ORIGINAL SUBMISSION

PureCircle

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OFFICE OF
FOOD ADDITIVE SAFETY

April 24, 2013

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

Dear Dr. Gaynor:

Re: GRAS Notification for Rebaudioside X

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting, as the notifier [PureCircle Ltd., 915 Harger Road, Suite 250, Oak Brook, Illinois, 60523], a Notice of the determination, on the basis of scientific procedures, that rebaudioside X, produced by PureCircle Ltd., as defined in the enclosed documents and CD-ROM, is GRAS under specific conditions of use as a food ingredient, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, which includes detailed information on the notified substance, a summary of the basis for the GRAS determination, as well as a consensus opinion of an independent panel of experts in support of the safety of rebaudioside X under the intended conditions of use, also are enclosed for review by the agency.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

Sidd Purkayastha, Ph.D
VP, Business Development & Regulatory Affairs
PureCircle Ltd.

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PureCircle

April 24, 2013

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

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PureCircle

April 22, 2013

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

Dear Dr. Gaynor:

Re: GRAS Notification for Rebaudioside X

Please be advised that Intertek Cantox is representing PureCircle Ltd. and is fully authorized to act on our behalf with respect to the Generally Recognized as Safe (GRAS) notification for rebaudioside X submitted on April 24, 2013.

Sincerely,

(b) (6)

Sidd Purkayastha, Ph.D
VP, Business Development & Regulatory Affairs
PureCircle Ltd.

cc: Dr. Ashley Roberts, Intertek Cantox

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RECEIVED
APR 25 2013
OFFICE OF
FOOD ADDITIVE SAFETY

April 24, 2013

Office of Food Additive Safety (HFS-265)
Center for Food Safety and Applied Nutrition
Food and Drug Administration, University Station
4300 River Rd
College Park, MD 20740

Dear Sir or Madam:

RE: Certification of Virus-Free Document – (GRAS ASSESSMENT OF REBAUDIOSIDE X)

I hereby certify that the enclosed electronic files for the GRAS Notification entitled, "GRAS ASSESSMENT OF REBAUDIOSIDE X Food Usage Conditions for General Recognition of Safety" were scanned for viruses prior to submission and is thus certified as being virus-free.

Software Used: McAfee VirusScan
Virus Definition: 8.8
Created on: April 21, 2013
Scan Engine: 5400.1158

Sincerely,

(b) (6)

Judy Vowles
Manager, Word Processing

GRAS ASSESSMENT

OF

REBAUDIOSIDE X

Food Usage Conditions for General Recognition of Safety

For:

PureCircle Ltd.
915 Harger Road, Suite 250
Oak Brook, Illinois
U.S.A. 60523

Evaluation by GRAS Expert Panel:

Samuel Cohen, Ph.D., M.D.
I. Glenn Sipes, Ph.D
John A. Thomas, Ph.D

April 22, 2013

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OFFICE OF
FOOD ADDITIVE SAFETY

GRAS ASSESSMENT

OF

REBAUDIOSIDE X

Food Usage Conditions for General Recognition of Safety

For:

PureCircle Ltd.
915 Harger Road, Suite 250
Oak Brook, Illinois
U.S.A. 60523

Evaluation by GRAS Expert Panel:

Samuel Cohen, Ph.D., M.D.
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John A. Thomas, Ph.D

April 22, 2013

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I. GRAS EXEMPTION CLAIM

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

PureCircle Ltd.s (herein PureCircle) rebaudioside X 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 an appropriately convened panel of experts who are qualified by scientific training and experience. The GRAS evaluation is based on scientific procedures as described in the following sections; the evaluation accurately reflects the conditions of the *Stevia rebaudiana*-derived sweeteners intended use in foods.

As the notifier, PureCircle accepts responsibility for the GRAS determination that has been made for rebaudioside X as described in the subject notification. Consequently, rebaudioside X meeting the conditions described herein is exempt from premarket approval requirements for food ingredients.

Signed,

(b) (6)

April 22, 2013

Sidd Purkayastha, Ph.D
PureCircle Ltd.
915 Harger Road, Suite 250
Oak Brook, Illinois
U.S.A. 60523

Date

B. Name and Address of Notifier

Sidd Purkayastha, Ph.D
PureCircle Ltd.
915 Harger Road, Suite 250
Oak Brook, Illinois
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Email: sidd.purkayastha@purecircle.com

[1] 62 FR 18938 (17 April 1997); available at http: www.gpo.gov fdsys pkg FR-1997-04-17 html 9-97-9706.htm.

C. Common Name Identity of the Notified Substance

Rebaudioside X, Reb X, Stevia Leaf Extract

D. Conditions of Intended Use in Food

PureCircle intends to market rebaudioside X, a steviol glycoside obtained by hot-water extraction of dried leaves from the *Stevia rebaudiana* Bertoni (*S. rebaudiana*) plant, as a general purpose sweetening agent, in accordance with current good manufacturing practices (cGMP), in the United States (U.S.) as a table-top sweetener and in a variety of traditional food products, excluding infant formulas and meat and poultry products.

Most other high intensity sweeteners (HIS), including aspartame, have been approved by the United States Food and Drug Administration (FDA) as general purpose sweeteners with no restriction on their use in specific foods or use-levels. Hence, the foods to which aspartame are added and the use level is controlled by technological properties (*e.g.*, sweetness potency). As the sweetening potency of rebaudioside X (200 to 350 times sweeter than 6 sucrose) is similar to that of aspartame (200 times sweeter than 6 sucrose), the uses for rebaudioside X primarily reflect those currently permitted for aspartame in the U.S.

E. Basis for the GRAS Determination

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR) § 170.30, rebaudioside X has been determined by PureCircle to be GRAS on the basis of scientific procedures (U.S. FDA, 2012a). This GRAS determination is based on data generally available in the public domain pertaining to the safety of steviol glycosides as discussed herein, and on consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of rebaudioside X as a component of food [see Appendix A, entitled, **EXPERT PANEL CONSENSUS STATEMENT CONCERNING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF REBAUDIOSIDE X FOR USE AS A GENERAL PURPOSE SWEETENING AGENT**].

F. Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the FDA upon request, or will be available for review and copying at reasonable times at the offices of: PureCircle USA, Inc., 915 Harger Road, Suite 250, Oak Brook, Illinois, U.S.A. 60523.

Should the FDA have any questions or additional information requests regarding this notification, PureCircle will supply these data and information.

II. INTRODUCTION

A. Objective

At the request of PureCircle, an Expert Panel (the Expert Panel) of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and to determine whether the intended uses of rebaudioside X as a general purpose sweetener are safe and suitable and would be GRAS based on scientific procedures. The Panel consisted of the following qualified scientific experts: Dr. Samuel Cohen, Ph.D., M.D. (University of Nebraska Medical Center), Dr. I. Glenn Sipes, Ph.D. (University of Arizona), and Dr. John A. Thomas, Ph.D. (Indiana University School of Medicine).

B. Foreword

On March 8, 2013, the Expert Panel, independently and collectively, critically evaluated a dossier that included a comprehensive summary of scientific information on steviol glycosides. The dossier was prepared from information available within the public domain and also included details pertaining to the method of manufacture and product specifications, supporting analytical data, intended use-levels of rebaudioside X in foods, consumption estimates for all intended uses, and an unpublished microbial metabolism study of rebaudioside X. In addition, the Expert Panel evaluated other information deemed appropriate or necessary. Summaries of the data evaluated by the Expert Panel deemed pertinent to the GRAS evaluation of rebaudioside X are provided in Sections III to V.

C. Summary of Regulatory History of Rebaudioside X and Steviol Glycosides

Although rebaudioside X *per se* is not currently permitted for addition to food and beverages in the U.S., the use of steviol glycosides as a sweetening agent is permitted in a number of global jurisdictions, including the U.S., Europe, Australia, New Zealand, Canada, Japan, Korea, China, Malaysia, India, Indonesia, Taiwan, Brazil, Argentina, Paraguay, Uruguay, Mexico, Peru, Colombia, Israel, Russia, Switzerland, Turkey, and Ukraine. In the U.S., FDA has raised no objections to 25 GRAS notices submitted since May 2008 for both highly-purified steviol glycosides and glucosylated steviol glycosides (U.S. FDA, 2008a,b, 2009a-d, 2010a-e; 2011a-i; 2012b-f).

III. CHEMISTRY □ MANUFACTURING OF REBAUDIOSIDE X

A. Chemistry of Rebaudioside X and Steviol Glycosides

Rebaudioside X purified from the hot water extraction of *S. rebaudiana* leaves is 200 to 350 times sweeter than sucrose and is consistent with the sweetness profile of steviol glycosides (JECFA, 2007a). Rebaudioside X will be marketed as an ingredient in products that will consist of not less than 95□ total steviol glycosides with rebaudioside X representing more than 50□ of the finished product and the remainder comprising the following 9 related steviol glycosides, in any combination and ratio: stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside A, steviolbioside, and rubusoside.

Rebaudioside X is a white to off-white powder that has a clean taste with no abnormal odor. The powder is sparingly soluble in water.

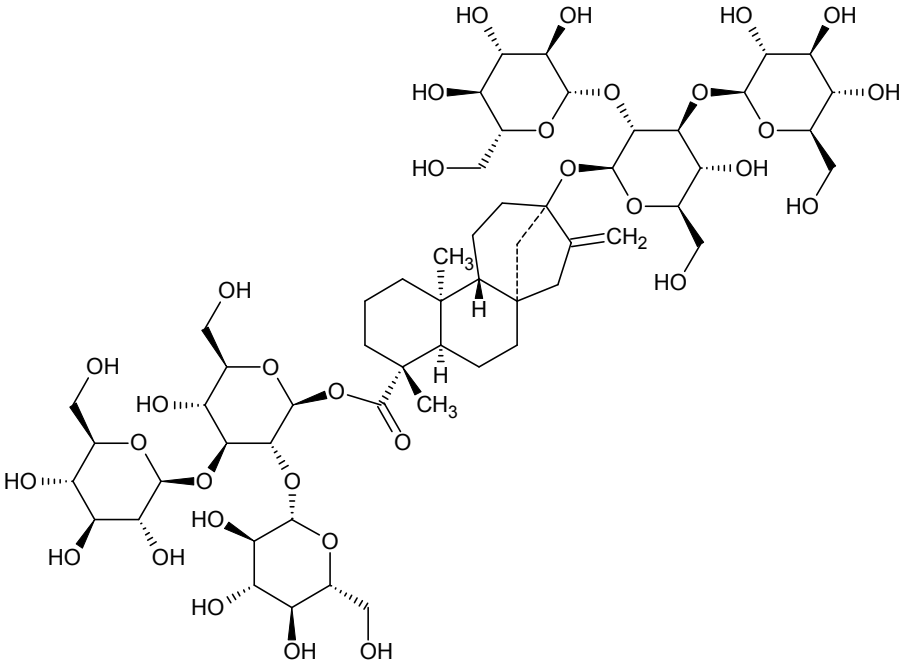
Chemical Name: 13-[(2-O-*β*-D-glucopyranosyl-3-O-*β*-D-glucopyranosyl-*β*-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-*β*-D-glucopyranosyl-3-O-*β*-D-glucopyranosyl-*β*-D-glucopyranosyl ester

Chemical Formula: $C_{56}H_{90}O_{33}$

Formula Weight: 1,291.3

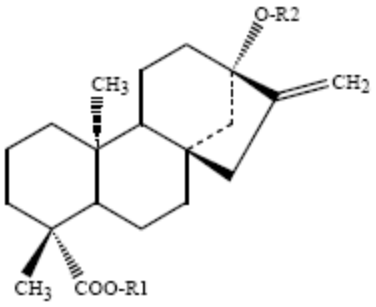
CAS Number: 1220616-44-3

Structural Formula:



All steviol glycoside constituents are glycosylated derivates of the aglycone steviol and as such, all share the same backbone structure (Figure 1) and differ only with respect to the type and number of glycoside units (glucose, rhamnose, or xylose) at positions R_1 and R_2. The chemical formulas (R_1 and R_2 groups) for each of the additional 9 steviol glycosides that may be present in a final rebaudioside X product, as well as the aglycone steviol, are summarized in Figure 1.

Figure 1. Chemical Structures for Various Steviol Glycosides



Compound Name	R_1	R_2
Stevioside	β-Glc	β-Glc-β-Glc(2→1)
Rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside C	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
Rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside F	β-Glc	β-Glc-β-Xly(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)
Steviol	H	H

Adapted from JECFA (2010)

B. Manufacturing Process

Rebaudioside X is manufactured in a facility certified under Food Safety System Certification (FSSC) 22000:2010. All raw materials and processing aids used in the manufacture of rebaudioside X are suitable food-grade materials and are used in accordance with applicable U.S. federal regulations as described in Table 1.

Table 1. Raw Materials and Processing Aids Used in the Manufacturing of Rebaudioside X

Material	Use	Regulatory Status
Stevia rebaudiana leaves	Reaction substrate	-
Water	Solvent	-
Calcium hydroxide	Processing aid	21 CFR §184.1205 (U.S. FDA, 2012a)
Ethanol	Processing aid	21 CFR §184.1293 & 21 CFR §172.340 (U.S. FDA, 2012a)
Methanol	Processing aid	21 CFR §172.859, 21 CFR §172.869, 21 CFR §173.250 & 21 CFR §172.867 (U.S. FDA, 2012a)
Activated carbon	Processing aid	-
Adsorption and ion-exchange resins (strong acidic and strong basic resins)	Processing aid	21 CFR §173.25 (U.S. FDA, 2012a)

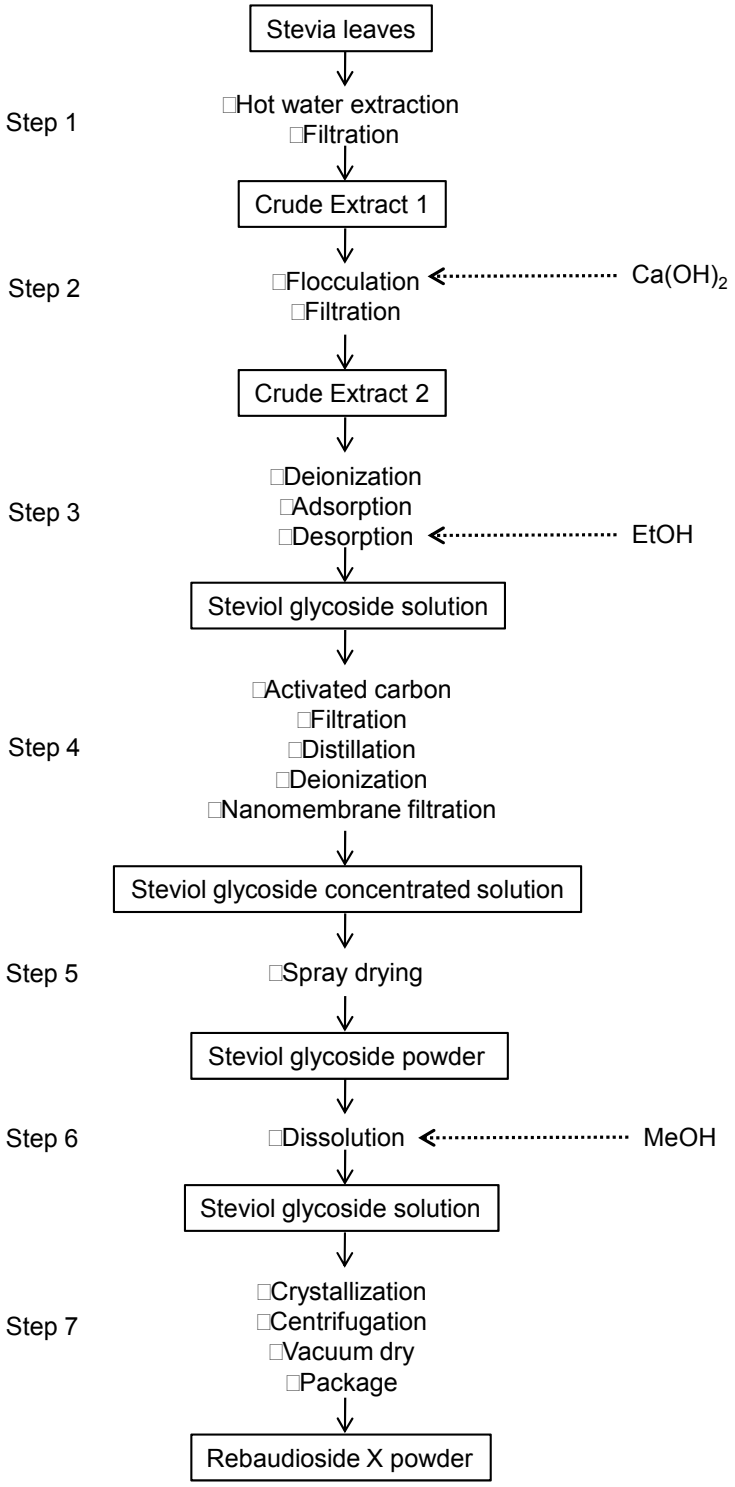
CFR, Code of Federal Regulations; U.S FDA, United States Food and Drug Administration

The production process of rebaudioside X is consistent with the methodologies for the manufacture of steviol glycosides as described in the Chemical and Technical Assessment (CTA) published by the Joint FAO WHO Expert Committee on Food Additives (JECFA) (2007a). *S. rebaudiana* leaves are placed in hot water in continuous countercurrent extractors. The filtrate is separated using mesh screens and collected in a holding tank. The filtrate is treated with calcium hydroxide to remove the mechanical particles, proteins, polysaccharides and coloring agents. A plate-and-frame filter press is used to separate the resulting precipitate from the filtrate. The filtrate is deionized by ion-exchange resins in (H^+) and (OH^-) form. The deionized filtrate is fed through columns packed with a macroporous adsorption resin that retains the glycosides. The resin is washed with deionized water, and the glycosides are desorbed from the resin using aqueous ethanol. The obtained glycoside solution is treated with activated carbon. The carbon is separated from the solution by using a plate-and-frame filter press. A standard evaporator is used to remove the ethanol from solution, and the resulting aqueous solution is deionized again by ion-exchange resins in (H^+) and (OH^-) forms. The refined solution is concentrated using a nanofiltration membrane. The concentrated solution is dried by a spray dryer. The resulting powder is dissolved in aqueous methanol. Rebaudioside X crystallization is carried out at low temperature for several hours. The crystallization process may be repeated several times so as to achieve the desired purity of rebaudioside X in the final product. As would be expected with fewer crystallization steps, the concentration of rebaudioside X in the final product would be lower with the final product containing a greater percentage of the 9 other steviol glycosides relative to rebaudioside X. The rebaudioside X crystals are separated by conventional centrifugation and dried in a rotary drum vacuum dryer. The obtained powder is sifted through stainless steel screens and passed through metal

detectors to be packed in aluminum foil bags. The bags are placed in high-density polyethylene (HDPE) drums sealed with tamper evident seals.

