued date: 11-12-2008

* THIS DOCUMENT CONTAINS CONFIDENTIAL INFORMATION THAT IS INTENDED ONLY FOR THE USE OF THE PARTY TO WHOM IT IS ADDRESSED. ANY DISCLOSURE, COPYING, DISTRIBUTION

Covance Laboratories Inc.
3301 Kinsman Blvd.
Madison, WI 53704
Tel: 608/241-4471 Fax: 608/241-7227

REPORT OF ANALYSIS

COVANCE
THE DEVELOPMENT SERVICES COMPANY

SAMPLE NUMBER: 80800777
BATCH NUMBER: 80800777
DATE ENTERED: 08/06/08
REPORT PRINTED: 08/14/08

FABRICE ROCCHICCIOLI
BLUE CALIFORNIA COMPANY
30111 TOMAS
RANCHO SANTA MARGARITA, CA 92688

GOOD & SWEET (REB-A) 99%: LOT #33308072119

PURCHASE ORDER NUMBER: 080408B

USP PESTICIDE SCREEN

USP PESTICIDE SCREEN

COMPOUND NAME	PASS/FAIL	MG/KG
ALACHLOR	PASS	< .02
ALDRIN AND DIELDRIN (SUM OF)	PASS	< .05
AZINPHOS-METHYL	PASS	< 1.0
BROMOPROPYLATE	PASS	< 3.0
CHLORDANE (SUM OF CIS-,TRANS-, OXYCHLORDANE)	PASS	< .05
CHLORFENVINPHOS	PASS	< .5
CHLORPYRIFOS	PASS	< .2
CHLORPYRIFOS-METHYL	PASS	< .1
CYPERMETHRIN	PASS	< 1.0
DDT+ISOMERS	PASS	< 1.0
DELTAMETHRIN	PASS	< .5
DIAZINON	PASS	< .5
DICHLORVOS	PASS	< 1.0
DITHIOCARBAMATES	PASS	< 2.0
ENDOSULFAN (ISOMERS+ENDOSULFAN SULFATE)	PASS	< 3.0
ENDRIN	PASS	< .05
ETHION	PASS	< 2.0
FENITROTHION	PASS	< .5
FENVALERATE	PASS	< 1.5
FONOFOS	PASS	< .05
HEPTACHLOR (HEPTACHLOR+HEPTACHLOR EPOXIDE)	PASS	< .05
HEXACHLOROCYCLOHEXANE ISOMERS (OTHER THAN GAMMA)	PASS	< .3
HEXACHLOROBENZENE	PASS	< .1
LINDANE (GAMMA-HEXACHLORCYCLOHEXANE)	PASS	< .6
MALATHION	PASS	< 1.0

Covance Laboratories Inc.
3301 Kinsman Blvd.
Madison, WI 53704
Tel: 608/241-4471 Fax: 608/241-7227

COVANCE
THE DEVELOPMENT SERVICES COMPANY

SAMPLE NUMBER: 80800777 PAGE 2

GOOD & SWEET (REB-A) 99%: LOT #33308072119

USP PESTICIDE SCREEN (CONTINUED)

METHIDATHION	PASS	<	.2
PARATHION	PASS	<	.5
PARATHION METHYL	PASS	<	.2
PERMETHRIN	PASS	<	1.0
PHOSALONE	PASS	<	.1
PIPERONYL BUTOXIDE	PASS	<	3.0
PRIMIPHOS-METHYL	PASS	<	4.0
PYRETHRINS I+II	PASS	<	3.0
QUINTOZENE (SUM OF PCNB+MPCPS +PENTACHLOROANILINE)	PASS	<	1.0

MI*= MATRIX INTERFERENCE

MATRIX INTERFERENCE IS CAUSED BY CO-ELUTING PEAKS IN THE SAMPLE THAT INHIBIT THE ABILITY TO IDENTIFY OR QUANTIFY THE COMPOUND AT THE SPECIFIC RETENTION TIME. WE THEREFORE CANNOT QUANTIFY THE PRESENCE OR ABSENCE OF SPECIFIC PESTICIDE RESIDUES THAT MAY COELUTE AT THE RETENTION TIMES WHERE THE MATRIX INTERFERENCES ARE OCCURRING.

METHOD REFERENCE
USP/NF METHOD 561, U.S. PHARMACOPEIA SUPPLEMENT 9, NOVEMBER 15, 1998, PP 4644-4646 (MODIFIED).

THIS IS A PARTIAL REPORT.
WHEN ALL ANALYSES ARE COMPLETED, YOU WILL RECEIVE A COMPLETE REPORT.
THE FOLLOWING ANALYSES ARE NOT COMPLETE:

STEVIA GLYCOSIDES
DULCOSIDE A
REBAUDIOSIDE A
REBAUDIOSIDE C
STEVIOSIDE
TOTAL STEVIA GLYCOSIDES

METHOD REFERENCES

USP PESTICIDE SCREEN
U.S. Pharmacopeia 31, General Chapter <561> "General Method for Pesticide Residues Analysis", USP 31/NF 26, Rockville, MD (2008).

ABC Testing

Advanced Botanical Consulting & Testing, Inc.
1169 Warner Ave., Tustin, CA 92780, Phone: (714) 259-0384 Fax: (714) 259-0385

Blue California Co.
30111 Tomas
Rancho Santa Margarita, CA 92688
Tel: (949) 635-1990 ext. 12
Fax: (949) 635-1987

ATTN: Fabrice
PO#: 012908A

Client Sample ID: Good N Sweet 99%
Lot# 33308010202
Lab #: 025201

Received Date: 01/30/2008

Report Date: 02/05/2008

Analyses	Results
Sweetness (Organoleptic)	**400 times**

Method: Organoleptic taste panel, compared to sucrose (table sugar)

Analyzed by: _____ Chemist

Approved by: _____ Wendi Wang, PhD, President

FEB-05-2008 02:39 AM ABC 714 259 0385 P.01

APPENDIX C

STABILITY TESTING STUDIES FOR BLUE CALIFORNIA REBAUDIOSIDE A

A Perfect Blend of Science and Nature

Determination of Reb-A by HPLC

Reference standard: Reb-A 95%(Sigma)

Principle: HPLC analysis was performed on a Shimadzu LC—10ATvp system equipped with SPD—10Avp detector. A Phenomenex Luna 5μ NH2 column (250mm×4.6mm) was used.