A schematic overview of the production process of rebaudioside X is illustrated below in Figure 2.

Figure 2. Schematic Overview of the Manufacturing Process for Rebaudioside X



C. Product Specifications Supporting Methods

1. Specifications for Steviol Glycosides Established by JECFA FCC

JECFA updated their specifications for steviol glycosides at the 73rd meeting and subsequently published the specifications in FAO JECFA Monographs 10 (JECFA, 2010). Additionally, the Food Chemicals Codex (FCC) has established specifications for rebaudioside A (FCC, 2012), based on those published by JECFA, as well as proposing specifications for steviol glycosides (USP, 2011). JECFA and FCC specifications describe steviol glycosides as a white to light powder, that has either a slight characteristic odor or is odorless, and having a sweetening potency of 200 to 300 times that of sucrose. The powder is freely to sparingly soluble in water and ethanol and has a pH range of 4.5 to 7 in a 1 solution. The identity of steviol glycosides is confirmed by the presence of stevioside and or rebaudioside A as the main peak in the chromatogram. The purity assay in both the JECFA and FCC specifications is defined as not less than 95 of the nine specified steviol glycosides (stevioside, rebaudiosides A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside). The final product loses not more than 6 moisture upon drying at 105C for 2 hours and contains not more than 1 total ash. Residual methanol and ethanol levels of not more than 200 and 5,000 mg kg, respectively, and arsenic and lead levels of not more than 1 mg kg, each, have been established.

2. Specifications for Rebaudioside X

The specifications established for rebaudioside X by PureCircle are based on those determined by JECFA and the FCC, as described above (JECFA, 2010; USP, 2011; FCC, 2012). Although rebaudioside X is not presently included as one of the nine steviol glycosides listed in the JECFA and FCC specifications, rebaudioside X has a similar structure to all other steviol glycosides, as presented in Section III.A, above, and can be detected in commercially available stevia extracts at concentrations between 0.1 and 0.4 . Since rebaudioside X is a steviol glycoside, the purity specification for a high purity rebaudioside X (not less than 95 total steviol glycosides) is consistent with the definition for purity established by JECFA. A summary of the specifications for rebaudioside X established by PureCircle including batch analyses of 5 non-consecutive lots is provided in Table 2. The methods of analysis used are based on internationally recognized standards.

Multi-residue pesticide screens that covered a range of commonly applied pesticides also were conducted on 5 non-consecutive lots of rebaudioside X (Lot Nos. PT090212, PT100412, PT110212, PT200212, and PT230212). No pesticide residues were detected in the finished product.

Table 2. Chemical Microbial Specifications for PureCircle s Rebaudioside X Batch Analyses for 5 Non-Consecutive Lots

Parameter	PureCircle Specification Rebaudioside X	Lot No. PT090212	Lot No. PT100412	Lot No. PT110212	Lot No. PT200212	Lot No. PT230212
Appearance	White to off-white powder	Conforms	Conforms	Conforms	Conforms	Conforms
Taste and Odor	Clean sweet taste with no abnormal odor	Conforms	Conforms	Conforms	Conforms	Conforms
Solubility	Sparingly soluble	Conforms	Conforms	Conforms	Conforms	Conforms
pH (1% solution)	4.5 to 7.0	6.9	4.97	5.87	5.00	5.14
Particle Size (%), through US mesh #20	>95	Conforms	Conforms	Conforms	Conforms	Conforms
Absorbance (370 nm)	≤ 0.1	0.012	0.012	0.013	0.012	0.016
Chemical Specifications						
Assay (%), anhydrous basis	≥95	99.75	99.61	99.41	99.52	99.35
Rebaudioside X(%), anhydrous basis	>50	96.60	96.45	96.23	96.32	96.14
Loss on Drying (%)	≤6	3.70	3.46	3.06	3.02	3.50
Total Ash (%)	<1	0.13	0.60	0.13	0.08	0.08
Residual Methanol (%)	<0.02	ND	0.007	0.007	0.006	0.007
Residual Ethanol (%)	<0.30	0.037	0.084	0.085	0.083	0.083
Arsenic (ppm)	<1	<0.005	0.014	0.007	0.009	0.010
Lead (ppm)	<1	0.212	0.300	0.250	0.213	0.238
Cadmium (ppm)	<1	<0.005	<0.005	<0.005	<0.005	<0.005
Mercury (ppm)	<1	<0.005	<0.005	<0.005	<0.005	<0.005
Microbial Specifications						
Total Plate Count (CFU/g)	<1,000	ND	20	ND	10	120
Yeast (CFU/g)	ND	ND	ND	ND	ND	ND
Mold (CFU/g)	ND	ND	ND	ND	ND	ND
Total Coliforms (MPN/g)	ND	ND	ND	ND	ND	ND
Escherichia coli count (MPN/g)	ND	ND	ND	ND	ND	ND
Thermophilic Acidophilus bacteria (CFU/g)	ND	ND	ND	ND	ND	ND
Guaiacol producing bacteria (in 1 g)	Negative	Negative	Negative	Negative	Negative	Negative
Staphylococcus aureus (CFU/g)	ND	ND	ND	ND	ND	ND

Table 2. Chemical Microbial Specifications for PureCircle s Rebaudioside X Batch Analyses for 5 Non-Consecutive Lots

Parameter	PureCircle Specification Rebaudioside X	Lot No. PT090212	Lot No. PT100412	Lot No. PT110212	Lot No. PT200212	Lot No. PT230212
Salmonella sp. (in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent
Listeria (in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent

ND, not detected; ppm, parts per million

As described in Section III.A, the concentration of rebaudioside X in the final steviol glycoside product is dependent on the number of crystallization steps conducted. With fewer crystallization steps, the level of rebaudioside X in the final product is lower but this is compensated through the presence of the other 9 steviol glycosides from the stevia leaf. Therefore, the final product still meets the JECFA purity definition. As a result, PureCircle analyzed 4 additional batches of a rebaudioside X-containing product to demonstrate variability in the steviol glycoside composition following different numbers of crystallization steps (see Table 3). Regardless of the number of crystallization steps employed during the manufacturing process, rebaudioside X is the main steviol glycoside present within the final rebaudioside X ingredient. Moreover, the finished ingredient consistently meets the purity specification for steviol glycosides (not less than 95 steviol glycosides) as presented above.

Table 3. Steviol Glycoside Distribution for 4 Non-Consecutive Lots of Rebaudioside X

Compound Name[1]	Lot No. PT040213	Lot No. PT110213	Lot No. PT010313	Lot No. PT070213
Rebaudioside D (%)	19.74	23.12	15.00	14.09
Rebaudioside X (%)	71.17	68.13	83.17	86.35
Rebaudioside A (%)	7.57	7.37	0.49	1.00
Rebaudioside B (%)	0.73	0.72	0.75	0.91
Total Steviol Glycoside (%)	99.20	99.34	99.41	99.36

[1] Stevioside, rebaudioside F, dulcoside A, rubusoside, and steviolbioside also were analyzed, but were not detected in these manufacturing lots.

D. Stability of Steviol Glycosides Rebaudioside X

The stability of steviol glycosides has been previously reviewed by a number of scientific advisory bodies involved in the evaluation of steviol glycoside safety [JECFA, the European Food Safety Authority (EFSA), and the Food Standards Australia New Zealand (FSANZ)] and also is discussed in several published studies (Chang and Cook, 1983; Kroyer, 1999). JECFA evaluated the stability of steviol glycosides under conditions mimicking their use in foods at the 68th meeting (JECFA, 2007a). The Committee noted that steviol glycosides do not undergo browning or caramelization when heated, and are reasonably stable under elevated temperatures used in food processing. Under acidic conditions (pH 2 to 4), steviol glycosides (approximately 90 to 94 purity), are stable for at least 180 days when stored at temperatures

up to 24□C. When exposed to elevated temperatures (80□C, in water, 8 hours), however, 4 and 8□ decomposition was observed in solutions of steviol glycosides at pH 4.0 and 3.0, respectively, indicating that the stability of steviol glycosides is pH and temperature dependent. When the temperature was increased to 100□C, expectedly higher rates of steviol glycoside decomposition (10 and 40□ at pH 4.0 and 3.0, respectively) were observed. Based on the above findings, as well as additional publicly available stability studies, JECFA concluded that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions.

Although the stability of rebaudioside X was not specifically addressed during JECFA□s evaluation, it is expected that the stability of rebaudioside X would be similar to other steviol glycosides given the similarity in structure. While expected, additional stability studies of rebaudioside X in a solution under normal and accelerated conditions, as well as at various pH levels and temperatures were conducted for confirmation. These studies, briefly summarized below, demonstrate that the stability of rebaudioside X is similar to other steviol glycosides, as previously concluded by JECFA.

1. Storage Stability

Stability was conducted on a batch of rebaudioside X (Lot PT200212a) at a concentration of 500 mg L. Samples were stored in sealed amber glass vials for up to 26 weeks either at 1) 25□C and ambient relative humidity (RH) (50 to 55□) or 2) 40□C and 75□ RH. Analyses for steviol glycosides were conducted in accordance with JECFA□s assay method (JECFA, 2010) and were measured upon study initiation, and after 12, 24, and 26 weeks of storage. Minimal degradation (□4□) of rebaudioside X was observed when stored as a solution under either condition. Additionally, minimal changes over the study period were observed in the other steviol glycosides detected.

2. pH Stability

The general stability of rebaudioside X (Lot No. PT090212a) was assessed in 0.1 M phosphate buffer at temperatures of 5, 25, or 40□C and at pH values from 2 to 8. Rebaudioside X was added to the solutions at target levels of 500 mg L and the stability was analyzed over 24 weeks under the respective conditions. The extent and rate of rebaudioside X degradation were shown to be dependent on pH, temperature, and time. Consistently, rebaudioside X at any pH level was most stable when stored at 5□C. Over the course of the study, minor degradation (□3□) was observed up to 6 weeks at pH 2, 3, 5, and 6 when stored at 5□C. After 6 weeks of storage, the rebaudioside X content increased slightly. Similar effects were observed at pH values of 3 and 6 when the solution was stored at 25□C and at pH levels of 4 to 6 when stored at 40□C.

Rebaudioside X started to degrade at pH 2 after 2 weeks of storage at both 25□C and 40□C resulting in more than 50□ of rebaudioside X being degraded after 6 weeks of storage at 40□C, in comparison to approximately 17□ and 3□ of rebaudioside X degraded when stored at 25□C and 5□C, respectively, at the same time point. After approximately 14 weeks of storage at 40□C, the degradation of rebaudioside X at pH 2 began to plateau, reaching a maximum of approximately 93□ of rebaudioside X degraded. Similarly, solutions of rebaudioside X at pH 3 stored at 40□C degraded over the 24 weeks of storage, with approximately 66□ of the

rebaudioside X sample remaining at study completion. Overall, at higher pH values (3 to 8), no significant degradation of rebaudioside X was observed over 24 weeks, regardless of the storage temperature of the solution, as previously stated. Similar to other steviol glycosides, the stability of rebaudioside X follows the same degradation pathway and is pH-, temperature-, and time-dependent. Therefore, the conclusions regarding the stability of steviol glycosides made by JECFA and other scientific bodies can be extended to include rebaudioside X.

3. Forced Degradation

Rebaudioside X, dissolved in a 0.1 M phosphate buffer, was subjected to forced degradation conditions at pH 2 heated to 80□C for 24 hours so as to generate degradation products, which could subsequently be identified. Beyond the identification of rebaudioside X in the sample, 8 degradation products were identified. Three (3) degradation products (molecular weights of 1309.3, 1291.3, and 804.9, respectively) were identified at study initiation in very low quantities (□0.5□) and increased over the duration of the study to approximately 7, 22, and 3□ , respectively. Low levels of rebaudioside B also were detected at study initiation (approximately 1□) and increased to approximately 9□ at study completion. DAQ2 (CC-00209), DAQ4 (CC-00210), steviol, and isosteviol, although not identified at study initiation, were identified at levels of up to approximately 10, 23, 1, and 7□ , respectively, at the end of the study.

IV. SELF-LIMITING LEVELS OF USE

The use of rebaudioside X in food is largely limited by the desired sweetness intended for a particular food or beverage product; therefore, the use of rebaudioside X as a general purpose sweetener in foods is self-limiting based on its organoleptic properties.

V. BASIS FOR GRAS DETERMINATION

The information summarized below demonstrates that rebaudioside X as a general purpose sweetener is GRAS, based on scientific procedures. Over the last few decades, the safety of steviol glycosides has been considered by several scientific bodies and regulatory agencies, including FDA, JECFA, the European Commission s Scientific Committee on Food (SCF), EFSA, FSANZ, and Health Canada. Interest in the use of steviol glycosides as sweeteners has encouraged extensive testing of the compounds and as such a large safety database exists. This database includes a thorough examination of the comparative metabolism and pharmacokinetics of steviol glycosides in experimental animals and humans, acute toxicity studies, short- and long-term toxicity and carcinogenicity studies, reproductive and developmental toxicology studies, *in vitro* and *in vivo* mutagenicity genotoxicity studies, and human studies. Although many earlier studies examining the safety of steviol glycosides were conducted with stevioside due to its predominance in *S. rebaudiana* leaves, the database pertaining to the safety of steviol glycosides was expanded following the completion of additional short-term toxicity, reproductive toxicity, *in vitro* and *in vivo* mutagenicity genotoxicity studies, and human studies conducted on rebaudioside A. Although the majority of toxicity studies have been conducted with either purified stevioside or rebaudioside A, the extensive database on the common metabolic fate of steviol glycosides has permitted the scientific bodies and regulatory agencies to extend their safety opinion to all steviol glycosides, rather than to just individual steviol glycosides.

The information reviewed by the Expert Panel, qualified by scientific training and experience to evaluate the safety of rebaudioside X as a general purpose sweetener, included a detailed summary of the conclusions made by global scientific and regulatory authorities regarding the safety of steviol glycosides and the data deemed pivotal in determining safety. Additionally, a detailed summary of the metabolic fate of steviol glycosides was reviewed. In order to corroborate previous conclusions regarding the shared metabolic pathway for steviol glycosides, a study examining the microbial metabolism of rebaudioside X in the presence of human fecal homogenates under anaerobic conditions also was reviewed (see Appendix B for full study report). Based on these data, the Expert Panel concluded that rebaudioside X was GRAS under the aforementioned intended conditions of use in food based on scientific procedures. A summary of the data reviewed by the Expert Panel is presented herein.

A. Intended Dietary Uses

Rebaudioside X, with a relative sweetening potency of 200 to 350 times that of sucrose, is intended for use as a general purpose sweetening agent, in accordance with cGMP. Although the solubility of rebaudioside X is described as sparingly soluble (see Table 2), the maximum inclusion levels within the relevant food categories are below these levels where rebaudioside X is no longer soluble.

Most other high-intensity sweeteners, including aspartame, have been approved by FDA as general purpose sweeteners, without their uses being restricted to specific foods or use-levels. Hence, the foods to which aspartame is added and the use-levels are controlled by technological properties (*e.g.*, sweetness potency). Considering that steviol glycosides are characterized by a sweetness profile that is, for the most part, comparable to that of aspartame

(*i.e.*, aspartame is approximately 200 times as sweet as sucrose), the uses and use levels of rebaudioside X are likely to primarily reflect those currently permitted for aspartame in the U.S.

B. Estimated Daily Intake

Numerous surveys have been completed in various global jurisdictions (U.S., Canada, Brazil, Australia New Zealand, and countries in the European Union) to assess daily consumption estimates of other well-established high-intensity sweeteners in the marketplace (*e.g.*, aspartame, cyclamate, saccharin, and sucralose). Renwick (2008) used the available post-market surveillance data for other high-intensity sweeteners as the basis for the assessment of dietary exposure for rebaudioside A by assuming full replacement of the currently approved intense sweeteners with the new sweetener. This intake assessment methodology yields intake estimates that while conservative, as it is unlikely that the new sweetener would entirely replace all other sweeteners in the marketplace, are realistic in that they reflect actual post-market intakes of high-intensity sweeteners. In order to estimate rebaudioside A intakes, Renwick (2008) first expressed the post-market surveillance intake estimates for intense sweeteners presently used in the global marketplace as sucrose equivalents in various population groups (for average and high-end non-diabetic and diabetic adult and child consumers) (see Table 4). The data used in these analyses were primarily derived from studies that used specifically designed food diaries combined with actual use-levels or approved levels in different foods and beverages (Renwick, 2008). In order to predict dietary exposure to rebaudioside A, the intake estimates for the high-intensity sweeteners (expressed as sucrose equivalents) were adjusted for the sweetness intensity of rebaudioside A relative to sucrose (approximately 200).

In the case of rebaudioside X, the same methodology as applied by Renwick (2008) was used to estimate intake values. Since rebaudioside X is 200 to 350 times as sweet as sucrose, the intake values for intense sweeteners presented in Table 4 were adjusted accordingly to derive intake estimates for the extract. The predicted intakes of rebaudioside X, expressed as steviol equivalents, are all below the current acceptable daily intake (ADI) defined by the JECFA for steviol glycosides (JECFA, 2007b) of up to 4 mg kg body weight as steviol.

Table 4. Estimated Consumption of Rebaudioside X Based on Post-Market Surveillance Intake Data for Currently Used High-Intensity Sweeteners

Target Population	Intakes of High-Intensity Sweeteners (expressed as sucrose equivalents) (mg kg bw day)		Consumption Estimates for Rebaudioside X[1] (mg kg bw day)		Consumption Estimates for Rebaudioside X as steviol equivalents[2] (mg kg bw day)	
	Average Consumer	High Consumer	Average Consumer	High Consumer	Average Consumer	High Consumer
Non-diabetic Adults	255	675	0.73-1.28	1.93-3.38	0.18-0.31	0.48-0.83
Diabetic Adults	280	897	0.80-1.40	2.56-4.49	0.20-0.35	0.63-1.11
Non-diabetic Children	425	990	1.21-2.13	2.83-4.95	0.30-0.52	0.70-1.22
Diabetic Children	672	908	1.92-3.36	2.59-4.54	0.47-0.83	0.64-1.12

[1] Rebaudioside X is approximately 200 to 350 times as sweet as sucrose.
[2] Calculated based on the molecular weight of rebaudioside X (*i.e.*, 1291.3 g/mol).

As part of their evaluation of the safety of steviol glycosides in 2008, JECFA considered various intake models for the estimation of dietary exposure to steviol glycosides, including the intake analysis conducted by Renwick (2008). Although higher intake estimates than those presented by Renwick (2008) were identified using other methodologies, including ones considering replacement of all sweeteners used in food (up to approximately 6 mg kg body weight day, expressed as steviol equivalents), it was noted by JECFA that such replacement estimates were highly conservative and that actual exposures to steviol glycosides (expressed as steviol equivalents) would be 20 to 30 of these values (1 to 2 mg kg body weight day, expressed as steviol equivalents). Furthermore, JECFA noted that the intake estimates based on post-market surveillance further confirmed the lower range.

C. Safety Data for Steviol Glycosides Rebaudioside X

1. Metabolic Fate of Steviol Glycosides

Steviol glycosides pass undigested through the upper portion of the gastrointestinal tract and enter the colon intact where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol (Hutapea *et al*., 1997; Gardana *et al*., 2003; Geuns, 2003; Koyama *et al*., 2003a,b; Geuns *et al*., 2007; Renwick and Tarka, 2008). All steviol glycosides are hydrolyzed to steviol prior to absorption (Wingard *et al*., 1980; Nakayama *et al*., 1986; Gardana *et al*., 2003; Koyama *et al*., 2003b).

Studies comparing the metabolic fate of rebaudioside A and stevioside demonstrate that both glycosides have similar pharmacokinetics in the rat; they are both metabolized in the gut to steviol prior to absorption followed by glucuronidation in the liver and excretion in the feces *via* the bile (Nakayama *et al*., 1986; Sung, 2002; Koyama *et al*., 2003a; Roberts and Renwick, 2008). In both rats and humans, steviol was shown to be metabolized to steviol glucuronide following absorption (Nakayama *et al*., 1986; Koyama *et al*., 2003a; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Roberts and Renwick, 2008; Wheeler *et al*., 2008). However, in humans, elimination of steviol glycosides, primarily as steviol glucuronide with very small amounts of the unchanged glycoside or steviol, occurs *via* the urine (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). Overall, the data demonstrate that rebaudioside A and stevioside have similar metabolism and pharmacokinetics in the rat. With the exception of having different numbers and types of sugar moieties, steviol glycosides share the same structural backbone, steviol. As such, all steviol glycosides are expected to follow the same metabolic pathway as demonstrated for rebaudioside A and stevioside. Therefore, the results of toxicology studies on either stevioside or rebaudioside A are applicable to the safety of all steviol glycosides.

The inter-species difference in the route of elimination of systemically absorbed steviol as steviol glucuronide (*via* the bile in rats and in the urine in humans) occurs as a result of the lower molecular weight threshold for biliary excretion in rats (325 Da) as compared to humans (500 to 600 Da; molecular weight of steviol glucuronide is 495 Da) (Renwick, 2007). Notably, in bile-duct ligated rats, excretion of steviol glucuronide occurred primarily in the urine (Wingard *et*

al., 1980). While the primary routes of elimination of steviol glucuronide differ between rats and humans, the metabolism and pharmacokinetics of steviol glycosides are quite similar, which confirms the rat as an acceptable model for risk assessment in humans. The difference in the route of elimination is considered to be of no toxicological significance due to the fact that the water soluble phase II metabolites are rapidly cleared in both species. Therefore, toxicology data generated in rats are applicable to assess the safety of steviol glycosides in humans given the similarities in metabolic fate. A general schematic of the metabolism of steviol glycosides is provided in Figure 3, below.

Figure 3. Schematic Diagram of Metabolic Fate of Steviol Glycosides

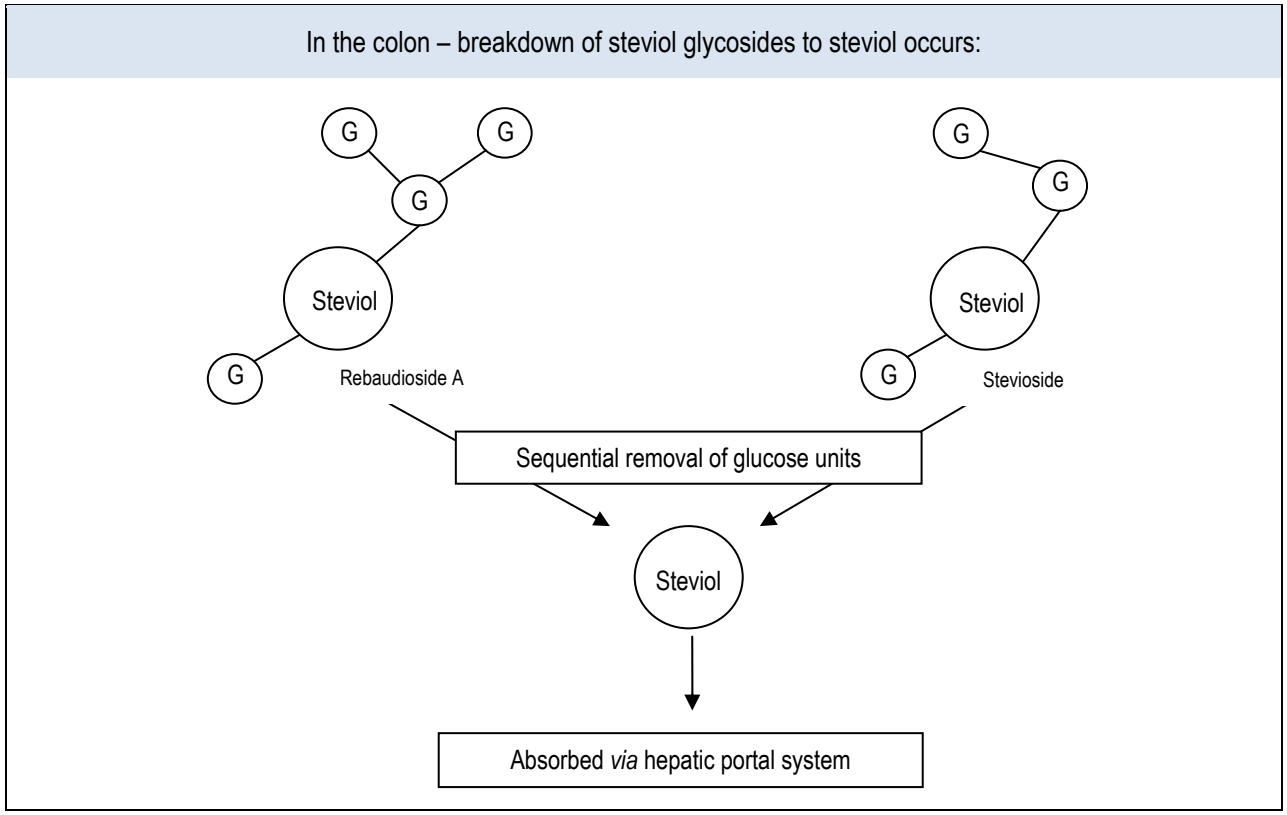
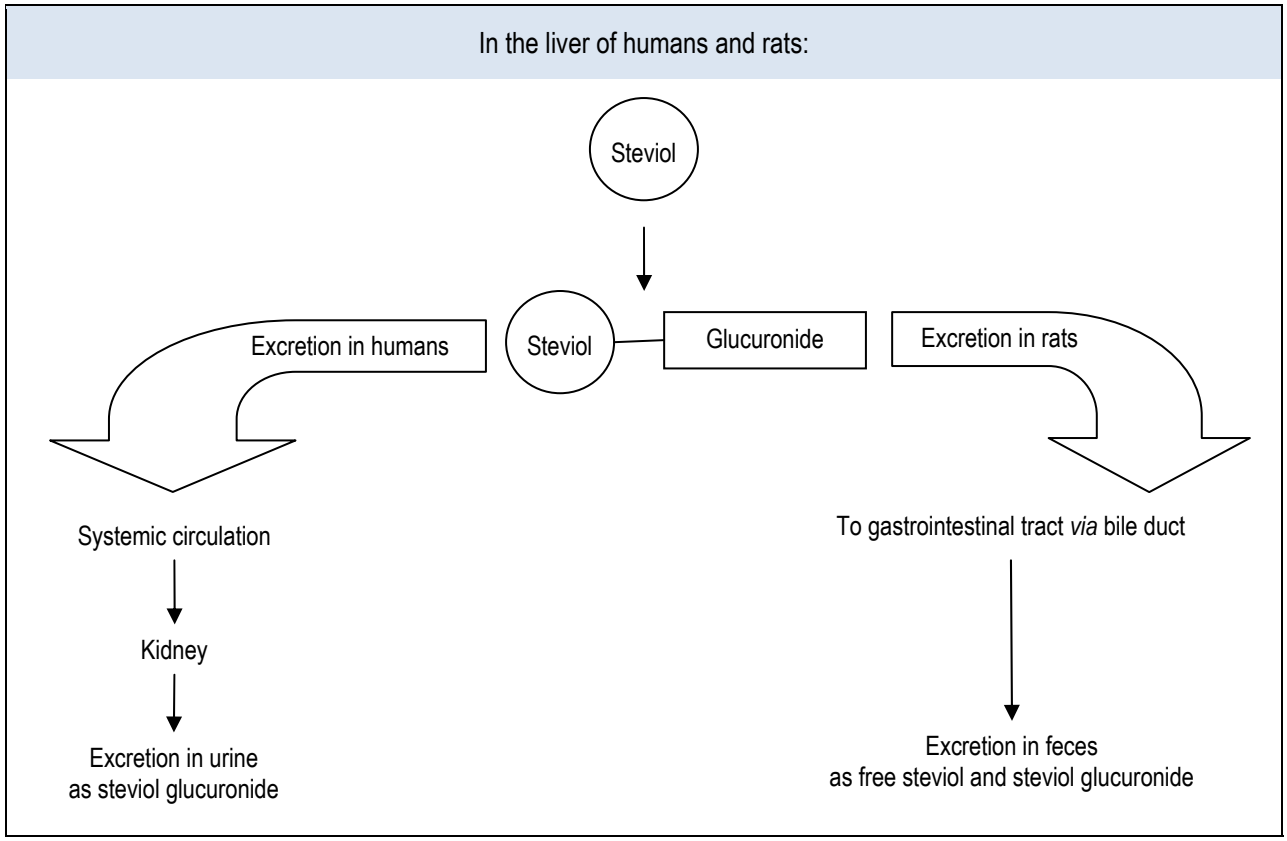


Figure 3. Schematic Diagram of Metabolic Fate of Steviol Glycosides



Adapted from Carakostas *et al.* (2008)

2. Metabolism of Rebaudioside X

In order to confirm the similarities in the microbial metabolism among all steviol glycosides as previously observed by Wingard *et al*. (1980), Hutapea *et al*. (1997), Gardana *et al*. (2003), Koyama *et al*. (2003b), 0.2 mg mL of rebaudioside X (Lot no. PT140312), rebaudioside A, and steviolbioside (a metabolic intermediate) were incubated individually with pooled fecal homogenates from 6 healthy male and 6 healthy female volunteers for up to 24 hours at 37°C under anaerobic conditions (BRI, 2013;see Appendix B). Rebaudioside X was rapidly hydrolyzed to steviol, with up to 83 and 91% of rebaudioside X degraded after 8 hours of incubation with male and female fecal homogenates, respectively. Within 16 hours of incubation, all of the rebaudioside X was degraded to steviol. Similarly, up to 77 and 100% of rebaudioside A was hydrolyzed to steviol by male and female fecal homogenates, respectively, within 8 hours of incubation, reaching complete degradation after 16 hours. Approximately 80% of the metabolic intermediate steviolbioside was hydrolyzed to steviol within 24 hours. This lower than expected result was determined to be due to the higher dilution factor used in the analysis rather than the efficiency of the microbial degradation of steviolbioside. When these results are taken into consideration with the results from other fecal homogenate studies as discussed above, the study conducted by Biotechnology Research Institute (BRI) (2013) confirms that rebaudioside X, like all other steviol glycosides, is hydrolyzed to steviol within 24 hours. Additionally, the study conducted by BRI (2013) corroborates the differences in

degradation rates of steviol glycosides. The slight increase in the rate of hydrolysis for rebaudioside A in comparison to rebaudioside X was determined to be the result of the structural differences between the 2 steviol glycosides, with rebaudioside A having 4 glucose moieties in comparison to the 6 glucose moieties within rebaudioside X.

Therefore, based on the overall similarities in the microbial metabolism of steviol glycosides, including rebaudioside X, following incubation with intestinal microflora, the comparable rates of hydrolysis, and the negligible changes in microflora produced after prolonged incubation with steviol glycosides previously reported in the literature, the toxicological effects of stevioside and rebaudioside A can be extrapolated to steviol glycosides, in general and rebaudioside X specifically, as all are converted to steviol (Renwick and Tarka, 2008; BRI, 2013).

3. Summary of Safety Opinions by Scientific Regulatory Authorities

JECFA reviewed the safety of steviol glycosides at four separate meetings (51st, 63rd, 68th, and 69th) in 1998, 2004, 2007, 2008. Although an ADI could not be established for steviol glycosides following the 51st meeting due to the limited data on stevioside and the need for specifications for commercial materials (JECFA, 1999), a temporary ADI of 2 mg kg body weight (expressed as steviol) was established at the Committee s 63rd meeting based on a no-observed-adverse-effect level (NOAEL) of 970 mg kg body weight day (383 mg kg body weight day as steviol) from a 2-year study in rats (Toyoda *et al.*, 1997) and a safety factor of 200 (JECFA, 2006). JECFA determined that the safety factor of 200 was appropriate due to the potential reproductive effects in animals and pharmacological effects in humans, and the lack of genotoxic findings for stevioside and rebaudioside A. The temporary ADI for steviol glycosides was further extended following the 68th meeting (JECFA, 2007b). At the 69th meeting of JECFA (2009), additional data pertaining to the metabolic fate of steviol glycosides in rats and humans (Roberts and Renwick, 2008; Wheeler *et al*., 2008), subchronic and reproductive developmental toxicity of rebaudioside A (Curry and Roberts, 2008; Curry *et al*., 2008; Nikiforov and Eapen, 2008), and the potential pharmacological effects of steviol glycosides in diabetic populations and individuals with normal or low-normal blood pressure (Maki *et al*., 2008a,b) were reviewed. The Committee concluded that the results of the animal studies and human studies evaluating the effects of steviol glycosides on blood pressure and blood glucose were sufficient to remove the additional safety factor of 2 and establish a full ADI of 4 mg kg body weight (expressed as steviol) for steviol glycosides (JECFA, 2009).

Following JECFA s most recent review of the specifications for steviol glycosides at their 73rd meeting in 2010, the specifications for steviol glycosides were revised to include 2 additional steviol glycosides, rebaudioside D and rebaudioside F, within the purity criteria[2] (JECFA, 2010). Although no specific studies have been conducted with these steviol glycosides individually, their inclusion within JECFA s purity specification further confirms that the safety of steviol glycosides is based on the recognition that all steviol glycosides are degraded to the aglycone steviol and that the safety demonstrated for one glycoside is relevant to all glycosides in general.

An overview of JECFA s main concerns raised at each meeting at which steviol glycosides were evaluated and the resolution, along with the respective ADI is presented in Table 5.

[2] Not less than 95 of the following 9 steviol glycosides, on a dried weight basis: stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside.