Reference standard preparation: Accurately weigh about 10.0 mg of Reb-A standard into a 50mL volumetric flask. Dissolve the standard in 20%Methanol.

Sample preparation: Accurately weigh about 20 mg of Reb-A Extract sample into a 50mL volumetric flask. Dissolve the sample in 20% methanol.

HPLC Parameters:

Column: Phenomenex Luna 5μ NH2 column (250mm×4.6mm)

Flow rate: 1mL/min

Injection volume: 20μL

Wavelength: 210nm

Runtime: 20min

Column temperature: 30°C

Retention time=9min

Mobile phase: Acetonitrile:Water=75:25

Calculation:

$$\text{Percent active compounds} = \frac{A_{sample} \times 0.95 \times W_{standard} \times 100\%}{A_{standard} \times W_{sample}}$$

Where: A_{sample} = sample's peak aero

$W_{standard}$ = standard weight in mg

$A_{standard}$ = standard's peak aero

W_{sample} = sample weight in mg

Ps: Also can detect Stevioside in same condition. And the peak of Reb-A will come out later than Stevioside's. The method of the determination of Stevioside provided below.

Determination of Stevioside by HPLC

Reference standard: Stevioside(Sigma)

Principle: HPLC analysis was performed on a Shimadzu LC—10ATvp system equipped with SPD—10Avp detector. A Phenomenex Luna 5μ NH2 column (250mm×4.6mm) was used.

Reference standard preparation: Accurately weigh about 10.0 mg of Stevioside standard into a 50mL volumetric flask. Dissolve the standard in 20%Methanol.

Sample preparation: Accurately weigh about 20 mg of Reb-A Extract sample into a 50mL volumetric flask. Dissolve the sample in 20% methanol.

HPLC Parameters:

Column: Phenomenex Luna 5μ NH2 column (250mm×4.6mm)

Flow rate: 1mL/min

Injection volume: 20μL

Wavelength: 210nm

Runtime: 20min

Column temperature: 30°C

Retention time=6min

Mobile phase: Acetonitrile:Water=75:25

Calculation:

$$\text{Percent active compounds} = \frac{A_{sample} \times 0.95 \times W_{standard} \times 100\%}{A_{standard} \times W_{sample}}$$

Where: A_{sample} = sample's peak aero

$W_{standard}$ = standard weight in mg

$A_{standard}$ = standard's peak aero

W_{sample} = sample weight in mg

Standard Curve

Precision respectively measure Stevioside reference solution for the volume of 2 μL, 4 μL, 8 μL, 12 μL, 16 μL, and inject into HPLC, record the area of the peaks, reunification and access curve equation

Inject volume	2μL	4μL	8μL	12μL	16μL
Peak area	16887.9	33820.3	67030.7	100828.5	134996.7

Linear equations	Y=30323X—20255□r=0.9937

Result: The standard curve of Stevioside means that the linear is good.

Precision respectively measure Stevioside reference solution for the volume of 2 μ L, 4 μ L, 8 μ L, 12 μ L, 16 μ L, and inject into HPLC, record the area of the peaks, reunification and access curve equation

Inject volume	2μL	4μL	8μL	12μL	16μL
Peak area	25570.0	51040.2	101987.6	153388.0	204600.0
Linear equations	Y=46041X—30805□r=0.9937□				

Result: The standard curve of Reb A means that the linear is good.

1, Reb-A 99% stability test (room temperature)
Leaving the packaged samples at room temperature, checking them every month. The results are below:

Sample title: Reb A 99%
Batch number:080101

Month / Item	Jan.2,08	Feb.1,08	Mar.2,08	Apr.2,08	May.1,08	Jun.2,08	Jul.1,08
Content (%)	99.0	99.3	99.4	99.1	99.2	99.2	99.3
Moisture content (%)	1.8	1.9	1.9	2.0	1.9	2.1	2.1

Sample title: Reb-A 99%
Batch number:080501

Month / Item	May.11,08	Jun.10,08	Jul.10,08
Content (%)	99.5	99.3	99.5
Moisture content (%)	0.7	0.9	0.9

Sample title: Reb-A 99%
Batch number:080502

Month / Item	May.17,08	Jun.15,08	Jul.14,08
Content (%)	101.1	100.9	100.9
Moisture content (%)	1.4	1.6	1.7

2, Reb-A 99% stability test (speed up temperature)
Put the packaged samples in a constant temperature and humidity room with relative humidity 75% and the temperature 40℃±2℃. Place three months.
Check the temperature and humidity at 0 month, 1 month, 2 month, and 3 month. The results are below:
Sample title: Reb-A 99%
Batch number:080101

Month / Item	Jan.2,08	Feb.1,08	Mar.2,08	Apr.2,08
Content (%)	99.0	99.2	99.3	99.2
Moisture content (%)	1.8	2.0	2.1	2.1

Sample title: Reb A 99%
Batch number:080501

Month / Item	May.11,08	Jun.10,08	Jul.10,08
Content (%)	99.5	99.4	99.7
Moisture content (%)	0.7	0.9	1.0

Sample title: Reb-A 99%
Batch number:080502

Month / Item	May.17,08	Jun.15,08	Jul.14,08
Content (%)	101.1	100.7	100.8
Moisture content (%)	1.4	1.6	1.8

• THIS DOCUMENT IS INTENDED ONLY FOR THE USE OF PARTY TO WHOM IT IS ADDRESSED AND MAY CONTAIN INFORMATION THAT IS PRIVILEGED, CONFIDENTIAL, AND PROTECTED FROM DISCLOSURE UNDER APPLICABLE LAW.
• *IF YOU ARE NOT THE ADDRESSEE, OR A PERSON AUTHORIZED TO DELIVER THE DOCUMENT TO THE ADDRESSEE, YOU ARE HEREBY NOTIFIED THAT ANY REVIEW, DISCLOSURE, DISSEMINATION, COPYING, OR OTHER ACTION BASED ON THE CONTENT OF THIS COMMUNICATION IS NOT AUTHORIZED.*
• THE INFORMATION SHOWN IN THIS DIAGRAM MAY BE UPDATED PERIODICALLY

Corporate Headquarters
30111 Tomas
Rancho Santa Margarita, CA 92688
Tel 949-635-1991
Fax: 949-635-1988

A Perfect Blend of Science and Nature

Determination of Reb-A by HPLC

Reference standard: Reb-A 95%(Sigma)

Principle: HPLC analysis was performed on a Shimadzu LC—10ATvp system equipped with SPD—10Avp detector. A Phenomenex Luna 5μ NH2 column (250mm×4.6mm) was used.