Table 5. Summary of the Joint FAO WHO Expert Committee on Food Additives (JECFA) Evaluations of Steviol Glycosides

Year (meeting)	Questions Concerns	ADI
1998 (51st)	• Need for specifications for the material of commerce. • Limited data; additional data required on: - human metabolism; - *in vivo* genotoxicity of steviol; - long-term toxicity and carcinogenicity in other species.	Could not be derived
2004 (63rd)	• Data required on pharmacological effects in patients with Type 2 diabetes and individuals with normal or below-normal blood pressure.	2 mg/kg bw (as steviol) (Temporary) • based on a NOAEL of 970 mg/kg bw/d (383 mg/kg body weight/day as steviol) and a safety factor of 200
2007 (68th)	• Data required on pharmacological effects in patients with Type 2 diabetes and individuals with normal or below-normal blood pressure.	2 mg/kg bw (as steviol) (Temporary) • based on a NOAEL of 970 mg/kg bw/d (383 mg/kg body weight/day as steviol) and a safety factor of 200
2008 (69th)	• No further questions.	4 mg/kg bw (as steviol) (full) • based on a NOAEL of 970 mg/kg bw/d (383 mg/kg body weight/day as steviol) and a safety factor of 100
2010 (73rd)	• Safety data not evaluated; specifications revised to include 2 additional steviol glycosides within the assay definition.	Not re-evaluated

ADI, acceptable daily intake; bw, body weight; d, day; NA, not applicable; NOAEL, no adverse effect level; NSE, no significant effects.

Following their own independent review of the safety of steviol glycosides, FSANZ, EFSA, and Health Canada corroborated JECFA's conclusion regarding safety and concurred with the ADI previously established by JECFA of 4 mg kg body weight for steviol glycosides, expressed as steviol equivalents (FSANZ, 2008; EFSA, 2010; Health Canada, 2012).

D. Summary and Basis for GRAS Conclusion

The GRAS determination for the use of rebaudioside X as a general purpose sweetener is based on scientific procedures. Rebaudioside X is produced using a multi-step process, beginning with the hot-water extraction of *S. rebaudiana* leaves, followed by initial purification and concentration steps to produce an intermediate steviol glycoside concentration, and finally by further refining, purification, and concentration steps to obtain rebaudioside X. This production process is consistent with the methodologies for the general manufacture of steviol glycosides as described by JECFA. Physical and chemical specifications, based on those established by JECFA for steviol glycosides, were established for rebaudioside X. Since the finished ingredient is obtained from a natural source (*S. rebaudiana* leaves), the potential presence of microbial contaminants was limited by establishing rigorous microbiological specification parameters. Batch samples of rebaudioside X are routinely tested to verify compliance with the established chemical and microbiological parameters. Additionally, since rebaudioside X is obtained from a plant source that may be subjected to various pesticides

during cultivation, the final ingredient also was subjected to a multi-residue pesticide screen. Results of the analysis showed the absence of any pesticide residues in rebaudioside X. Although JECFA concluded that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions, PureCircle undertook a series of studies to confirm the stability of rebaudioside X in a solution under normal and accelerated conditions and under various pH levels stored at various temperatures. Similar to the conclusions by JECFA for other steviol glycosides, rebaudioside X was shown to be stable at pH values above 3.0 and under normal processing and storage conditions.

PureCircle intends to market rebaudioside X as a sweetening agent for use as a general purpose sweetener as either a high-purity material or as a component of a steviol glycoside material containing more than 50 rebaudioside X. All rebaudioside X products will therefore consist of not less than 95 steviol glycosides with rebaudioside X representing more than 50 of the finished product and the remainder comprising the following 9 related steviol glycosides, in any combination and ratio: stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside A, steviolbioside, and rubusoside. Steviol glycosides (steviol conjugated with glucose, xylose, and or rhamnose) are natural constituents of the *S. rebaudiana* plant. Rebaudioside X can be detected in commercially available stevia extracts at concentrations between 0.1 and 0.4 . Based on its intended uses as a general purpose sweetening agent, rebaudioside X will be added to a variety of food products, excluding infant formulas and meat and poultry products, consistent with the current uses of other related high-intensity sweeteners that are already in the market (*e.g.*, aspartame).

Intakes of rebaudioside X were estimated based on post-market surveillance data for other high-intensity sweeteners and adjusting for relative sweetness intensity (200 to 350 times sweeter than sucrose); the mean intake of rebaudioside X was predicted to range from 0.73 mg kg body weight day for non-diabetic adults to 3.36 mg kg body weight day for diabetic children, equivalent to 0.18 and 0.83 mg steviol equivalents kg body weight day for non-diabetic adults and diabetic children, respectively. Predicted intakes for heavy consumers ranged from 1.93 mg kg body weight day for non-diabetic adults to 4.95 mg kg body weight day for non-diabetic children, equivalent to 0.48 and 1.22 mg steviol equivalents kg body weight day for non-diabetic adults and non-diabetic children, respectively. Accordingly, the highest intake estimate for rebaudioside X of 1.22 mg kg body weight day, as steviol equivalents, derived for non-diabetic children under the proposed conditions of use is well below the current ADI for steviol glycosides of 4 mg kg body weight, expressed as steviol, as set by JECFA.

Steviol glycosides are naturally occurring constituents of the stevia plant, *S. rebaudiana* (Bertoni). As a result of their characteristically sweet taste, extracts of the stevia plant have a long history of human consumption. The safety of steviol glycosides has been the subject of numerous reviews over the last couple of decades, and recently multiple jurisdictions including the U.S., EU, Australia and New Zealand, and Canada have concluded that preparations containing at least 95 steviol glycosides are safe when used in accordance with cGMP. Based on the similar metabolic pathway for all steviol glycosides in rats and humans, an ADI of 0 to 4 mg kg body weight, as steviol equivalents, was established by EFSA, FSANZ, Health Canada, and JECFA on the basis of a NOAEL of 970 mg kg body weight day (383 mg kg body weight day, as steviol) from a 2-year study in rats (Toyoda *et al.*, 1997) and a safety factor of 100, to account for intra- and inter-species differences. In order to confirm whether the safety conclusions for steviol glycosides would also extend to rebaudioside X, an *in vitro* study examining the microbial metabolism of rebaudioside X in the presence of human fecal

homogenates was conducted (BRI, 2013). The results of this study corroborate the findings from previously published studies demonstrating that all steviol glycosides sharing the same steviol backbone are degraded by fecal microbes to steviol in the gastrointestinal tract. Therefore, it was determined that JECFA's ADI for steviol glycosides also would extend to rebaudioside X.

The Expert Panel convened on behalf of PureCircle, independently and collectively, critically evaluated the data and information summarized above, and concluded that the intended uses of rebaudioside X meeting appropriate food-grade specifications and manufactured according to cGMP, are safe and suitable and are GRAS based on scientific procedures. It is also PureCircle's opinion that other qualified scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, PureCircle has concluded that rebaudioside X is GRAS under the intended conditions of use on the basis of scientific procedures; therefore, it is excluded from the definition of a food additive and may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

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Appendix A

Expert Panel Statement

Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of Rebaudioside X as a General Purpose Sweetener

March 8, 2013

PureCircle Ltd. (herein PureCircle) intends to market rebaudioside X as a sweetening agent for use as a general purpose sweetener. Rebaudioside X consists of not less than 95 steviol glycosides with rebaudioside X representing more than 50 of the finished product and the remainder comprising the following 9 related steviol glycosides, in any combination and ratio: stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside, steviolbioside, and rubusoside. Based on its intended uses as a general purpose sweetening agent, rebaudioside X will be added to a variety of food products, consistent with the current uses of other related high-intensity sweeteners (HIS) that are already in the market (*e.g.*, aspartame).

At the request of PureCircle, an Expert Panel (the Panel) of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether the intended uses of rebaudioside X as a sweetening agent are safe and suitable and would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Panel consisted of Dr. Samuel Cohen, Ph.D., M.D. (University of Nebraska Medical Center), Dr. I. Glenn Sipes, Ph.D. (University of Arizona), and Dr. John A. Thomas, Ph.D. (Indiana University School of Medicine).

The Panel, independently and collectively, critically evaluated a dossier that included a comprehensive summary of scientific information on steviol glycosides prepared from information available within the public domain and also included details pertaining to the method of manufacture and product specifications of rebaudioside X, supporting analytical data, intended use-levels of rebaudioside X in foods, consumption estimates for all intended uses, and an unpublished microbial metabolism study of rebaudioside X. In addition, the Panel evaluated other information deemed appropriate or necessary.

Following its independent, critical evaluation of such data and information, the Panel convened on March 8, 2013 *via* teleconference and unanimously concluded that the intended uses described herein for rebaudioside X, meeting appropriate food-grade specifications as described in the supporting dossier [**Documentation Supporting the Evaluation of Rebaudioside X as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener**] and manufactured according to current Good Manufacturing Practice (cGMP), are

safe and suitable and GRAS based on scientific procedures. A summary of the basis for the Panel s conclusion is provided below.

The ingredient that is the subject of this GRAS evaluation is a steviol glycoside extracted from the leaves of *Stevia rebaudiana*. Rebaudioside X can be detected in commercially available *S. rebaudiana* extracts at concentrations between 0.1 and 0.4 and is produced using a multi-step process. The production of rebaudioside X begins with the hot-water extraction of *S. rebaudiana* leaves, followed by initial purification and concentration steps to produce an intermediate steviol glycoside concentrate, and finally by further refining, purification, and concentration steps to obtain rebaudioside X. This production process is consistent with the methodologies for the general manufacture of steviol glycosides as described by the Joint FAO WHO Expert Committee on Food Additives (JECFA) (2007a). Physical and chemical specifications, based on those determined by JECFA for steviol glycosides (JECFA, 2010), were established for rebaudioside X. Since the finished ingredient is obtained from a natural source (*S. rebaudiana* leaves), the potential presence of microbial contaminants was limited by establishing rigorous microbiological specification parameters. Batch samples of rebaudioside X are routinely tested to verify compliance with the set chemical and microbiological parameters. Additionally, since rebaudioside X is obtained from a plant source that may be subjected to various pesticides during cultivation, the final ingredient also was subjected to a multi-residue pesticide screen. Results of the analyses showed the absence of any pesticide residues in rebaudioside X. Although JECFA concluded that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions (JECFA, 2007a), PureCircle undertook a series of studies to confirm the stability of rebaudioside X in a solution under normal and accelerated conditions and under various pH levels stored at various temperatures. Similar to the conclusions by JECFA for other steviol glycosides, rebaudioside X was shown to be stable at pH values above 3.0 and under normal processing and storage conditions.

The Panel understands that rebaudioside X, as mentioned previously, is proposed for use as a general purpose sweetener that will be added to a variety of food products, consistent with the current uses of other related HIS that are already in the market (*e.g.*, aspartame). Based on post-market surveillance data for other HIS and adjusting for relative sweetness intensity of rebaudioside X (200 to 350 times sweeter than sucrose), the estimated intakes of rebaudioside X were calculated for adults (diabetic and non-diabetic) and children (diabetic and non-diabetic). The mean intake of rebaudioside X was predicted to range across all groups from 0.73 mg kg body weight day for non-diabetic adults to 3.36 mg kg body weight day for diabetic children, equivalent to 0.18 and 0.83 mg steviol equivalents kg body weight day for non-diabetic adults and diabetic children, respectively. Predicted intakes for heavy consumers ranged across all groups from 1.93 mg kg body weight day for non-diabetic adults to 4.95 mg kg body weight day for non-diabetic children, equivalent to 0.48 and 1.22 mg steviol equivalents kg body weight day for non-diabetic adults and non-diabetic children, respectively. Accordingly,

the highest intake estimate for rebaudioside X of 1.22 mg kg body weight day, as steviol equivalents, derived for non-diabetic children under the proposed conditions of use is well below the current Acceptable Daily Intake (ADI) for steviol glycosides of 4 mg kg body weight, expressed as steviol, as set by JECFA (2009).

The Panel reviewed the available safety data on steviol glycosides during their GRAS assessment, extrapolating to the safety of rebaudioside X; this information included a detailed summary of the conclusions made by global scientific and regulatory authorities regarding the safety of steviol glycosides and the data deemed pivotal in determining safety. Additionally, a detailed summary of the metabolic fate of steviol glycosides in general was reviewed. In order to corroborate previous conclusions regarding the shared metabolic pathway for steviol glycosides, a study examining the microbial metabolism of rebaudioside X in the presence of human fecal homogenates under anaerobic conditions also was presented.

Steviol glycosides pass undigested through the upper portion of the gastrointestinal tract and enter the colon intact where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol (Hutapea *et al*., 1997; Gardana *et al*., 2003; Koyama *et al*., 2003a,b; Geuns *et al*., 2003, 2007; Renwick and Tarka, 2008). All steviol glycosides are hydrolyzed to steviol prior to absorption (Wingard *et al*., 1980; Nakayama *et al*., 1986; Gardana *et al*., 2003; Koyama *et al*., 2003b). In order to confirm the similarities in microbial metabolism among steviol glycosides, an *in vitro* study examining the degradation of rebaudioside X and rebaudioside A in the presence of human fecal homogenates was conducted (BRI, 2013). The results of this study corroborate the findings from previously published studies demonstrating that all steviol glycosides sharing the same steviol backbone are degraded by fecal microbes to steviol in the gastrointestinal tract.

Studies comparing the metabolic fate of rebaudioside A and stevioside demonstrate that both glycosides have similar pharmacokinetics in the rat; they are both metabolized in the gut to steviol prior to absorption followed by glucuronidation in the liver and excretion in the feces *via* the bile (Nakayama *et al*., 1986; Sung, 2002; Koyama *et al*., 2003a; Roberts and Renwick, 2008). In both rats and humans, steviol was shown to be metabolized to steviol glucuronide following absorption (Nakayama *et al*., 1986; Koyama *et al*., 2003a; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Roberts and Renwick, 2008; Wheeler *et al*., 2008). However, in humans, elimination of steviol glycosides, primarily as steviol glucuronide with very small amounts of the unchanged glycoside or steviol, occurs *via* the urine (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). Overall, the data demonstrate that rebaudioside A and stevioside have similar metabolism and pharmacokinetics in the rat. With the exception of having different numbers and types of sugar moieties, steviol glycosides share the same structural backbone, steviol. As such, all steviol glycosides, including rebaudioside X are expected to follow the same metabolic pathway as demonstrated for rebaudioside A and stevioside. Therefore, the results of

toxicology studies on either stevioside or rebaudioside A are applicable to the safety of all steviol glycosides.

The inter-species difference in the route of elimination of systemically absorbed steviol as steviol glucuronide (*via* the bile in rats and in the urine in humans) occurs as a result of the lower molecular weight threshold for biliary excretion in rats (325 Da) as compared to humans (500 to 600 Da; molecular weight of steviol glucuronide is 495 Da) (Renwick, 2007). Notably, in bile-duct ligated rats, excretion of steviol glucuronide occurred primarily in the urine (Wingard *et al*., 1980). While the primary routes of elimination of steviol glucuronide differ between rats and humans, the metabolism and pharmacokinetics of steviol glycosides are quite similar, which confirms the rat as an acceptable model for risk assessment in humans. The difference in the route of elimination is considered to be of no toxicological significance due to the fact that the water soluble phase II metabolites are rapidly cleared in both species. Therefore, toxicology data generated in rats are applicable to assess the safety of steviol glycosides in humans given the similarities in metabolic fate.

As a result of the characteristically sweet taste of steviol, extracts of the stevia plant have a long history of human consumption. The safety of steviol glycosides has been the subject of numerous reviews over the last couple of decades, and recently multiple jurisdictions including the United States (U.S.), European Union (EU), Australia and New Zealand, and Canada have concluded that preparations containing at least 95 steviol glycosides are safe when used in accordance with cGMP (FSANZ, 2008; U.S. FDA, 2008a,b, 2009a-d, 2010a-e; 2011a-i; 2012a-e; EU, 2011; Health Canada, 2012). Furthermore, JECFA has conducted several safety reviews of steviol glycosides over 4 separate meetings between 1998 and 2008 (JECFA, 1999, 2006, 2007b, 2009). Specifically, based on the similar metabolic pathway for all steviol glycosides in rats and humans, as presented above, JECFA established an ADI of 0 to 4 mg kg body weight, as steviol equivalents, based on a no-observed-adverse-effect level (NOAEL) of 970 mg kg body weight day (383 mg kg body weight day as steviol) from a 2-year study in rats (Toyoda *et al.*, 1997) and a safety factor of 100, to account for intra- and inter-species differences (JECFA, 2009). Based on the similar metabolic fate of several steviol glycosides, including rebaudioside X, it was determined that JECFA s ADI for steviol glycosides also would extend to rebaudioside X.

The scientific evidence examined by the Panel demonstrates that under the conditions of intended use, rebaudioside X would not produce any adverse health effects.

CONCLUSIONS

We, the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that the intended uses of rebaudioside X, meeting appropriate food-grade specifications presented in the supporting dossier [**Documentation Supporting the Evaluation of Rebaudioside X as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener**] and produced consistent with current Good Manufacturing Practices (cGMP), are safe and suitable and are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

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Appendix B

Final Study Report for BRI (2003)

CONFIDENTIAL

FINAL REPORT

Study Title: *In Vitro* Metabolism of Rebaudioside X in Pooled Human Intestinal Fecal Homogenate under Physiological Anaerobic Conditions

Testing Facility:	**BRI Biopharmaceutical Research Inc.** **101-8898 Heather Street,** **Vancouver, BC** **Canada V6P 3S8**
BRI Study Number:	**TAR-2012-001**
Sponsor:	**The Tarka Group, Inc.** **210 N. Old Stone House Road** **Carlisle, PA 17015-8517** **USA**
Sponsor Representative:	**Stanley M. Tarka, Jr., Ph.D.** **The Tarka Group, Inc.** **210 N. Old Stone House Road** **Carlisle, PA 17015-8517** **USA** **Phone: +1 (717) 243-9216** **Fax: +1 (702) 993-5458** **Email: tarkagroup@comcast.net**
Sponsor Reference Number:	**Not Available**
BRI Study Protocol No.:	**Protocol-TAR-2012-001**
BRI Report No.:	**RPT-TAR-2012-001 (v2.0)**
Final Report (v2.0) Issuance Date:	**January 16, 2013**

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4 List of Abbreviations

%CV	percent coefficient of variation
%Bias	percent deviation from expected values
°C	degree Celsius
µg/mL	microgram per milliliter
µL	microliter
µM	micromolar
Abs	absolute
ACN	acetonitrile
AD	amendment and deviation
BHI	brain-heart infusion broth
CoA	certificate of analysis
conc	Concentration
CRO	Contract research organization
diH_2O	deionized water
DMSO	dimethyl sulfoxide
GLP	good laboratory practices
GP	general practices
HFH	human fecal homogenate
HPLC	high performance liquid chromatography
IS / ITS	internal standard
LC/MS	liquid chromatography / mass spectrometry
LLOQ	lower limit of quantitation
MeOH	methanol
mg	milligram
mg/mL	milligram per milliliter
min	minute
mL	milliliter
mL/min	milliliter per minute
MSDS	material safety data sheet
n/ap	not applicable
n/av	not available
ng/mL	nanogram per milliliter
NH_4OH	ammonium hydroxide
QA	quality assurance
QC	quality control
R^2	coefficient of determination
RCF	relative centrifugal force
Rep	replicate
RFS	reference standard
RT	retention time
SD	standard deviation
SOP	standard operating procedure
std	standard
sys suit	system suitability
ULOQ	upper limit of quantitation
v	version
v/v	volume to volume
x *g*	times gravity

5 GLP Compliance Statement

The study was conducted at BRI Biopharmaceutical Research Inc (BRI), Unit 101, 8898 Heather Street, Vancouver, British Columbia, Canada V6P3S8

This laboratory study was performed under my supervision in accordance with the Study Protocol described herein. This study was conducted in accordance with the Good Laboratory Practice Regulations/Standards/Guidelines below:

- ☑ United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, current.
- ☑ Organization for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Series on Principles of Good Laboratory Practice and Compliance Monitoring, Monograph No.1 to 15, 1997.
- ☑ Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Study Director:

(b) (6)

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6 BRI Quality Assurance Statement

The Quality Assurance Unit (QAU) of BRI has reviewed the data in this study and this report for accuracy and adherence to the Study Protocol, the applicable GLP regulations stipulated in the protocol and BRI Standard Operating Procedures. The final report accurately describes the procedures employed during the course of this study and accurately reflects the raw data.

Date(s) of Inspection	Phase Audited	Date(s) of Reports to Study Director	Date(s) of Reports to Testing Facility Management
14 and 18Jun2012	Study Protocol	14 and 19Jun2012	24Jun2012
18 to 20Jul, 27Aug, 01Oct2012, and 07Jan2013	In Phase / In Vitro Incubation	07Aug, 04Sep, 09Oct2012, and 07Jan2013	08Jan2013
09Aug2012	In Phase / Assay Performance	13Aug2012	08Jan2013
12Dec2012, 08Jan2013	In-Process Data	12Dec2012, 08Jan2013	08Jan2013
19Dec2012, and 08Jan2013	Draft Report	20Dec2012, and 08Jan2013	08Jan2013
08Jan2013	Final Report	08Jan2013	08Jan2013
16Jan2013	Amended Final Report (v2.0)	16Jan2013	16Jan2013

(b) (6)

Alan Yeung, B.Sc.
Quality Assurance Officer
BRI Biopharmaceutical Research Inc.

16 Jan 2013
Date

7 Amendment Summary Table

Report Version Number	Issuance Date	Description and Rationale for Amendment(s)
1.0	08Jan2013	Original Study Report
2.0	16Jan2013	The report was amended based on corrections and comments from the Study Sponsor dated 11Jan2013 upon review of the final study report (v1.0). The amendment includes the following items: 1) Corrections for the structures of R1 and R2 for rebaudioside X, and R2 for steviolbioside and rebaudioside A in the Table of Section 9 on page 12; 2) Addition to Section 12.2 on page 18 to clarify that the 2 mg/ml incubation samples were not assayed; 3) Addition of a reference to ADF-256 in Appendix 7 in the last paragraph of Section 14 on page 20; and 4) Corrections from ‘2mg/mL’ to ‘0.2mg/mL’ for the BRI sample ID’s in Table 8 on pages 33 to 34.

8 Summary

8.1 Purpose & Rationale

The purpose of this study was to investigate the *in vitro* metabolism and hydrolysis of rebaudioside X in a pooled human fecal homogenate preparation at 37°C under anaerobic conditions to provide metabolism kinetic time-course data.

8.2 Summary of Results

Fecal samples were collected from twelve healthy subjects (six males and six females). Pooled human homogenates were prepared under anaerobic conditions based on pooling of fecal homogenate from 3 subjects to provide 2 male and 2 female pooled fecal homogenates.

Using a qualified LC/MS assay method it was determined that the Test Article rebaudioside X at a concentration of 0.2 mg/mL was completely metabolized to steviol, in both male and female fecal homogenates during a 24- hour incubation time period, with between 46% and 91% being converted within the first 8 hours.

Rebaudioside A (0.2 mg/mL) evaluated as a metabolic activity positive control was likewise shown to be completely metabolized to steviol after 24 hours. Again, the majority, ranging between 52% and 101% in each of the pooled homogenate samples, was converted within the first 8 hours. To confirm the metabolism-based formation of steviol metabolite in the pooled fecal homogenate, the stability of rebaudioside A and rebaudioside X was determined in control brain-heart infusion buffer that was used in preparation of the pooled fecal homogenate, and the results indicated the absence of steviol following incubation for 24 hours.

There were no observable differences in the overall degree or rate of metabolism of either rebaudioside X or rebaudioside A between males and females, indicating a similarity in metabolism between genders. The results may indicate a slight increase in the rate of formation of steviol from the conversion of rebaudioside A than rebaudioside X, which might be expected given that rebaudioside X contains additional glucose moieties that are required to be cleaved during the metabolic process.

Stability of steviol as the metabolite end product was confirmed in the pooled fecal homogenate. Steviol control samples were assayed and steviol was observed to remain stable during the 24-hour incubation period.

Furthermore, steviolbioside was also evaluated as a metabolic intermediate and a mean 77% to 82% molar conversion to steviol as a final anaerobic metabolite was observed within 24 hours. This metabolic yield of steviol metabolite from steviolbioside was observed consistently lower than 100%. It is likely that a higher dilution factor used in the preparation of steviolbioside samples may have contributed to these results.

Collectively, these results indicated that the fecal anaerobic incubation conditions used in this study were suitable for evaluating metabolism of rebaudioside X and rebaudioside A used as a positive control.

9 Introduction

In vitro studies have confirmed that steviol glycosides including stevioside and rebaudioside A are metabolized by the gut microflora to the aglycone steviol prior to absorption. Steviol (Figure 1) is the glycoside terpene aglycone of the principle and secondary steviol glycoside sweetener components of stevia extract, derived from *Stevia rebaudiana* [ref 1].

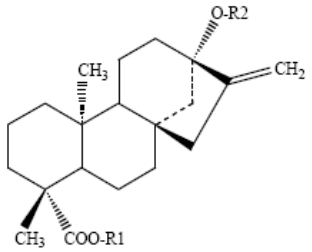

Figure 1. Steviol

Test Article Compound Name	R1	R2
Steviol	H	H
Rebaudioside A (CAS 58543-16-1)	ß-Glc	ß-Glc-ß-Glc (2→1) | ß-Glc (3→1)
Rebaudioside X	β-Glc-β-Glc (2→1) | β-Glc (3→1)	β-Glc-β-Glc (2→1) | β-Glc (3→1)
Steviolbioside	H	β-Glc-β-Glc (2→1)

A copy of the Study Protocol-TAR-2012-001 for this study is provided in **Appendix 5**.

9.1 Experimental Design

Prior to this study, a number of experimental parameters including anaerobic culture bacterial density and anaerobic buffers have been optimized. As a result, an optimal human fecal anaerobic culture for investigating the metabolism of rebaudioside X and rebaudioside A involved the use of 12.5X pooled fecal homogenate in anaerobic phosphate buffer diluted with 3 volumes of Brain-Heart Infusion Broth (BHI) to achieve 50X pooled fecal homogenate with 2 %v/v Oxyrase™ added at the start of incubation.

Rebaudioside A and steviolbioside were independently studied with rebaudioside A being used as a positive control while steviolbioside was evaluated as an intermediate hydrolysis product. Both rebaudioside A and steviolbioside were studied to ensure that the experimental incubation conditions were suitable for evaluating the metabolism of rebaudioside X in this study. The stability of steviol as a metabolite once formed was also monitored so as to establish the stability of the metabolite end product during the 24-hour incubation period.

For evaluation of metabolic stability of the Test Article, steviol concentrations in the incubated samples were being used to determine the molar equivalent conversion of Test Article and the positive control articles under anaerobic conditions.

9.2 Justification for Selection of Test System, Species and Dose Levels

The *in vitro* evaluation of steviol glycosides metabolism in human fecal homogenates at 37°C under anaerobic conditions has been previously reported in the published literature and is generally recognized as an acceptable experimental method for predicting their metabolic fate *in vivo.*

9.3 Study Schedule

Study initiation date (Protocol Finalized):	19Jun2012
Experimental start date :	17Jul2012
Experimental completion date:	28Oct2012

10 Test Article and Control Article Information

10.1 Test Article

The Test Article, rebaudioside X, and the positive control, rebaudioside A, along with the hydrolysis intermediate steviolbioside and the metabolite end product steviol, were provided by PureCircle with their respective Certificates of Analysis (COA) (**Appendix 1**, **Appendix 2**, **Appendix 3** and **Appendix 4** respectively).

10.2 Characterization of Test Articles and Control Articles

Test Article:	Identity:	Rebaudioside X (**Appendix 1**)
	Description:	White to off-white powder
	Batch/Lot No.:	PT140312
	Expiry Date:	14Mar2014
	Purity (wt/wt):	97.33% on anhydrous basis
	Loss of Drying (wt/wt):	5.56%
	Stability:	Information from Sponsor
	Storage Conditions:	4°C
	Handling Precautions:	Universal chemical safety precaution, refer to MSDS
	Supplier:	PureCircle
	BRI ID:	RFS-1294
Control Article 1:	Identity:	Rebaudioside A (**Appendix 2**)
	Description:	White to off-white powder
	Batch/Lot No.:	DR0311329
	Expiry Date:	08Mar2013
	Purity (wt/wt):	99.30% on anhydrous basis
	Loss of Drying (wt/wt):	0.29%
	Stability:	Information from Sponsor
	Storage Conditions:	4°C
	Handling Precautions:	Universal chemical safety precaution, refer to MSDS
	Supplier:	PureCircle
	BRI ID:	RFS-1297

Control Article 2:	Identity:	Steviolbioside (**Appendix 3**)
	Description:	White to off-white powder
	Batch/Lot No.:	PT081111
	Expiry Date:	08Nov2013
	Purity (wt/wt):	96.81% on anhydrous basis
	Loss of Drying (wt/wt):	5.28%
	Stability:	Information from Sponsor
	Storage Conditions:	4°C
	Handling Precautions:	Universal chemical safety precaution, refer to MSDS
	Supplier:	PureCircle
	BRI ID:	RFS-1295
Control Article 3:	Identity:	Steviol (**Appendix 4)**
	Description:	White to off-white powder
	Batch/Lot No.:	PT270312
	Expiry Date:	27Mar2014
	Purity:	97.37% Adjusted for water and solvent
	Water Content :	1.79%
	Stability:	Information from Sponsor
	Storage Conditions:	4°C
	Handling Precautions:	Universal chemical and radioisotope safety precautions, refer to MSDS
	Supplier:	PureCircle
	BRI ID:	RFS-1296, RFS-1345

All Test Article and control article concentrations were corrected for their purity, loss of drying or water and solvent content values where applicable.

10.3 Characterization of Vehicle

Vehicle for Rebaudioside X:	Identity:	Formamide
	Description:	Clear liquid at room temperature
	Batch/Lot No.:	69996TMV
	Expiry Date:	26Jan2017
	Purity:	≥99.5%
	Storage Conditions:	Room temperature
	Handling Precautions:	Universal chemical safety precaution, refer to MSDS
	Supplier:	Sigma Aldrich
	BRI ID:	LCI-2725
Vehicle for Rebaudioside A:	Identity:	Deionized water (diH_2O)
	Description:	Clear liquid at room temperature
	Batch/Lot No.:	n/ap; freshly collected in-house at BRI
	Expiry Date:	Used Fresh
	Purity:	Not Applicable
	Storage Conditions:	Room temperature
	Supplier:	BRI in house
	BRI ID:	Not Applicable

Vehicle for Steviol:	Identity:	Dimethyl Sulfoxide (DMSO)
	Description:	Clear liquid at room temperature
	Batch/Lot No.:	SHBB8179V
	Expiry Date:	25Jun2017
	Purity:	≥99.9%
	Storage Conditions:	Room temperature
	Handling Precautions:	Universal chemical safety precaution, refer to MSDS
	Supplier:	Sigma Aldrich
	BRI ID:	LCI-2944
Vehicle for Steviolbioside:	Identity:	Formamide and DMSO (1:1, v/v)
	Description:	Clear liquid at room temperature
	Batch/Lot No.:	n/ap; freshly prepared in-house at BRI
	Expiry Date:	Used Fresh
	Purity:	Not Applicable
	Storage Conditions:	Room temperature
	Handling Precautions:	Universal chemical safety precaution, refer to MSDS of Formamide and DMSO
	Supplier:	Prepared in house at BRI
	BRI ID:	LN-12-215

10.4 Preparation of Test Article and Control Article Solutions

The Test Article, rebaudioside X, and the positive control rebaudioside A, along with the metabolic hydrolysis intermediate steviolbioside and the metabolic end product steviol, were separately prepared in formamide, deionized water, a mixture of formamide and DMSO (1:1, v/v), and DMSO respectively at a nominal concentration of 200 mg/mL. These stock solutions were further diluted in the corresponding vehicle to a nominal concentration of 20 mg/mL to provide 100X spiking stock solution for testing at 0.2 mg/mL.

The spiking stock solution of rebaudioside A at 200 mg/mL was prepared immediately before use to avoid precipitation of the solute from the solution. This stock solution was further diluted 100X to 2 mg/mL before being stored at nominal -80°C prior to LC/MS verification of its concentration. The spiking stock solutions of rebaudioside A at 200 mg/mL and 20 mg/mL were also stored at nominal -80°C but not assayed by LC/MS since the solute precipitated and could not be dissolved again following the storage. All spiking stock solutions of rebaudioside X, steviolbioside, and steviol at both 200 mg/mL and 20 mg/mL were also stored at nominal -80°C prior to LC/MS verification of their concentrations.

10.5 Analysis of Test Article Solutions

10.5.1 Analysis of Achieved Stock Solution Concentrations

The verification of the Test Article and the control article stock solution concentrations was conducted based on an LC/MS method developed and qualified in the current study.

10.5.2 Frozen Storage Stability

Verification of the frozen storage stability of the Test Article and the control article stock solutions is not required for this study.

10.5.3 Stock Solution Analytical Results

Based on an LC/MS assay, the assay results of the Test Article rebaudioside X and the hydrolysis intermediate steviolbioside dose solutions at 20 mg/mL were verified to be within 15% of their expected concentrations. The positive control rebaudioside A was known to precipitate in solution at the dosing concentrations during storage. Hence, this dose solution was diluted to 2 mg/mL immediately after dosing to avoid precipitation during storage before assay. The assay result of this 2 mg/mL rebaudioside A solution was within 15% of its expected concentration.

The assay result of the steviol dose solution at 20 mg/mL was negatively biased from its expected concentration by 33.5% due to steviol precipitation during storage. However, this lower steviol dose concentration had no impact to the study results since the steviol stability samples were only used for stability determination (ie. % steviol remaining) based on comparison with 0 hr samples dosed with the same solution.

10.6 Archive Sampling

Sampling of the Test Article and the control article stock solutions for archive storage is not required for this study. Any unused portion of the stock solutions will be discarded following completion of this study.

11 Test System

11.1 Human Fecal Materials

Human fecal materials were collected from six healthy male and six healthy female volunteer donors without known gastrointestinal disease and without previous ingestion of stevia-based natural sweeteners, laxatives and antimicrobial drugs at least 7 days prior to the morning of fecal sample collection.

Upon voiding, approximately 10 to 20 g of fecal material from each donor was immediately collected in a pre-labelled 100 mL screw-capped polypropylene specimen container pre-filled with 50 mL anaerobic 0.2 M phosphate buffer (pH7.0). The initial stool-to-buffer ratio of the collected sample was determined based on the tare-weight of the screw-capped stool sample container containing the initial volume of anaerobic buffer. All 12 fecal materials were stored at room temperature before homogenate preparation within 48 hours of collection.

11.2 Anaerobic Potassium Phosphate Buffer (pH 7.0)

Identity:	Anaerobic Potassium Phosphate Buffer (pH 7.0)
BRI ID:	SS-7360-1
Batch/Lot No.:	27Sep2012
Expiry Date:	03Oct2012
Source:	BRI in house

11.3 Brain Heart Infusion Broth (BHI)

Identity:	Brain-Heart Infusion Broth	
BRI ID:	LCI-2989	LCI-3022
Batch/Lot No.:	20328	20810
Expiry Date:	28Mar2013	08Oct2013
Source:	Oxyrase Inc.	

11.4 Oxyrase for Broth

Identity:	Oxyrase™ for Broth
BRI ID:	LCI-2990
Batch/Lot No.:	POB20315
Expiry Date:	15Sep2013
Source:	Oxyrase Inc.