Reference standard preparation: Accurately weigh about 10.0 mg of Reb-A standard into a 50mL volumetric flask. Dissolve the standard in 20%Methanol.

Sample preparation: Accurately weigh about 20 mg of Reb-A Extract sample into a 50mL volumetric flask. Dissolve the sample in 20% methanol.

HPLC Parameters:

Column: Phenomenex Luna 5μ NH2 column (250mm×4.6mm)

Flow rate: 1mL/min

Injection volume: 20μL

Wavelength: 210nm

Runtime: 20min

Column temperature: 30°C

Retention time=9min

Mobile phase: Acetonitrile:Water=75:25

Calculation:

$$\text{Percent active compounds} = \frac{A_{sample} \times 0.95 \times W_{standard} \times 100\%}{A_{standard} \times W_{sample}}$$

Where: A_{sample} = sample's peak aero

$W_{standard}$ = standard weight in mg

$A_{standard}$ = standard's peak aero

W_{sample} = sample weight in mg

Ps: Also can detect Stevioside in same condition. And the peak of Reb-A will come out later than Stevioside's. The method of the determination of Stevioside provided below.

Determination of Stevioside by HPLC

Reference standard: Stevioside(Sigma)

Principle: HPLC analysis was performed on a Shimadzu LC—10ATvp system equipped with SPD—10Avp detector. A Phenomenex Luna 5μ NH2 column (250mm×4.6mm) was used.

Reference standard preparation: Accurately weigh about 10.0 mg of Stevioside standard into a 50mL volumetric flask. Dissolve the standard in 20%Methanol.

Sample preparation: Accurately weigh about 20 mg of Reb-A Extract sample into a 50mL volumetric flask. Dissolve the sample in 20% methanol.

HPLC Parameters:

Column: Phenomenex Luna 5μ NH2 column (250mm×4.6mm)

Flow rate: 1mL/min

Injection volume: 20μL

Wavelength: 210nm

Runtime: 20min

Column temperature: 30℃

Retention time=6min

Mobile phase: Acetonitrile:Water=75:25

Calculation:

$$\text{Percent active compounds} = \frac{A_{sample} \times 0.95 \times W_{standard} \times 100\%}{A_{standard} \times W_{sample}}$$

Where: A_{sample} = sample's peak aero

$W_{standard}$ = standard weight in mg

$A_{standard}$ = standard's peak aero

W_{sample} = sample weight in mg

Standard Curve

Precision respectively measure Stevioside reference solution for the volume of 2 μL, 4 μL, 8 μL, 12 μL, 16 μL, and inject into HPLC, record the area of the peaks, reunification and access curve equation

Inject volume	2μL	4μL	8μL	12μL	16μL
Peak area	16887.9	33820.3	67030.7	100828.5	134996.7

Linear equations	Y=30323X—20255 r=0.9937

Result: The standard curve of Stevioside means that the linear is good.

Precision respectively measure Stevioside reference solution for the volume of 2 μ L, 4 μ L, 8 μ L, 12 μ L, 16 μ L, and inject into HPLC, record the area of the peaks, reun:fication and access curve equation

Inject volume	2μL	4μL	8μL	12μL	16μL
Peak area	25570.0	51040.2	101987.6	153388.0	204600.0
Linear equations	Y=46041X—30805 r=0.9937				

Result: The standard curve of Reb-A means that the linear is good.

1, Reb A 99% stability test (room temperature)
Leaving the packaged samples at room temperature, checking them every month. The results are below:

Sample title: Reb-A 99%
Batch number:080101

Month / Item	Jan.2,08	Feb.1,08	Mar.2,08	Apr.2,08	May.1,08	Jun.2,08	Jul.1,08
Content (%)	99.0	99.3	99.4	99.1	99.2	99.2	99.3
Moisture content (%)	1.8	1.9	1.9	2.0	1.9	2.1	2.1

Sample title: Reb-A 99%
Batch number:080501

Month / Item	May.11,08	Jun.10,08	Jul.10,08
Content (%)	99.5	99.3	99.5
Moisture content (%)	0.7	0.9	0.9

Sample title: Reb-A 99%
Batch number:080502

Month / Item	May.17,08	Jun.15,08	Jul.14,08
Content (%)	101.1	100.9	100.9
Moisture content (%)	1.4	1.6	1.7

2, Reb-A 99% stability test (speed up temperature)
Put the packaged samples in a constant temperature and humidity room with relative humidity 75% and the temperature 40℃±2℃. Place three months.
Check the temperature and humidity at 0 month, 1 month, 2 month, and 3 month. The results are below:
Sample title: Reb-A 99%
Batch number:080101

Month / Item	Jan.2,08	Feb.1,08	Mar.2,08	Apr.2,08
Content (%)	99.0	99.2	99.3	99.2
Moisture content (%)	1.8	2.0	2.1	2.1

Sample title: Reb-A 99%
Batch number:080501

Month / Item	May.11,08	Jun.10,08	Jul.10,08
Content (%)	99.5	99.4	99.7
Moisture content (%)	0.7	0.9	1.0

Sample title: Reb-A 99%
Batch number:080502

Month / Item	May.17,08	Jun.15,08	Jul.14,08
Content (%)	101.1	100.7	100.8
Moisture content (%)	1.4	1.6	1.8

• THIS DOCUMENT IS INTENDED ONLY FOR THE USE OF PARTY TO WHOM IT IS ADDRESSED AND MAY CONTAIN INFORMATION THAT IS PRIVILEGED, CONFIDENTIAL, AND PROTECTED FROM DISCLOSURE UNDER APPLICABLE LAW
• IF YOU ARE NOT THE ADDRESSEE, OR A PERSON AUTHORIZED TO DELIVER THE DOCUMENT TO THE ADDRESSEE, YOU ARE HEREBY NOTIFIED THAT ANY REVIEW, DISCLOSURE, DISSEMINATION, COPYING OR OTHER ACTION BASED ON THE CONTENT OF THIS COMMUNICATION IS NOT AUTHORIZED
• THE INFORMATION SHOWN IN THIS DIAGRAM MAY BE UPDATED PERIODICALLY