11.5 Human Fecal Homogenate Preparation

In an anaerobic chamber, each of the 12 fecal materials was accurately weighed and suspended in a known volume of buffer with a disposable wooden stick. The fecal materials were pooled by appropriate volume based on 3 subjects per donor gender in a clean 50-mL screw-capped test tube and added with appropriate volumes (%v/v) of 0.2 M potassium phosphate buffer (pH 7.0) to provide a 12.5X diluted pooled human fecal homogenate. Each of the screw-capped homogenates was mixed followed by centrifugation at 500 x*g* for 1 min at 37°C. In the anaerobic chamber, the supernatant of the fecal homogenate was transferred to a clean container, and diluted with 3 volumes of Brain-Heart Infusion Broth (BHI) into a 50X diluted pooled human fecal homogenate. Following the addition of Oxyrase™ for Broth (2%,v/v), the 50X diluted pooled human fecal homogenate was pre-incubated at 37°C overnight, prior to use in the *in vitro* incubations. A total of 2 male and 2 female pooled human fecal homogenate supernatant samples were each prepared from 3 subjects.

Pooled Human Fecal Homogenate ID	Donor Gender	Human Fecal Material Lot No.	Human Fecal Material BRI ID
M1	M	1	STM-1743
	M	4	STM-1746
	M	5	STM-1747
M2	M	8	STM-1750
	M	9	STM-1751
	M	11	STM-1753
F1	F	2	STM-1744
	F	6	STM-1748
	F	7	STM-1749
F2	F	12	STM-1754
	F	13	STM-1755
	F	14	STM-1756

In parallel, one volume of 0.2 M potassium phosphate buffer (pH 7.0) was mixed with 3 volumes of Brain Heart Infusion Broth (BHI). Following the addition of Oxyrase™ for Broth (2%. v/v), this BHI matrix was pre-incubated at 37°C overnight prior to being used as a control in the *in vitro* incubation.

12 Experimental Procedures

All procedures were carried out according to applicable BRI Standard Operating Procedures, the Study Protocol and Protocol Amendments approved for this study.

12.1 *In Vitro* Metabolic Stability

In vitro metabolic stability samples were generated from experiments as summarised below.

Sample Type	Test Materials	Test Concentration	Incubation Time Points
Assay Stability Control	Steviol	2 mg/mL and 0.2 mg/mL of each sample	37°C for 0, 8, 16, and 24 hours (n=3 replicates)
Hydrolysis intermediate Control	Steviolbioside		
Metabolism Positive Control	Rebaudioside A		
Test Article	Rebaudioside X		
Stability Controls in BHI	Rebaudioside A and Rebaudioside X		

12.2 Incubation and Sample Preparation Procedure

Metabolic incubations were carried out in triplicates at 37°C in sealed glass screw-capped test tubes to maintain an anaerobic condition during the incubation.

The positive control, rebaudioside A, along with steviol and the metabolic intermediate control, steviolbioside, and as well as the Test Article, rebaudioside X, was each incubated independently at test concentrations of 0.2 mg/mL and 2 mg/mL under an anaerobic environment for 0, 8, 16, and 24 hours at 37°C±5°C in a calibrated environmental chamber. At the end of each incubation time point, an equal volume of methanol containing 5% ammonium hydroxide was added to the fecal homogenate sample to stop the enzyme reaction with gentle vortex mixing and sonication for 5 min. The samples were centrifuged at 3270 *xg* for 10 minutes to remove protein components. The supernatant was transferred to a 1.5 mL microcentrifuge tube and then stored at nominal –80°C until LC/MS assay.

While the incubations were performed at two test concentrations, 0.2 mg/mL and 2 mg/mL, only the samples from 0.2 mg/mL were assayed by LC/MS for steviol while the samples from 2 mg/mL were not assayed for steviol concentration per request from the Study Sponsor. This decision was made by the Sponsor because the 0.2mg/mL samples provided clear and definitive results indicating the complete metabolic conversion to steviol, and therefore, it was unnecessary to analyze the 2 mg/mL samples.

12.3 LC/MS Analytical Methodology

12.3.1 LC/MS Assay Reference Standard and Internal Standard

Reference Standard:	Steviol
BRI ID:	RFS-1296
Supplier:	PureCircle
Batch Number:	PT270312
Purity:	97.37% (Based on supplier's COA adjusted for water and solvent)
Internal Standard:	Abietic Acid
BRI ID:	ITS-114
Supplier:	Sigma Aldrich
Lot Number:	BCBG8881V
Purity:	82.1% (Based on supplier's COA)

Reference standard concentrations were corrected for the purity. Correction for purity was not required for the internal standard.

12.3.2 LC/MS Assay Blank Matrix

For LC/MS assay of steviol in the male and female pooled fecal homogenate samples, steviol calibration standards and QC samples were prepared in a pooled fecal homogenate assay blank matrix composed of an equal mixture of the four pooled human fecal homogenate matrices, M1, M2, F1 and F2 as described in **Section 11.5.** Following *in vitro* incubation for 24 hours, the blank fecal homogenate matrices were pooled and stored at nominal 2-8°C before being used as a blank assay matrix for preparation of calibration standards and QC samples. During preparation of calibration standards and QC samples, an equal volume of methanol containing 5% ammonium hydroxide was added to mimic the assay matrix of the incubation test samples.

A set QC samples (n=3 at each concentration) was prepared in a blank matrix of equal volume of BHI and methanol containing 5% ammonium hydroxide, to confirm the absence of matrix effect of the assay method when the stability control samples of rebaudioside A and rebaudioside X in BHI were run with calibration standards prepared in fecal homogenates.

12.3.3 Summary of LC/MS Assay Procedures

All calibration standards and QC samples were prepared fresh on the day of assay. Test samples were equilibrated to room temperature followed by sonication for 5 min and vortex mixing. Samples of expected steviol concentration higher than the assay ULOQ were diluted with a mixture of equal volume of pooled human fecal homogenate and 5% ammonium hydroxide in methanol.

An aliquot of the internal standard solution was spiked into each sample except for blanks. Following vortex mixing and centrifugation to remove protein precipitates. The supernatant was transferred to LC vials for LC/MS analysis.

12.3.4 LC/MS Instrumentation

A Micromass Quattro®-LC triple quadrupole mass spectrometer, inclusive of the HPLC unit, controlled by Micromass Masslynx® software Version 4.0 was used.

- BRI ID: 446 Micromass Quattro®-LC triple quadrupole mass spectrometer
- BRI ID: 459 Agilent Model 1100 G1329A Autosampler
- BRI ID: 443 Agilent Model 1100 G1312A Binary Pump
- BRI ID: 460 Agilent Model 1100 G1330B Thermostat
- BRI ID: 573 Agilent Model 1100 G1316A Column Compartment

12.3.5 Chromatographic and Mass Spectral Parameters

- Gradient elution using two mobile phases
- Mobile Phase A: 2 mM ammonium acetate in deionized water
- Mobile Phase B: 2 mM ammonium acetate and 1% DCM in a mixture of 90% acetonitrile and 10% deionized water
- Column: ACE 3 C18, 30 X 2.1 mm (BRI ID: LC-231)
- Injection volume: 5 µL
- MS mode: ESI negative SIR mode

For further details, refer to **Appendix 8** of this report.

13 Data Management

13.1 Data Collection and Statistical Methods

Data collection, chromatographic peak integration and regression analysis were performed using Micromass Masslynx® Version 4.0. Descriptive statistics such as those described in the appendix of the Study Protocol (**Appendix 5**) were performed using Microsoft Excel 2007.

Analytical data were printed on hardcopy and electronic data backup was performed via BRIPHARM Windows Server 2008 following BRI Standard Operating Procedures.

13.2 Data Archiving

All experimental raw data, related documentation, and the study report will be archived at BRI's archives (103-8898 Heather Street, Vancouver, BC, Canada) following procedures described in BRI Standard Operating Procedures for a period of at least 5 years following completion of this study (final report issuance date). BRI agrees to give the Study Sponsor sufficient advance notification of any relocation of the archived materials after the 5-year holding period to the Study Sponsor, or to an alternative storage facility. Retention of records after the time stated above will be subject to further contractual agreement between BRI and the Study Sponsors.

14 Protocol Amendments

During the conduct of this study, the protocol was amended to change the source and preparation procedure of human fecal homogenate (HFH), the preparation of stock solutions, the procedure for spiking incubation samples, the incubation time points, and the addition of BHI buffer control samples (Amendment No. 2; ADF-256; **Appendix 7**). In addition, the following changes were also implemented:

i) The vehicles used in preparation of the Test Article and the control article were changed from 0.2M potassium phosphate buffer (pH 7.0) containing 10% DMSO to those listed in Section 10.3 to ensure solubility.
ii) The verification of assay recovery was not necessary as this LC/MS assay did not involve any extraction step.
iii) The precision criteria for verifying assay lower limit of quantitation was corrected to 20 %CV to follow BRI SOP requirement for bioanalytical assays.
iv) The LIMS was not used for sample logistics, data handling and processing.
v) The LC/MS assay for dosing solutions was qualified for each of the Test Article and control articles, instead of for steviol alone. This was necessary to reliably assay the different Test Article and control articles in the dosing solutions.

All of the above protocol amendments and changes were necessary adjustments and had no impact to the results of the study.

In addition, during the conduct of this study, an investigation for the lower than expected conversion of the positive control and variability of metabolite formation was performed and described in Protocol Amendment No. 1 (ADF-252; **Appendix 6**). The changes proposed as a result of the investigation were not implemented in the final experiments but further refined into

the amendments made in Protocol Amendment No. 2 (ADF-256; **Appendix 7**) described above which were subsequently implemented.

15 Results and Discussion

15.1 Steviol LC/MS Assay Batch Acceptance Summary

LC/MS system suitability samples were prepared and evaluated in each LC/MS assay batch based on six repeated injections of a sample prepared at a nominal concentration of 150 ng/mL. In order to meet batch acceptance criteria, a peak area response ratio of steviol to internal standard was observed within 10 %CV. Chromatographic retention times were also observed within ±0.5 min of the value expected for steviol and the internal.

Assay calibration of steviol was performed at a nominal concentration range of 50 ng/mL to 5000 ng/mL in human fecal homogenate (HFH). Calibration samples were prepared in duplicate (except for the batch for stability control samples in BHI where calibration standards in singleton were prepared) by *ex vivo* spiking steviol in a pooled human fecal homogenate preparation. The calibration standard curve data was best described by linear regression (origin excluded) with 1/x weighting factor. In order to meet batch acceptance criteria, deviations of the calculated against the expected concentrations were within ±15% for more than 75% of the total number of calibration standards (non-zero points of both curves combined); except a maximum of ±20% deviation was acceptable at the LLOQ. The coefficient of determination (R^2) was greater than or equal to 0.99. Calibration standards with back-interpolated concentrations exceeding ±15% deviation were not included in regression analysis. A representative steviol LC/MS assay calibration standard curve is presented in **Figure 1**.

QC samples were prepared using a stock solution independent of that used for calibration standards. QC samples were prepared in six replicates at nominal concentrations of 150, 2000, and 4000 ng/mL. In order to meet batch acceptance criteria, a minimum of 67% of the total number of QC samples within an assay batch was required to occur within a ±15% deviation. For each concentration level, a minimum of 4 out of 6 replicates were required to occur with absolute deviations within 15%, and not all of the QC samples in the same assay group were affected by deviations. In addition, the absolute mean % deviation and %CV at each concentration level was observed to be within 15%. Analytical data outliers as determined by an outlier statistical test were excluded from mean calculation of mean % deviation and %CV if applicable. The QC samples in BHI matrix were prepared in the same way but in triplicates, with the same batch acceptance criteria applied proportionally.

Assay LLOQ was evaluated at concentration of 50 ng/mL in six replicates, with a minimum of 4 out of 6 replicates required to occur with absolute deviations within 20% and a mean %CV within 20%. Carryover was monitored and evaluated as per BRI SOP-GP-016 (v1.0) following each high calibration standard, QC, and test sample. Carryover observed within 20% of the assay LLOQ was considered acceptable. In addition, dilution integrity at factors of 10X and 40X, applied to the test samples, were evaluated to support dilution of samples for assay.

The LC/MS assay batch performance data from the assays of rebaudioside X, rebaudioside A, and steviolbioside samples are presented in **Tables 1**, **2** and **3**, respectively. The LC/MS assay batch performance data from the assay of steviol stability control samples is summarized in **Table 4**. The steviol LC/MS assay performance data for rebaudioside X and rebaudioside A in BHI buffer stability control samples is presented in **Table 5**. All assay batches were observed to meet pre-defined batch acceptance criteria as outlined above. Representative LC/MS chromatograms of

steviol in human fecal homogenate sample matrices are presented in Figures 2 to 8, including a blank sample (**Figure 2**), blank sample with internal standard (**Figure 3**), LLOQ sample (**Figure 4**), low QC sample (**Figure 5**), mid QC sample (**Figure 6**), high QC sample (**Figure 7**) and assay ULOQ sample (**Figure 8**).

15.2 Metabolism of Rebaudioside X, Rebaudioside A, Steviolbioside and Steviol in Pooled Human Fecal Homogenates

The metabolism and hydrolysis of rebaudioside X in pooled human fecal homogenate was evaluated by incubating the Test Article under anaerobic conditions at nominal 37°C over a time-course of 0, 8, 16, and 24 hr. In parallel, rebaudioside A as a positive control, along with steviolbioside as a hydrolysis intermediate control, and steviol as a stability control were also evaluated in triplicate samples under identical conditions.

Assessment on the rate and extent of metabolism for rebaudioside X and for each of the controls was based on evaluating the molar equivalent of steviol formed as a common metabolite end-product over the incubation time-course of 24 hours. The steviol LC/MS assay results from rebaudioside X (**Tables 6** and **7**), rebaudioside A (**Tables 8** and **9**), and steviolbioside (**Tables 10** and **11**) samples are separately summarized. The steviol LC/MS assay results from the steviol stability control samples are summarized in **Tables 12** and **13**. The steviol LC/MS assay results from the rebaudioside X and rebaudioside A in BHI buffer stability control samples are summarized in **Table 14**.

The results from this *in vitro* metabolism study indicated that rebaudioside X was rapidly metabolized in pooled human homogenate under anaerobic conditions at 37°C with almost complete molar equivalent conversion to steviol as a metabolite end-product at the end of 8 hours.

For the positive control, rebaudioside A and the hydrolysis intermediate steviolbioside, similar rates and extent of metabolism were observed as evident by the abundant formation of steviol within the first 8 hours of incubation. The metabolic yield of steviol metabolite from steviolbioside was observed consistently at a mean of 79.8% and was lower than expected, however, it is possible that the higher dilution factor used in this analysis may have contributed to these results. Collectively, the results from the positive control rebaudioside A and the hydrolysis intermediate steviolbioside indicated that the anaerobic incubation conditions used in this study were suitable for *in vitro* evaluation of these steviol glycosides.

The % molar equivalent formation of steviol observed from the two pooled male and two pooled female fecal homogenate samples (**Table 15**) indicate that no gender difference was apparent in the metabolism of rebaudioside X, rebaudioside A, and steviolbioside.

Steviol as the metabolite end-product was observed to remain stable over the incubation time course of 24 hours (**Table 16**). Furthermore, the absence of detectable levels of steviol in the Brain-Heart Infusion buffer control samples containing rebaudioside A or rebaudioside X over the incubation time-course similarly confirmed the chemical stability of these steviol glycosides and further supported the formation of steviol from a fecal metabolism-based reaction (**Table 17**).

16 Conclusion

Pooled human homogenates were prepared under anaerobic conditions based on pooling of fecal homogenate from 3 subjects to provide 2 male and 2 female pooled fecal homogenates from a total of 6 males and 6 females healthy subjects.

A qualified LC/MS assay has been successfully implemented for quantitation of steviol as a metabolite end-product in human fecal homogenate samples. Multiple assay batches were satisfactorily performed for the quantitation of steviol in human fecal homogenate incubation samples.

The Test Article rebaudioside X at 0.2 mg/mL was completely metabolized to steviol, with a mean of 110% molar conversion to the final anaerobic metabolite within 24 hours. To confirm the metabolism-based formation of steviol metabolite in the pooled fecal homogenate, the stability of rebaudioside A and rebaudioside X in the brain-heart infusion buffer used in the preparation of the pooled fecal homogenate indicated the absence of steviol following incubation for 24 hours.

Rebaudioside A was evaluated as a metabolic activity positive control and was completely metabolized to steviol after 24 hours, with a mean of 99% molar conversion.

There were no observable differences in metabolism of either rebaudioside A or rebaudioside X between males and females.

To monitor the stability of steviol in the pooled fecal homogenate as a metabolite end product, steviol stability control samples were assayed and steviol was observed to remain stable at a mean of 97.7% remaining after 24 hours.

Likewise, an intermediate metabolite steviolbioside was also evaluated as a hydrolysis intermediate and a mean of 80% molar conversion to steviol as a final anaerobic metabolite was observed within 24 hours. This metabolic yield of steviol metabolite from steviolbioside was observed consistently lower than 100%. However it is possible that the higher dilution factor used in this analysis might have contributed to these results. Collectively, these results indicated that the anaerobic incubation conditions used in this study were suitable for evaluating the anaerobic metabolism of these steviol glycosides

17 Reference

[1] Harriet Wallin. Steviol glycosides. Chemical and Technical Assessment. 63rd JECFA. 2004

18 Tables

Table 1 Summary of Steviol LC/MS Assay Batch Acceptance Summary from the Assay of Rebaudioside X Samples

Calibration Samples	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
50 ng/mL-1	50.0	53.1	6.2
100 ng/mL-1	100	100	-0.5
200 ng/mL-1	200	205	2.5
500 ng/mL-1	500	479	-4.2
1000 ng/mL-1	1000	996	-0.4
2000 ng/mL-1	2000	1920	-4.0
4000 ng/mL-1	4000	3980	-0.5
5000 ng/mL-1	5000	5200	4.0
50 ng/mL-2	50.0	51.3	2.6
100 ng/mL-2	100	97.6	-2.4
200 ng/mL-2	200	211	5.5
500 ng/mL-2	500	473	-5.4
1000 ng/mL-2	1000	1000	0.0
2000 ng/mL-2	2000	1930	-3.5
4000 ng/mL-2	4000	3890	-2.8
5000 ng/mL-2	5000	5110	2.2

Calibration Curve : y = 0.000960747*x - 0.00433958
R^2 = 0.9990

QC Samples	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
QC 150-1	150	152	1.3
QC 150-2	150	155	3.3
QC 150-3	150	155	3.3
QC 150-4	150	151	0.7
QC 150-5	150	156	4.0
QC 150-6	150	150	0.0
Mean		153	2.1
%CV		1.6	
QC 2000-1	2000	2080	4.0
QC 2000-2	2000	2080	4.0
QC 2000-3	2000	2150	7.5
QC 2000-4	2000	2100	5.0
QC 2000-5	2000	2290	14.5
QC 2000-6	2000	2070	3.5
Mean		2130	6.4
%CV		4.0	
QC 4000-1	4000	4270	6.8
QC 4000-2	4000	4300	7.5
QC 4000-3	4000	4300	7.5
QC 4000-4	4000	4400	10.0
QC 4000-5	4000	4330	8.3
QC 4000-6	4000	4420	10.5
Mean		4340	8.4
%CV		1.4	

Table 2 Summary of Steviol LC/MS Assay Batch Acceptance Summary from the Assay of Rebaudioside A Samples

Calibration Samples	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
50 ng/mL-1	50.0	50.5	1.0
100 ng/mL-1	100	104	4.0
200 ng/mL-1	200	210	5.0
500 ng/mL-1	500	469	-6.2
1000 ng/mL-1	1000	966	-3.4
2000 ng/mL-1	2000	1790	-10.5
4000 ng/mL-1	4000	3750	-6.3
5000 ng/mL-1	5000	4920	-1.6
50 ng/mL-2	50.0	51.9	3.8
100 ng/mL-2	100	99.3	-0.7
200 ng/mL-2	200	200	0.0
500 ng/mL-2	500	486	-2.8
1000 ng/mL-2	1000	1030	3.0
2000 ng/mL-2	2000	2050	2.5
4000 ng/mL-2	4000	4330	8.3
5000 ng/mL-2	5000	5180	3.6

Calibration Curve : y = 0.000930744*x - 0.00203858
R^2 = 0.9965

QC Sample	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
QC 150-1	150	154	2.7
QC 150-2	150	160	6.7
QC 150-3	150	153	2.0
QC 150-4	150	156	4.0
QC 150-5	150	155	3.3
QC 150-6	150	151	0.7
Mean		155	3.2
%CV		2.0	
QC 2000-1	2000	2010	0.5
QC 2000-2	2000	1960	-2.0
QC 2000-3	2000	2080	4.0
QC 2000-4	2000	2020	1.0
QC 2000-5	2000	2210	10.5
QC 2000-6	2000	2160	8.0
Mean		2070	3.7
%CV		4.6	
QC 4000-1	4000	3900	-2.5
QC 4000-2	4000	4160	4.0
QC 4000-3	4000	4170	4.3
QC 4000-4	4000	4350	8.8
QC 4000-5	4000	4490	12.3
QC 4000-6	4000	4290	7.3
Mean		4230	5.7
%CV		4.8	

Table 3 Summary of Steviol LC/MS Assay Batch Acceptance Summary from the Assay of Steviolbioside Samples

Calibration Samples	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
50 ng/mL-1	50.0	57.9	15.8
100 ng/mL-1	100	107	7.0
200 ng/mL-1	200	188	-6.0
500 ng/mL-1	500	441	-11.8
1000 ng/mL-1	1000	905	-9.5
2000 ng/mL-1	2000	1750	-12.5
4000 ng/mL-1	4000	3940	-1.5
5000 ng/mL-1	5000	5150	3.0
50 ng/mL-2	50.0	59.2	18.4
100 ng/mL-2	100	107	7.0
200 ng/mL-2	200	197	-1.5
500 ng/mL-2	500	436	-12.8
1000 ng/mL-2	1000	971	-2.9
2000 ng/mL-2	2000	1970	-1.5
4000 ng/mL-2	4000	4190	4.8
5000 ng/mL-2	5000	5230	4.6

Calibration Curve : y = 0.000932843*x - 0.0139324
R^2 = 0.9962

QC Samples	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
QC 150-1	150	146	-2.7
QC 150-2	150	155	3.3
QC 150-3	150	149	-0.7
QC 150-4	150	158	5.3
QC 150-5	150	155	3.3
QC 150-6	150	150	0.0
Mean		152	1.4
%CV		3.0	
QC 2000-1	2000	2120	6.0
QC 2000-2	2000	1920	-4.0
QC 2000-3	2000	2040	2.0
QC 2000-4	2000	2040	2.0
QC 2000-5	2000	1940	-3.0
QC 2000-6	2000	2080	4.0
Mean		2020	1.2
%CV		3.9	
QC 4000-1	4000	4480	12.0
QC 4000-2	4000	4560	14.0
QC 4000-3	4000	4360	9.0
QC 4000-4	4000	4370	9.3
QC 4000-5	4000	4840	21.0
QC 4000-6	4000	4290	7.3
Mean		4480	12.1
%CV		4.4	

Table 4 Summary of Steviol LC/MS Assay Batch Acceptance Summary from the Assay of Steviol Stability Samples

Calibration Samples	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
50 ng/mL-1	50.0	59.2	18.4
100 ng/mL-1	100	108	8.0
200 ng/mL-1	200	198	-1.0
500 ng/mL-1	500	433	-13.4
1000 ng/mL-1	1000	971	-2.9
2000 ng/mL-1	2000	1910	-4.5
4000 ng/mL-1	4000	4090	2.3
5000 ng/mL-1	5000	5290	5.8
50 ng/mL-2	50.0	55.8	11.6
100 ng/mL-2	100	103	3.0
200 ng/mL-2	200	187	-6.5
500 ng/mL-2	500	455	-9.0
1000 ng/mL-2	1000	944	-5.6
2000 ng/mL-2	2000	1870	-6.5
4000 ng/mL-2	4000	3940	-1.5
5000 ng/mL-2	5000	5100	2.0

Calibration Curve : y = 0.000950835*x - 0.0136887
R^2 = 0.9975

QC Samples	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
QC 150-1	150	148	-1.3
QC 150-2	150	163	8.7
QC 150-3	150	138	-8.0
QC 150-4	150	171	14.0
QC 150-5	150	155	3.3
QC 150-6	150	144	-4.0
Mean		153	2.1
%CV		8.0	
QC 2000-1	2000	2220	11.0
QC 2000-2	2000	1960	-2.0
QC 2000-3	2000	1960	-2.0
QC 2000-4	2000	1800	-10.0
QC 2000-5	2000	1920	-4.0
QC 2000-6	2000	2080	4.0
Mean		1990	-0.5
%CV		7.2	
QC 4000-1	4000	3950	-1.3
QC 4000-2	4000	3950	-1.3
QC 4000-3	4000	4040	1.0
QC 4000-4	4000	4040	1.0
QC 4000-5	4000	3900	-2.5
QC 4000-6	4000	4300	7.5
Mean		4030	0.8
%CV		3.6	

Table 5 Summary of Steviol LC/MS Assay Batch Acceptance Summary from the Assay of Rebaudioside X and Rebaudioside A in BHI Buffer Stability Samples

Calibration Samples	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
50 ng/mL-1	50.0	55.2	10.4
100 ng/mL-1	100	103	3.0
200 ng/mL-1	200	201	0.5
500 ng/mL-1	500	460	-8.0
1000 ng/mL-1	1000	949	-5.1
2000 ng/mL-1	2000	1910	-4.5
4000 ng/mL-1	4000	4040	1.0
5000 ng/mL-1	5000	5130	2.6

Calibration Curve : y = 0.000990232*x - 0.00990299
R^2 = 0.9987

QC Samples	Expected Conc (ng/mL)	Reported Conc (ng/mL)	% Bias
BHI QC 150-1	150	146	-2.7
BHI QC 150-2	150	141	-6.0
BHI QC 150-3	150	142	-5.3
Mean		143	-4.7
%CV		1.9	
BHI QC 2000-1	2000	1890	-5.5
BHI QC 2000-2	2000	1940	-3.0
BHI QC 2000-3	2000	1940	-3.0
Mean		1920	-3.8
%CV		1.5	
BHI QC 4000-1	4000	3960	-1.0
BHI QC 4000-2	4000	4050	1.3
BHI QC 4000-3	4000	3910	-2.3
Mean		3970	-0.7
%CV		1.8	

Table 6 Summary of Steviol LC/MS Assay Results from Rebaudioside X Pooled Male-1 (M-1) and Male-2 (M-2) Fecal Homogenate Samples

Pooled Fecal Homogenate from Male #1

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb X Metabolized
0.2mg/mL RX in M1 0h-1	0	2	18.1521	36.3042	0.114	<LLOQ	n/ap
0.2mg/mL RX in M1 0h-2	0	2	14.1968	28.3937	0.0892	<LLOQ	
0.2mg/mL RX in M1 0h-3	0	2	17.3194	34.6388	0.109	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RX in M1 8h-1	8	20	1141.7570	22835.1393	71.7	71.7	46.3
0.2mg/mL RX in M1 8h-2	8	20	1148.8348	22976.6958	72.2	72.2	46.6
0.2mg/mL RX in M1 8h-3	8	20	1139.6753	22793.5051	71.6	71.6	46.2
					Mean	**71.8**	**46.4**
					%CV	**0.4**	
0.2mg/mL RX in M1 16h-1	16	20	2931.9265	58638.5298	184	184	118.8
0.2mg/mL RX in M1 16h-2	16	20	2822.7406	56454.8123	177	177	114.3
0.2mg/mL RX in M1 16h-3	16	20	2823.8856	56477.7112	177	177	114.3
					Mean	**179**	**115.8**
					%CV	**2.3**	
0.2mg/mL RX in M1 24h-1	24	20	2792.4517	55849.0337	175	175	113.0
0.2mg/mL RX in M1 24h-2	24	20	2845.3272	56906.5442	179	179	115.6
0.2mg/mL RX in M1 24h-3	24	20	2887.6901	57753.8016	181	181	116.9
					Mean	**178**	**115.1**
					%CV	**1.7**	

Pooled Fecal Homogenate from Male #2

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb X Metabolized
0.2mg/mL RX in M2 0h-1	0	2	32.9323	65.8645	0.207	<LLOQ	
0.2mg/mL RX in M2 0h-2	0	2	32.6200	65.2400	0.205	<LLOQ	n/ap
0.2mg/mL RX in M2 0h-3	0	2	31.3710	62.7420	0.197	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RX in M2 8h-1	8	20	2004.5231	40090.4625	126	126	81.4
0.2mg/mL RX in M2 8h-2	8	20	2046.1574	40923.1479	129	129	83.3
0.2mg/mL RX in M2 8h-3	8	20	2051.5699	41031.3970	129	129	83.3
					Mean	**128**	**82.6**
					%CV	**1.4**	
0.2mg/mL RX in M2 16h-1	16	20	2601.8708	52037.4163	163	163	105.2
0.2mg/mL RX in M2 16h-2	16	20	2709.7036	54194.0715	170	170	109.8
0.2mg/mL RX in M2 16h-3	16	20	2645.1705	52903.4091	166	166	107.2
					Mean	**166**	**107.4**
					%CV	**2.1**	
0.2mg/mL RX in M2 24h-1	24	20	2574.2881	51485.7622	162	162	104.6
0.2mg/mL RX in M2 24h-2	24	20	2714.2833	54285.6669	170	170	109.8
0.2mg/mL RX in M2 24h-3	24	20	2736.7658	54735.3170	172	172	111.1
					Mean	**168**	**108.5**
					%CV	**3.1**	

Table 7 Summary of Steviol LC/MS Assay Results from Rebaudioside X Pooled Female-1 (F-1) and Female-2 (F-2) Fecal Homogenate Samples

Pooled Fecal Homogenate from Female #1

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb X Metabolized
0.2mg/mL RX in F1 0h-1	0	2	36.4712	72.9424	0.229	<LLOQ	
0.2mg/mL RX in F1 0h-2	0	2	36.2630	72.5260	0.228	<LLOQ	n/ap
0.2mg/mL RX in F1 0h-3	0	2	35.7426	71.4852	0.224	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RX in F1 8h-1	8	20	2267.3395	45346.7891	142	142	91.7
0.2mg/mL RX in F1 8h-2	8	20	2238.5077	44770.1545	141	141	91.0
0.2mg/mL RX in F1 8h-3	8	20	2247.3550	44947.1001	141	141	91.0
					Mean	**141**	**91.3**
					%CV	**0.4**	
0.2mg/mL RX in F1 16h-1	16	20	2569.0838	51381.6765	161	161	103.9
0.2mg/mL RX in F1 16h-2	16	20	2728.4390	54568.7799	171	171	110.4
0.2mg/mL RX in F1 16h-3	16	20	2706.3728	54127.4567	170	170	109.8
					Mean	**167**	**108.0**
					%CV	**3.3**	
0.2mg/mL RX in F1 24h-1	24	20	2630.5985	52611.9692	165	165	106.5
0.2mg/mL RX in F1 24h-2	24	20	2722.1939	54443.8771	171	171	110.4
0.2mg/mL RX in F1 24h-3	24	20	2585.9457	51718.9141	162	162	104.6
					Mean	**166**	**107.2**
					%CV	**2.8**	

Pooled Fecal Homogenate from Female #2

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb X Metabolized
0.2mg/mL RX in F2 0h-1	0	2	39.0733	78.1467	0.245	<LLOQ	
0.2mg/mL RX in F2 0h-2	0	2	41.2591	82.5182	0.259	<LLOQ	n/ap
0.2mg/mL RX in F2 0h-3	0	2	40.8428	81.6856	0.257	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RX in F2 8h-1	8	20	1959.8704	39197.4074	123	123	79.4
0.2mg/mL RX in F2 8h-2	8	20	2082.8996	41657.9928	131	131	84.6
0.2mg/mL RX in F2 8h-3	8	20	2014.8276	40296.5522	127	127	82.0
					Mean	**127**	**82.0**
					%CV	**3.1**	
0.2mg/mL RX in F2 16h-1	16	20	2695.1316	53902.6316	169	169	109.1
0.2mg/mL RX in F2 16h-2	16	20	2730.0003	54600.0056	171	171	110.4
0.2mg/mL RX in F2 16h-3	16	20	2630.0780	52601.5607	165	165	106.5
					Mean	**168**	**108.7**
					%CV	**1.8**	
0.2mg/mL RX in F2 24h-1	24	20	2663.1773	53263.5456	167	167	107.8
0.2mg/mL RX in F2 24h-2	24	20	2691.0722	53821.4448	169	169	109.1
0.2mg/mL RX in F2 24h-3	24	20	2618.6286	52372.5722	164	164	105.9
					Mean	**167**	**107.6**
					%CV	**1.5**	

Table 8 Summary of Steviol LC/MS Assay Results from Rebaudioside A Pooled Male-1 (M-1) and Male-2 (M-2) Fecal Homogenate Samples

Pooled Fecal Homogenate from Male #1

BRI Sample ID (Note 1)	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb A Metabolized
0.2mg/mL RA in M1 0h-1	0	2	71.5971	143.1942	0.450	0.450	0.2
0.2mg/mL RA in M1 0h-2	0	2	68.9111	137.8222	0.433	0.433	0.2
0.2mg/mL RA in M1 0h-3	0	2	69.2334	138.4668	0.435	0.435	0.2
					Mean	**0.439**	**0.2**
					%CV	**2.1**	
0.2mg/mL RA in M1 8h-1	8	20	1711.8978	34237.9554	108	108	52.2
0.2mg/mL RA in M1 8h-2	8	20	1660.0038	33200.0761	104	104	50.3
0.2mg/mL RA in M1 8h-3	8	20	1725.0056	34500.1113	108	108	52.2
					Mean	**107**	**51.6**
					%CV	**2.2**	
0.2mg/mL RA in M1 16h-1	16	20	3274.6261	65492.5217	206	206	99.6
0.2mg/mL RA in M1 16h-2	16	20	3318.7843	66375.6861	208	208	100.6
0.2mg/mL RA in M1 16h-3	16	20	3196.1942	63923.8841	201	201	97.2
					Mean	**205**	**99.1**
					%CV	**1.8**	
0.2mg/mL RA in M1 24h-1	24	20	3289.1306	65782.6122	207	207	100.1
0.2mg/mL RA in M1 24h-2	24	20	3195.5496	63910.9912	201	201	97.2
0.2mg/mL RA in M1 24h-3	24	20	3094.5551	61891.1017	194	194	93.8
					Mean	**201**	**97.0**
					%CV	**3.2**	

Pooled Fecal Homogenate from Male #2

BRI Sample ID (Note 1)	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb A Metabolized
0.2mg/mL RA in M2 0h-1	0	2	113.2842	226.5684	0.711	0.711	0.3
0.2mg/mL RA in M2 0h-2	0	2	114.2512	228.5023	0.718	0.718	0.3
0.2mg/mL RA in M2 0h-3	0	2	116.0777	232.1553	0.729	0.729	0.4
					Mean	**0.719**	**0.3**
					%CV	**1.3**	
0.2mg/mL RA in M2 8h-1	8	20	2478.8111	49576.2225	156	156	75.4
0.2mg/mL RA in M2 8h-2	8	20	2582.9214	51658.4277	162	162	78.3
0.2mg/mL RA in M2 8h-3	8	20	2501.6960	50033.9208	157	157	75.9
					Mean	**158**	**76.6**
					%CV	**2.0**	
0.2mg/mL RA in M2 16h-1	16	20	3151.6062	63032.1244	198	198	95.7
0.2mg/mL RA in M2 16h-2	16	20	3225.0958	64501.9163	203	203	98.2
0.2mg/mL RA in M2 16h-3	16	20	3276.3451	65526.9028	206	206	99.6
					Mean	**202**	**97.8**
					%CV	**2.0**	
0.2mg/mL RA in M2 24h-1	24	20	3201.1365	64022.7298	201	201	97.2
0.2mg/mL RA in M2 24h-2	24	20	3301.2714	66025.4287	207	207	100.1
0.2mg/mL RA in M2 24h-3	24	20	3189.3180	63786.3597	200	200	96.7
					Mean	**203**	**98.0**
					%CV	**1.9**	