Corporate Headquarters
30111 Tomas
Rancho Santa Margarita, CA 92688
Tel 949-635-1991
Fax: 949-635-1988

APPENDIX D

BLUE CALIFORNIA PROPOSED FOOD USES FOR REBAUDIOSIDE A

Proposed Food Categories for Use of Rebaudioside A

FDA DEFINED FOOD CATEGORY	INTENDED FOOD PRODUCTS
21 CFR 170.3(n)(1)	baked products
21 CFR 170.3(n)(3)	beverages, nonalcoholic
21 CFR 170.3(n)(4)	breakfast cereals
21 CFR 170.3(n)(7)	coffee and tea
21 CFR 170.3(n)(9)	confections & frostings
21 CFR 170.3(n)(10)	dairy product analogs
21 CFR 170.3(n)(12)	fats & oils
21 CFR 170.3(n)(20)	frozen dairy desserts
21 CFR 170.3(n)(21)	fruit & water ices
21 CFR 170.3(n)(30)	milk, whole & skim
21 CFR 170.3(n)(31)	milk products
21 CFR 170.3(n)(35)	processed fruits/fruit juices
21 CFR 170.3(n)(36)	processed vegetables/vegetable juice
21 CFR 170.3(n)(37)	snack foods
21 CFR 170.3(n)(38)	soft candy, candy bars, etc.
21 CFR 170.3(n)(42)	sugar substitutes
21 CFR 170.3(n)(43)	sweet sauces, toppings & syrups
	table top sweeteners
	meal replacement
	medical foods

APPENDIX E

SUMMARY OF STEVIOL GLYCOSIDES SAFETY STUDIES REVIEWED BY JECFA

The literature on steviol glycosides (other than on purified rebaudioside A) and on steviol that was relied upon in the JECFA reviews are summarized below.

A. Absorption, Distribution, Metabolism and Excretion (ADME) Studies

Many studies in rats (Wingard et al., 1980; Nakayama et al., 1986; Koyama et al., 2003a) and other animal models, including chickens (Geuns et al. 2003c), hamsters (Hutapea et al., 1999), and pigs (Geuns et al., 2003b) indicate that stevioside is not readily absorbed from the GI tract. Transport of steviol was more than an order of magnitude faster than stevioside or Rebaudioside A in an *in vitro* system using human colon carcinoma cell line (Geuns, 2003b).

There is evidence from *in vitro* metabolism studies that bacteria in the colon of rats and humans can transform various stevia glycosides into steviol (Gardana et al., 2003). Steviol was shown to be more readily transported with *in vitro* intestinal preparations than various steviosides (Geuns et al. 2003b, Koyama et al., 2003b). Slow absorption of steviol was indicated by detection in the plasma of rats given oral stevioside (Wang et al., 2004). However, Sung (2002) did not detect plasma steviol after oral doses of steviosides when administered to rats. In studies with human and rat liver extracts, it was demonstrated that steviol can be converted to various glucuronides (Koyama et al., 2003b).

Excretion of metabolites of stevioside after oral doses has been found in urine and feces in rats (Sung, 2002) and hamsters (Hutapea et al., 1999). Oral doses in pigs led to the detection of metabolites in feces but not in urine (Geuns, et al., 2003b).

In a study using 10 healthy human subjects, blood, urine and fecal metabolites were measured after subjects received 3 doses of 250 mg of purified stevioside (>97%) 3 times a day for 3 days. Urine was collected for 24 hours on day 3 and blood and fecal samples were also taken on day 3. Free steviol was detected in feces but not in blood or urine. Steviol glucuronide was detected in blood, urine and feces. 76% of the total steviol equivalents dosed were recovered in urine and feces. Based on the measurements, the author concluded that there was complete conversion in the colon to steviol which was absorbed and rapidly converted to the glucuronide (Geuns, et al., 2006).

B. Subchronic Toxicity Studies

Several subchronic studies with oral administration of steviol glycosides have been conducted in rats (Aze et al., 1991, Mitsuhashi 1976, Akashi and Yokoyama, 1975).

The most recent and the most well documented subchronic study was a 13-week toxicity study was carried out in Fischer 344 rats given doses of 0, 0.31, 0.62, 1.25, 2.5, or 5% in the diet (equivalent to 160, 310, 630, 1300, and 2500 mg/kg bw/day) to determine the appropriate doses for a two-year study of carcinogenicity. The rats were randomly allocated to six groups, each consisting of 10 males and 10 females. None of the animals died during the administration period, and there was no difference in body-weight gain between the control and treated groups during administration or in food consumption in the latter part of the study. The activity of lactic dehydrogenase and the incidence of single-cell necrosis in the liver were increased in all groups of treated males. The authors considered these

effects to be nonspecific because of the lack of a clear dose-response relationship, the relatively low severity, and their limitation to males. Other statistically significant differences in hematological and biochemical parameters were also considered to be of minor toxicological significance. The authors concluded that a concentration of 5% in the diet was a suitable maximum tolerable dose of stevioside for a two-year study in rats (Aze et al., 1991).

In earlier 3-month rat studies reviewed by Geuns (2003a)---the sample purity, doses, strain of rat were not reported---a no effect level was determined to be in excess of 2500 mg/kg bw/day and 7% of the diet, apparently due to lack of effects at highest dose tested in both studies (Akashi and Yokoyama, 1975).

C. Reproductive and Developmental Studies

S. rebaudiana has been used by Indians in Paraguay as an oral contraceptive (Mazzei-Planas and Kuc, 1968; Schvartzman et al., 1977). Crude stevia leaf extract has been shown to inhibit fertility in rats (Mazzei-Planas and Kuc, 1968). Several reproductive studies have been done with orally administered purified steviol glycosides. No effect on fertility or reproductive parameters was seen in a three generation study in hamsters at doses up to 2500 mg/kg (sample purity 90% stevioside; Yodyingyuad et al., 1991). There was an absence of statistically significant effects at doses up to 3% (equivalent to 3000 mg/kg bw/day; sample purity 96% stevioside; Mori et al., 1981). Similar results were observed in an additional rat study that was reviewed by Geuns (2003a) where limited information is available in English (sample purity 95.6% stevioside[1] Usami et al., 1995).

D. Mutagenicity and Genotoxicity on Steviol Glycosides

Many mutagenicity and genotoxicity studies on stevioside and they are summarized in Table E-1. All showed an absence of adverse genetic activity with the exception of the comet assay performed by Nunes et al. (2007).

Table E-1. Mutagenicity and Genotoxicity Studies on Stevia Extracts and Various Steviol Glycosides

End-Point	Test System	Material	Purity (%)	Concentration / Dose	Result	Reference
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[e] 1 mg/plate[f]	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	Not specified	Negative[a]	Medon et al. (1982)
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	*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/mL	Negative[a,b]	Oh et al. (1999)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	83	8 mg/mL 12 mg/mL	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[e]	Ishidate et al. (1984)
Chromosomal aberration	CHL/IU Chinese hamster lung fibroblasts	Rebaudio-side A	NS	1.2—55 mg/mL	Negative[a]	Nakajima (2000a)
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)
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)
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	Rebaudio-side A	NS	500-2000 mg/kg bw per day for 2 days	Negative[d]	Nakajima (2000b)
Comet Assay	Wistar rats (Blood, liver and brain cells examined)	Stevioside	88.62%	Wistar rats treated w/ 4 mg/ml stevioside solution via oral administration for 45 days.	Positive Stevioside generated DNA lesions in the blood, liver (36 x higher than control), brain (2.5 x higher than control) and spleen (3.4 x higher than control).	Nunes et al., 2007

NS = Not specified. [a] With and without metabolic activation (source not specified in original monograph). [b] Inadequate detail available. [c] Sacrificed at 3 hours and 24 hours. [d] Sacrificed at 30 hours after 2nd administration. [e] Without metabolic activation.

E. Chronic Toxicity Studies on Steviol Glycosides

There have been three chronic rat studies conducted on steviol glycosides. No treatment related increase in tumor incidence was seen in any of these studies. In the most recent and best documented study (additional study details were presented to JECFA in 2006) , the apparent no adverse effect level (NOAEL) in F344 rats was the dietary level of 2.5% (test sample purity 96%, Toyoda et al.,1997). At 5% of the diet, statistically significant decreases in body weight, percent survival and kidney weight were seen. The author attributed these effects to various factors. The decrease in body weight was attributed to an inhibition of glucose utilization. The decrease in survival seemed to have been caused by an unusual late onset of large granular lymphocyte leukemia in high dose males. The author reported that this tumor is rather common in F344 rats and that the overall incidence in male rats was within the historical control range experienced in the particular laboratory. The decrease in kidney weight may have been due to a decrease in chronic inflammation found in the histopathological examination. JECFA agreed that 2.5% level is the NOAEL and calculated this dose to be equivalent to 970 in males (JECFA, 2006).

F. Clinical Studies and Other Reports in Humans

Several pharmacological and biochemical effects have been reported for crude extracts of stevia leaves and purified steviol glycosides. These include effects on glucose uptake, insulin secretion and blood pressure (Geuns, 2003a). Stevioside is used in South America as a treatment for Type II diabetes. The effects of purified steviol glycosides on glucose metabolism and blood pressure have been explored further in clinical studies (Hawke, 2002; Gregersen et al., 2004).

Aqueous extracts of 5 g of *S. rebaudiana* leaves were administered to 16 volunteers at 6-h intervals for three days, and glucose tolerance tests were performed before and after administration. Another six volunteers were given an aqueous solution of arabinose in order to eliminate possible effects of stress. The extract increased glucose tolerance and significantly decreased plasma glucose concentrations during the test and after overnight fasting in all volunteers (Curi et al., 1986).

In a multi-center randomized, double-blind, placebo-controlled trial of hypertensive Chinese men and women (aged 28–75 years), 60 patients were given capsules containing 250 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 750 mg of stevioside per day (equivalent to 11 mg/kg bw/day as calculated by FSANZ, 2008) and followed up at monthly intervals for one year. Forty-six patients were given a placebo. After 3 months, systolic and diastolic blood pressure in men and women receiving stevioside decreased significantly and the effect persisted over the year. Blood biochemistry parameters, including lipids and glucose, showed no significant changes. Three patients receiving stevioside and one receiving the placebo withdrew from the study as a result of side-effects (nausea, abdominal fullness, dizziness). In addition, four patients receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved and the patients remained in the study (Chan et al., 2000).

A follow-up multi-center randomized, double-blind, placebo-controlled trial was conducted in hypertensive Chinese men and women (aged 20–75 years). Eighty-five patients were

given capsules containing 500 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 1500 mg of stevioside per day (equivalent to 21 mg/kg bw /day, as calculated by FSANZ, 2008). Eighty-nine patients were given a placebo. Three patients in each group withdrew during the course of the study. There were no significant changes in body mass index or blood biochemistry parameters throughout the study. In the group receiving stevioside, mean systolic and diastolic blood pressure was significantly decreased compared with the baseline, commencing from about 1 week after the start of treatment. After 2 years, 6 out of 52 patients (11.5%) in the group receiving stevioside had left ventricular hypertrophy compared with 17 of 50 patients (34%) in the group receiving the placebo ($p < 0.001$). Eight patients in each group reported minor side-effects (nausea, dizziness and asthenia), which led two patients in each group to withdraw from the study. Four patients in the group receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved and the patients remained in the study (Hsieh et al., 2003).

In a paired cross-over study, 12 patients with Type II diabetes were given either 1 g of stevioside (stevioside, 91%; other stevia glycosides, 9%) or 1 g of maize starch (control group), which was taken with a standard carbohydrate-rich test meal. Blood samples were drawn at 30 minutes before and for 240 minutes after ingestion of the test meal. Stevioside reduced postprandial blood glucose concentrations by an average of 18% and increased the insulinogenic index by an average of 40%, indicating beneficial effects on glucose metabolism. Insulin secretion was not significantly increased. No hypoglycemic or adverse effects were reported by the patients or observed by the investigators. Systolic and diastolic blood pressure was not altered by stevioside administration (Gregersen et al., 2004).

Forty-eight hyperlipidemic volunteers were recruited to a randomized, double-blind trial designed to investigate the hypolipidemic and hepatotoxic potential of steviol glycoside extract. The extract used in this study was a product containing stevioside (73 ± 2%), rebaudioside A (24 ± 2%) and other plant polysaccharides (3%). The subjects were given two capsules, each containing 50 mg of steviol glycoside extract or placebo, twice daily (i.e., 200 mg/day, equivalent to 3.3 mg/kg bw/day assuming an average body weight of 60 kg), for 3 months. One volunteer receiving placebo and three volunteers receiving steviol glycoside failed to complete the study for personal reasons, not related to adverse reactions. At the end of the study, both groups showed decreased serum concentrations of total cholesterol and of low-density lipoproteins. Analyses of serum concentrations of triglycerides, liver-derived enzymes and glucose indicated no adverse effects. The authors questioned the subjects' compliance with the dosing regime, in view of the similarity of effect between treatment and placebo (Anonymous, 2004a). In a follow-up study, 12 patients were given steviol glycoside extract in incremental doses of 3.25, 7.5 and 15 mg/kg bw/day, for 30 days per dose. Preliminary results indicated no adverse responses in blood and urine biochemical parameters (Anonymous, 2004b).

FSANZ (2008) summarized a new clinical study that was reported to JECFA and reviewed in 2007. At doses of stevioside of 11 mg/kg bw/day for a 3-month period in type 1 and 2 diabetics and non-diabetics with normal/low BP, no significant differences were observed in mean BP between control and treated subjects for all three groups (Barriocanal et al., 2008). In the same study, normal healthy human subjects or in type I or II diabetics

administered oral doses of stevioside at 11 mg/kg bw/day or those with mild to moderate hypertension no effect on blood glucose and insulin concentrations were observed.

G. Studies On Metabolites: Steviol

There have been a number of studies conducted on steviol, and the results are provided below.

1. Acute Toxicity

In male and female mice and rats given steviol (purity, 90%) orally, the LD_{50} was > 15 g/kg bw, and 1/15 animals died within 14 days of administration. The LD_{50} values in hamsters given steviol orally were 5.2 g/kg bw in males and 6.1 g/kg bw in females. Histopathological examination of the kidneys revealed severe degeneration of the proximal tubular cells, and these structural alterations were correlated with increased serum blood urea nitrogen and creatinine. The authors concluded that the cause of death was acute renal failure (Toskulkao et al., 1997).

2. Mutagenicity and Genotoxicity

Several mutagenicity and genotoxicity studies have been conducted on steviol. The studies reviewed by JECFA are summarized in Table E-2.

3. Developmental Toxicity Studies: Steviol

Groups of 20 pregnant golden hamsters were given steviol (purity, 90%) at doses of 0, 250, 500, 750, or 1000 mg/kg bw/day (only 12 animals at the highest dose) by gavage in corn oil on days 6-10 of gestation. A significant decrease in body weight gain and increased mortality (1/20, 7/20, and 5/12) were observed at the three highest doses, and the number of live fetuses per litter and mean fetal weight decreased in parallel. Histopathological examination of the maternal kidneys showed a dose-dependent increase in the severity of effects on the convoluted tubules (dilatation, hyaline droplets). No dose-dependent teratogenic effects were seen. The NOEL was 250 mg/kg bw/day for both maternal and developmental toxicity (Wasuntarawat et al., 1998).

Table E-2. Mutagenicity and Genotoxicity Studies on Steviol

STUDY	*IN VIVO/IN VITRO*	SYSTEM	TEST SAMPLE PURITY	AUTHOR CONCLUSION	RESULTS AND REMARKS
Sekihashi et al., 2002[a]	*in vivo/in vitro*	Comet Assay	Not reported	Negative	In *in vitro* study, steviol at 62.5, 125, 250 and 500 µg/ml did not damage DNA of TK6 and WTK1 cells in presence or absence of S9 mix. In *in vivo* study, mice sacrificed 3 or 24 hours after one-time oral administration of 250, 500, 1000 or 2000 mg/kg of steviol. Stomach, colon, kidneys, testis and liver DNA not damaged as shown by comet assay. An identical *in vivo* experiment with stevia extract performed, which also gave negative results via comet assay.
Oh et al., 1999[b]	*in vitro*	Cell Mutation and DNA damage	Not reported	Negative	Steviol gave negative results for cell mutation and DNA damage in cultured cells.
Terai, et al, 2002[a]	*in vitro*	Bacterial Mutagenicity	Not Reported	Positive	Steviol found to be mutagenic in Aroclor induced rat liver S9 fraction. 15-oxo-steviol found to be mutagenic at 10% level of steviol. Specific mutagenicity of lactone derivative in presence of S9 mixture 10x lower than that of derivative without S9 mixture.
Temcharoen et al., 1998[c]	*in vitro*	Bacterial Mutagenicity	Not Reported	Positive	Mutagenic effects of steviol and/or metabolites found in *Samonella Typhimurium* TM677 by tranversions, transitions, duplications, and deletions at the guanine phosphoribosyltransferase (gpt) gene. Magnitude of increase of these mutations over the control not reported.
Klongpanich-pak et al., 1997[c]	*in vitro*	Bacterial Mutagenicity	Not Reported	Negative	Steviol and stevioside inactive in TA strains of *Salmonella Typhimurium, e. coli WP2, uvrA/PKM101* and rec assay using *Bacillius subtilis* even when microsomal activated fraction present. Magnitude of increase of these mutations over the control not reported
Matsui et al., 1989[a]	*In vitro*	Bacterial Mutagenicity	Not Reported	Negative	Testing of Southern Blot technique with probe for gpt gene DNA of *E. coli*. The chromosomal DNA of TM677 and steviol-induced TM677 mutants digested by restriction enzymes and probed. No significant differences found in fragment length between wild-type and mutant DNA.
Matsui et al., 1996[a]	*In vitro*	Bacterial Mutagenicity, Mammalian Cells	Not Reported	Both	Steviol weakly positive in umu test, either with or without metabolic activation. Steviol negative in reverse mutation and other bacterial assays even in presence of S9 activation. The magnitude of increase over control in umu test not discussed.
Matsui et al., 1996[a]	*In vivo*	Mouse micronucleus	Not Reported	Negative	Steviol did not increase number of micronuclei observed in this study.
Procinska et al., 1991[c]	*In vitro*	Bacterial Mutagenicity	Not Reported	Negative	The direct mutagenic activity of 15-oxo-steviol was refuted.
Compadre et al., 1988[a]	*In vitro*	Bacterial Mutagenicity, Mass Spec	Not Reported	Positive	Mass spectral analysis of steviol and analogues under conditions known to produce a mutagenic response. 15-oxo-steviol, a product of the metabolite, 15-alpha-hydroxysteviol was found to be direct-acting mutagen. Magnitude of increase over control in assay not discussed.

STUDY	*IN VIVO/IN VITRO*	SYSTEM	TEST SAMPLE PURITY	AUTHOR CONCLUSION	RESULTS AND REMARKS
Pezzuto et al., 1985[d]	*In vitro*	Bacterial Mutagenicity	Not Reported	Positive	Using *Salmonella Typhimurium* TM677 strain, steviol found to be highly mutagenic in presence of 9000 x g supernatant from livers of Aroclor 1254-pretreated rats. This mutagenicty dependent on pretreatment of rats with Aroclor and NADPH addition, as unmetabolized steviol was inactive. None of other metabolites tested was mutagenic. Authors conclude that structural features of requisite importance for the expression of mutagenic activity may include a hydroxy group at position 13 and an unsaturated bond joining the carbon atoms at positions 16 and 17.
Matsui et al, 1996[c]	*In vitro*	Mutagenicity and Chromosome aberration (Chinese hamster lung fibroblasts)	Not reported	Positive	Gene mutation and chromosomal aberration found in Chinese hamster lung fibroblasts after metabolic activation of steviol. In hamsters, several metabolites of stevioside found that have not been found in rats or humans. Therefore, experimental relevance should be questioned when hamsters are used.
Temacharoen et al., 2000[c]	*In vivo*	Micronucleus (rat)	90%	Negative (see remarks)	Very high doses (8 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals. However, some cytotoxic effects were seen in females, but these not discussed further.
Temacharoen et al., 2000[c]	*In vivo*	Micronucleus (mouse)	90%	Negative (see remarks)	Very high doses (8 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals. However, some cytotoxic effects seen in the females, but were not discussed further.
Temacharoen et al., 2000[c]	*In vivo*	Micronucleus (hamster)	90%	Negative (see remarks)	Very high doses (4 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals. However, some cytotoxic effects were seen in the females, but were not discussed further.

[a] Abstract Only; [b] As Reported in JECFA 2006; [c] As Reviewed by Geuns 2003a; [d] Full Article.

References

Akashi, H., Yokoyama, Y., 1975. Security of dried-leaf extracts of Stevia. Toxicological tests. Food Industry 18, 34—43.

Anonymous, 2004a. Evaluation of the ingestion of stevioside, orally, in humans through a randomized clinical study of the type blind double. Subproject 1: Investigation of the hypolipidemic and hepatotoxic potential of the stevioside using doses usually consumed of the stevioside as sweetener. Unpublished report of a study conducted by the State University of Maringá and the Academical Hospital of Maringá. Submitted to WHO by State University of Campinas, Brazil.

Anonymous, 2004b. Evaluation of the ingestion of stevioside, orally, in humans through a randomized clinical study of the type blind double. Subproject 2: Investigation of the antihypertensive potential, insulintropic, hypolipidemic and toxic (hepatotoxic potential, nephrotoxic and of interference in the endocrine system) of the stevioside using doses above the usually consumed, but previously respecting values used in humans. Unpublished report of a study conducted by the State University of Maringá and the Academical Hospital of Maringá. Submitted to WHO by State University of Campinas, Brazil.

Aze, Y., Toyoda K., Imaida, K., Hayashi, S., Imazawa, T., Hayashi, Y., Takahashi, M., 1991. Subchronic oral toxicity study of stevioside in F344 rats. Bull. Natl. Inst. Hyg., 48-54 (in Japanese).

Barriocanal, L.A., Palacios, M., Benitez, G., Benitez, S., Jimenez, J.T., 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, 37-41.

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

Compadre, C.M., Hussain, R.A., Nanayakkara, N.P., Pezzuto, J.M., Kinghorn, A.D., 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.

Curi, R., Alvarez, M., Bazotte, R.B., Botion, L.M., Godoy, J.L., Bracht, A., 1986. Effect of Stevia rebaudiana on glucose tolerance in normal adult humans. Braz.J. Med. Biol. Res.19, 771-774 (In Portugese, English abstract only).

FSANZ, (Food Standards Australia New Zealand), 2008. Final Assessment Report, Application A540, Steviol Glycosides As Intense Sweeteners.

Gardana, C., Simonetti, P., Canzi, E., Zanchi, R., Pieta, P., (2003). Metabolism of stevioside and rebaudioside A from Stevia rebaudiana extracts by human microflora. J. Agri. Food Chem., 51, 6618–6622.

Geuns, J.M.C., 2003a. Stevioside. Phytochemistry 64, 913-921.

Geuns, J.M.C., Augustijns, P., Mols, R., Buyse, J.G.,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, J.M.C., Malheiros, R.D., Moraes, V.M.B., Decuypere, E.M.P., Compernolle, F. Buyse, J.G., 2003c. Metabolism of stevioside by chickens. J. Agri. Food Chem. 51, 1095-1101.

Geuns, J.M., Buyse, J., Vankeirsbilck, A., Temme, E.H., Compernolle, F., Toppet, S., 2006. Identification of steviol glucuronide in human urine. J. Agric. Food Chem. 5: 2794-2798.

Gregersen, S., Jeppensen, P.B., Holst, J.J.,Hermansen, K., 2004. Antihyperglycemic effects of stevioside in type 2 diabetic subjects. Metabolism, 53, 73–76.

Hawke, J., 2002. The Bittersweet Story of the Stevia Herb. *Nexus Magazine* vol.10 no. 2. Available at: http://www.nexusmagazine.com/articles/stevia.html.

Hsieh, M., Chan, P., Sue, Y., Liu, J., Liang, T., Huang, T., Tomlinson, B., Chow, M.S., Kao, P.,Chen, Y., 2003. Efficacy and tolerability of oral stevioside in patients with mild essential hypertension: A two-year, randomized, placebo-controlled study. Clin. Therap. 25, 2797–2808.

Hutapea, A.M., Tolskulkao, C., Wilairat, P., Buddhasukh, D., 1999. High-performance liquid chromatographic separation and quantitation of stevioside and its metabolites. J. Liq.Chromatogr. & Rel. Technol. 22, 1161–1170.

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

Kerr, W.E., Mello, M.L.S., Bonadio, E., 1983. Mutagenicity tests on the stevioside from Stevia rebaudiana. (Bert.) Bertoni. Brazil. J. Genetics 1, 173–176.

Klongpanichpak, S., Toskulkao, D., Temcharoen, P., Apibal, S., Glinsukon, T., 1997. Lack of mutagenicity of stevioside and steviol in *Salmonella typhimurium* TA98 and TA100. J. Med. Assoc. Thailand 80, 121-128.

Koyama, E., Sakai, N., Ohori, Y., Kitazawa, K., Izawa, O., Kakegawa, K., Fujino, A., 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., Ohori, Y., Kitazawa, K., Izawa, O., Kakegawa, K., Fujino, A., Ui, M., 2003b. In vitro metabolism of the glycosidic sweeteners, *Stevia* mixture and enzymically modified *Stevia* in human intestinal microflora. Food Chem. Toxicol. 41, 359–374.

Matsui, M., Matsui, K., Nohmi, T., Mizusawa, H.,Ishidate, M., 1989. Mutagenicity of steviol: An analytical approach using a Southern blotting system. Mutat. Res., 203, 377.

Matsui, M., Matsui, K., Kawasaki, Y., Oda, Y., Noguchi, T., Kitagawa, Y., Sawada, M., Hayashi, M., Nohmi, T., Yoshihira, K., Ishidate, M. & Sofuni, T., 1996. Evaluation of the genotoxicity of stevioside and steviol using six in vitro and one in vivo mutagenicity assays. Mutagenesis 11, 573–579.

Mazzei-Planas, G. and Kuc, J., 1968. Contraceptive properties of Stevia rebaudiana. Science162, 1007.

Medon, P.J., Pezzuto, J.M., Hovanec-Brown, J.M., Nanayakkara, N.P., Soejarto, D.D., Kamath, S.K., Kinghorn, A.D., 1982. Safety assessment of some Stevia rebaudiana sweet principles. Fed. Proc. 41, 1568.

Mitsuhashi, H., 1976. Safety of Stevioside. In: Tama Biochemical Co. Ltd. Report on Safety of Stevia, pp. 9-10.

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

Nakajima, (initials unknown), 2000a. Chromosome aberration assay of rebaudioside A in cultured mammalian cells. Test number 5001 (079–085). Ministry of Health and Welfare, Japan.

Nakajima, (initials unknown), 2000b. Micronucleus test of rebaudioside A in mice. Test number 5002 (079-086). Unpublished report of a study conducted at the Biosafety Research Center, Japan. Submitted to WHO by Ministry of Health and Welfare, Japan.

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

Nunes, A.P., Ferreira-Machado, S.C., Nunes, R.M., Dantas, F.J., De Mattos, J.C., Caldiera de Araujo, A., 2007. Analysis of genotoxic potentiality of stevioside by comet assay. Food Chem. Toxicol. 45, 662-666.

Oh, H., Han, E., Choi, D., Kim, J., Eom, M., Kang, I., Kang, H.,Ha, K., 1999. *In vitro* and *in vivo* evaluation of genotoxicity of stevioside and steviol, natural sweetener. J. Pharm. Soc. Korea 43, 614–622.

Pezzuto, J.M., Compadre, C.M., Swanson, S.M., Nanayakkara, D., Kinghorn, A.D., 1985. Metabolically activated steviol, the aglycone of stevioside, is mutagenic. Proc. Natl. Acad. Sci. USA 82, 2478–2482.

Procinska, E., Bridges, B.A.,Hanson, J.R., 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.

Schvartzman, J.B., Krimer, D.B., Moreno-Azorero, R., 1977. Cytological effects of some medicinal plants used in the control of fertility. Experientia 33, 663-665.

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

Sasaki, Y.F., Kawaguchi, S., Kamaya, A., Ohshita, M., Kabasawa, K., Iwama, K., Taniguchi, K.,Tsuda, S., 2002. The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutat. Res. 519, 103–119.

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

Suttajit, M., Vinitketkaumnuen, U., Meevatee U.,Buddhasukh, D.,1993. Mutagenicity and human chromosomal effect of stevioside, a sweetener from Stevia rebaudiana Bertoni. Environ. Health Perspect.101, 53–56.

Temcharoen, P., Pimbua, J., Glinsukon, T., Rojanapo, W., Apibal, S., 1998. Mutagenic activity of steviol to Salmonella typhimurium TM 677: Comparison of the activity of S9 liver fractions from five laboratory animal species. Bull. Health Sci. & Tech. 1, 38–45.

Temcharoen, P., Suwannatrai, M., Klongpanichpak, S., Apibal, S., Glinsukon, T., Toskulkao, D., 2000. Evaluation of the effect of steviol on chromosomal damage using micronucleus test in three laboratory animal species. J. Med. Assoc. Thailand 83, 101-108.

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., 1007–1010.

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.

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

Usami, M., Sakemi, K., Kawashima, K., Tsuda, M.,Ohno, Y., 1995. Teratogenicity study of stevioside in rats. Bull. Natl Inst. Hyg. Sci. 113, 31-35 (in Japanese).

Wang, L.Z., Goh, B.C., Fan, L.,Lee, H.S., 2004. Sensitive high-performance liquid chromatography/ mass spectrometry method for determination of steviol in rat plasma. Rapid Commun. Mass Spectrom. 18, 83-86.

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

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

WHO, 2006. Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additive Series; 54. Safety evaluation of certain food additives, Steviol Glycosides, pp.117-144.

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

APPENDIX F

Qualifications of Expert Panel Members

Richard C. Kraska, Ph.D., DABT

Robert S. McQuate, Ph.D.

Wayne R. Bidlack, Ph.D.

Pages 000095-000111 removed under Freedom of Information exemption 6.

SUBMISSION END