Table 9 Summary of Steviol LC/MS Assay Results from Rebaudioside A Pooled Female-1 (F-1) and Female-2 (F-2) Fecal Homogenate Samples

Pooled Fecal Homogenate from Female #1

BRI Sample ID (Note 1)	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb A Metabolized
0.2mg/mL RA in F1 0h-1	0	2	148.8471	297.6943	0.935	0.935	0.5
0.2mg/mL RA in F1 0h-2	0	2	135.5245	271.0489	0.851	0.851	0.4
0.2mg/mL RA in F1 0h-3	0	2	142.6156	285.2311	0.896	0.896	0.4
					Mean	**0.894**	**0.4**
					%CV	**4.7**	
0.2mg/mL RA in F1 8h-1	8	20	3002.9080	60058.1595	189	189	91.4
0.2mg/mL RA in F1 8h-2	8	20	3149.1351	62982.7016	198	198	95.7
0.2mg/mL RA in F1 8h-3	8	20	3122.3823	62447.6458	196	196	94.8
					Mean	**194**	**94.0**
					%CV	**2.4**	
0.2mg/mL RA in F1 16h-1	16	20	3256.3611	65127.2225	205	205	99.1
0.2mg/mL RA in F1 16h-2	16	20	3326.1977	66523.9546	209	209	101.1
0.2mg/mL RA in F1 16h-3	16	20	3301.3789	66027.5775	207	207	100.1
					Mean	**207**	**100.1**
					%CV	**1.0**	
0.2mg/mL RA in F1 24h-1	24	20	3222.0875	64441.7494	202	202	97.7
0.2mg/mL RA in F1 24h-2	24	20	3173.2018	63464.0369	199	199	96.2
0.2mg/mL RA in F1 24h-3	24	20	3277.9568	65559.1351	206	206	99.6
					Mean	**202**	**97.8**
					%CV	**1.7**	

Pooled Fecal Homogenate from Female #2

BRI Sample ID (Note 1)	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb A Metabolized
0.2mg/mL RA in F2 0h-1	0	2	207.7248	415.4495	1.30	1.30	0.6
0.2mg/mL RA in F2 0h-2	0	2	206.4355	412.8710	1.30	1.30	0.6
0.2mg/mL RA in F2 0h-3	0	2	207.0801	414.1602	1.30	1.30	0.6
					Mean	**1.30**	**0.6**
					%CV	**0.0**	
0.2mg/mL RA in F2 8h-1	8	20	3474.6811	69493.6219	218	218	105.4
0.2mg/mL RA in F2 8h-2	8	20	3227.5670	64551.3391	203	203	98.2
0.2mg/mL RA in F2 8h-3	8	20	3232.6167	64652.3336	203	203	98.2
					Mean	**208**	**100.6**
					%CV	**4.2**	
0.2mg/mL RA in F2 16h-1	16	20	3455.4492	69108.9834	217	217	104.9
0.2mg/mL RA in F2 16h-2	16	20	3530.0132	70600.2634	222	222	107.3
0.2mg/mL RA in F2 16h-3	16	20	3593.6182	71872.3640	226	226	109.3
					Mean	**222**	**107.2**
					%CV	**2.0**	
0.2mg/mL RA in F2 24h-1	24	20	3501.8636	70037.2730	220	220	106.4
0.2mg/mL RA in F2 24h-2	24	20	3326.1977	66523.9546	209	209	101.1
0.2mg/mL RA in F2 24h-3	24	20	3386.6870	67733.7395	213	213	103.0
					Mean	**214**	**103.5**
					%CV	**2.6**	

Table 10 Summary of Steviol LC/MS Assay Results from Steviolbioside Pooled Male-1 (M-1) and Male-2 (M-2) Fecal Homogenate Samples

Pooled Fecal Homogenate from Male #1

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% SB Conversion
0.2mg/mL SB in M1 0h-1	0	2	311.3411	622.6823	1.96	1.96	0.6
0.2mg/mL SB in M1 0h-2	0	2	311.0195	622.0391	1.95	1.95	0.6
0.2mg/mL SB in M1 0h-3	0	2	312.3059	624.6119	1.96	1.96	0.6
					Mean	**1.96**	**0.6**
					%CV	**0.3**	
0.2mg/mL SB in M1 8h-1	8	80	1000.6318	80050.5466	251	251	80.7
0.2mg/mL SB in M1 8h-2	8	80	979.1920	78335.3598	246	246	79.1
0.2mg/mL SB in M1 8h-3	8	80	995.8079	79664.6295	250	250	80.3
					Mean	**249**	**80.0**
					%CV	**1.1**	
0.2mg/mL SB in M1 16h-1	16	80	969.9729	77597.8294	244	244	78.4
0.2mg/mL SB in M1 16h-2	16	80	964.2913	77143.3049	242	242	77.8
0.2mg/mL SB in M1 16h-3	16	80	1050.3722	84029.7799	264	264	84.8
					Mean	**250**	**80.3**
					%CV	**4.9**	
0.2mg/mL SB in M1 24h-1	24	80	1020.9997	81679.9740	256	256	82.3
0.2mg/mL SB in M1 24h-2	24	80	984.0160	78721.2768	247	247	79.4
0.2mg/mL SB in M1 24h-3	24	80	993.3423	79467.3830	250	250	80.3
					Mean	**251**	**80.7**
					%CV	**1.8**	

Pooled Fecal Homogenate from Male #2

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% SB Conversion
0.2mg/mL SB in M2 0h-1	0	2	384.2366	768.4732	2.41	2.41	0.8
0.2mg/mL SB in M2 0h-2	0	2	380.6990	761.3980	2.39	2.39	0.8
0.2mg/mL SB in M2 0h-3	0	2	374.2671	748.5341	2.35	2.35	0.8
					Mean	**2.38**	**0.8**
					%CV	**1.3**	
0.2mg/mL SB in M2 8h-1	8	80	948.1042	75848.3389	238	238	76.5
0.2mg/mL SB in M2 8h-2	8	80	1038.7947	83103.5791	261	261	83.9
0.2mg/mL SB in M2 8h-3	8	80	986.5887	78927.0992	248	248	79.7
					Mean	**249**	**80.0**
					%CV	**4.6**	
0.2mg/mL SB in M2 16h-1	16	80	1008.1358	80650.8619	253	253	81.3
0.2mg/mL SB in M2 16h-2	16	80	1040.1883	83215.0662	261	261	83.9
0.2mg/mL SB in M2 16h-3	16	80	1003.9550	80316.4005	252	252	81.0
					Mean	**255**	**82.1**
					%CV	**1.9**	
0.2mg/mL SB in M2 24h-1	24	80	926.7716	74141.7280	233	233	74.9
0.2mg/mL SB in M2 24h-2	24	80	1011.3517	80908.1400	254	254	81.6
0.2mg/mL SB in M2 24h-3	24	80	1026.4668	82117.3466	258	258	82.9
					Mean	**248**	**79.8**
					%CV	**5.4**	

Table 11 Summary of Steviol LC/MS Assay Results from Steviolbioside Pooled Female-1 (F-1) and Female-2 (F-2) Fecal Homogenate Samples

Pooled Fecal Homogenate from Female #1

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% SB Conversion
0.2mg/mL SB in F1 0h-1	0	2	415.2171	830.4343	2.61	2.61	0.8
0.2mg/mL SB in F1 0h-2	0	2	414.2523	828.5047	2.60	2.60	0.8
0.2mg/mL SB in F1 0h-3	0	2	424.8651	849.7301	2.67	2.67	0.9
					Mean	**2.63**	**0.8**
					%CV	**1.4**	
0.2mg/mL SB in F1 8h-1	8	80	988.4111	79072.8901	248	248	79.7
0.2mg/mL SB in F1 8h-2	8	80	963.3265	77066.1215	242	242	77.8
0.2mg/mL SB in F1 8h-3	8	80	897.3990	71791.9221	225	225	72.3
					Mean	**238**	**76.6**
					%CV	**5.0**	
0.2mg/mL SB in F1 16h-1	16	80	963.6481	77091.8493	242	242	77.8
0.2mg/mL SB in F1 16h-2	16	80	956.0370	76482.9580	240	240	77.1
0.2mg/mL SB in F1 16h-3	16	80	990.9839	79278.7125	249	249	80.0
					Mean	**244**	**78.3**
					%CV	**1.9**	
0.2mg/mL SB in F1 24h-1	24	80	1017.6765	81414.1201	256	256	82.3
0.2mg/mL SB in F1 24h-2	24	80	1031.6124	82528.9915	259	259	83.2
0.2mg/mL SB in F1 24h-3	24	80	982.1936	78575.4859	247	247	79.4
					Mean	**254**	**81.6**
					%CV	**2.5**	

Pooled Fecal Homogenate from Female #2

BRI Sample ID	Timepoint (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% SB Conversion
0.2mg/mL SB in F2 0h-1	0	2	460.8840	921.7680	2.89	2.89	0.9
0.2mg/mL SB in F2 0h-2	0	2	531.0994	1062.1989	3.34	3.34	1.1
0.2mg/mL SB in F2 0h-3	0	2	664.5624	1329.1248	4.17	4.17	1.3
					Mean	**3.47**	**1.1**
					%CV	**18.7**	
0.2mg/mL SB in F2 8h-1	8	80	969.4369	77554.9498	244	244	78.4
0.2mg/mL SB in F2 8h-2	8	80	975.5472	78043.7780	245	245	78.7
0.2mg/mL SB in F2 8h-3	8	80	907.9045	72632.3636	228	228	73.3
					Mean	**239**	**76.8**
					%CV	**4.0**	
0.2mg/mL SB in F2 16h-1	16	80	947.2466	75779.7314	238	238	76.5
0.2mg/mL SB in F2 16h-2	16	80	1035.1500	82811.9973	260	260	83.6
0.2mg/mL SB in F2 16h-3	16	80	983.1584	78652.6693	247	247	79.4
					Mean	**248**	**79.8**
					%CV	**4.5**	
0.2mg/mL SB in F2 24h-1	24	80	938.9923	75119.3845	236	236	75.8
0.2mg/mL SB in F2 24h-2	24	80	963.0049	77040.3937	242	242	77.8
0.2mg/mL SB in F2 24h-3	24	80	966.9713	77357.7033	243	243	78.1
					Mean	**240**	**77.2**
					%CV	**1.6**	

Table 12 Summary of Steviol LC/MS Assay Results from Steviol Pooled Male-1 (M-1) and Male -2 (M-2) Stability Control Samples

Negative Control in HFH- Male #1

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Steviol Remaining
0.2mg/mL ST in M1 0h-1	0	80	2138.4243	171073.9466	537	537	
0.2mg/mL ST in M1 0h-2	0	80	2095.6198	167649.5880	526	526	
0.2mg/mL ST in M1 0h-3	0	80	2125.0677	170005.4121	534	534	
					Mean	**532**	**100.0**
					%CV	**1.1**	
0.2mg/mL ST in M1 8h-1	8	80	2070.0634	165605.0692	520	520	
0.2mg/mL ST in M1 8h-2	8	80	2092.2544	167380.3510	526	526	
0.2mg/mL ST in M1 8h-3	8	80	2144.8397	171587.1797	539	539	
					Mean	**528**	**99.2**
					%CV	**1.8**	
0.2mg/mL ST in M1 16h-1	16	80	1951.7463	156139.7046	490	490	
0.2mg/mL ST in M1 16h-2	16	80	2032.2019	162576.1525	511	511	
0.2mg/mL ST in M1 16h-3	16	80	2089.3096	167144.7685	525	525	
					Mean	**509**	**95.7**
					%CV	**3.5**	
0.2mg/mL ST in M1 24h-1	24	80	2060.9135	164873.0810	518	518	
0.2mg/mL ST in M1 24h-2	24	80	2062.0704	164965.6313	518	518	
0.2mg/mL ST in M1 24h-3	24	80	1986.9785	158958.2798	499	499	
					Mean	**512**	**96.2**
					%CV	**2.1**	

Negative Control in HFH- Male #2

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Steviol Remaining
0.2mg/mL ST in M2 0h-1	0	80	2280.9307	182474.4525	573	573	
0.2mg/mL ST in M2 0h-2	0	80	2270.9394	181675.1550	570	570	
0.2mg/mL ST in M2 0h-3	0	80	2337.1970	186975.7592	587	587	
					Mean	**577**	**100.0**
					%CV	**1.6**	
0.2mg/mL ST in M2 8h-1	8	80	2279.7738	182381.9022	573	573	
0.2mg/mL ST in M2 8h-2	8	80	2371.9033	189752.2662	596	596	
0.2mg/mL ST in M2 8h-3	8	80	2015.4798	161238.3810	506	506	
					Mean	**558**	**96.7**
					%CV	**8.4**	
0.2mg/mL ST in M2 16h-1	16	80	2346.4520	187716.1611	589	589	
0.2mg/mL ST in M2 16h-2	16	80	2267.8895	181431.1589	570	570	
0.2mg/mL ST in M2 16h-3	16	80	2281.0358	182482.8661	573	573	
					Mean	**577**	**100.0**
					%CV	**1.8**	
0.2mg/mL ST in M2 24h-1	24	80	1997.0749	159765.9909	502	502	
0.2mg/mL ST in M2 24h-2	24	80	2265.7861	181262.8858	569	569	
0.2mg/mL ST in M2 24h-3	24	80	2043.3500	163468.0002	513	513	
					Mean	**528**	**91.5**
					%CV	**6.8**	

Table 13 Summary of Steviol LC/MS Assay Results from Steviol Pooled Female-1 (F-1) and Female -2 (F-2) Stability Control Samples

Negative Control in HFH- Female #1

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Steviol Remaining
0.2mg/mL ST in F1 0h-1	0	80	1957.8462	156627.6967	492	492	
0.2mg/mL ST in F1 0h-2	0	80	1927.5570	154204.5634	484	484	
0.2mg/mL ST in F1 0h-3	0	80	2043.8759	163510.0685	513	513	
					Mean	**496**	**100.0**
					%CV	**3.0**	
0.2mg/mL ST in F1 8h-1	8	80	1997.4956	159799.6456	502	502	
0.2mg/mL ST in F1 8h-2	8	80	2038.5121	163080.9720	512	512	
0.2mg/mL ST in F1 8h-3	8	80	1974.1477	157931.8136	496	496	
					Mean	**503**	**101.4**
					%CV	**1.6**	
0.2mg/mL ST in F1 16h-1	16	80	2094.1475	167531.7968	526	526	
0.2mg/mL ST in F1 16h-2	16	80	2114.9713	169197.7010	531	531	
0.2mg/mL ST in F1 16h-3	16	80	2083.9459	166715.6720	524	524	
					Mean	**527**	**106.3**
					%CV	**0.7**	
0.2mg/mL ST in F1 24h-1	24	80	2034.2002	162736.0120	511	511	
0.2mg/mL ST in F1 24h-2	24	80	1874.6562	149972.4937	471	471	
0.2mg/mL ST in F1 24h-3	24	80	2114.3402	169147.2190	531	531	
					Mean	**504**	**101.6**
					%CV	**6.1**	

Negative Control in HFH- Female #2

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Steviol Remaining
0.2mg/mL ST in F2 0h-1	0	80	2058.4946	164679.5669	517	517	
0.2mg/mL ST in F2 0h-2	0	80	2157.1447	172571.5776	542	542	
0.2mg/mL ST in F2 0h-3	0	80	2252.3242	180185.9376	566	566	
					Mean	**542**	**100.0**
					%CV	**4.5**	
0.2mg/mL ST in F2 8h-1	8	80	2215.3041	177224.3302	557	557	
0.2mg/mL ST in F2 8h-2	8	80	2057.7584	164620.6713	517	517	
0.2mg/mL ST in F2 8h-3	8	80	2115.6023	169248.1829	531	531	
					Mean	**535**	**98.7**
					%CV	**3.8**	
0.2mg/mL ST in F2 16h-1	16	80	2232.9728	178637.8246	561	561	
0.2mg/mL ST in F2 16h-2	16	80	2089.6251	167170.0095	525	525	
0.2mg/mL ST in F2 16h-3	16	80	2144.6294	171570.3524	539	539	
					Mean	**542**	**100.0**
					%CV	**3.3**	
0.2mg/mL ST in F2 24h-1	24	80	2200.5802	176046.4181	553	553	
0.2mg/mL ST in F2 24h-2	24	80	2123.1746	169853.9663	533	533	
0.2mg/mL ST in F2 24h-3	24	80	2240.3348	179226.7807	563	563	
					Mean	**550**	**101.5**
					%CV	**2.8**	

Table 14 Summary of Steviol LC/MS Assay Results from Rebaudioside X and Rebaudioside A BHI Buffer Stability Control Samples

Rebaudioside A in BHI Data Summary

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc (ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb A Metabolized
0.2mg/mL RA in BHI 0h-1	0	2	10.2587	20.5174	0.0644	<LLOQ	
0.2mg/mL RA in BHI 0h-2	0	2	10.2268	20.4537	0.0642	<LLOQ	n/ap
0.2mg/mL RA in BHI 0h-3	0	2	10.5957	21.1914	0.0665	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RA in BHI 8h-1	8	2	10.2697	20.5394	0.0645	<LLOQ	
0.2mg/mL RA in BHI 8h-2	8	2	10.1841	20.3681	0.0640	<LLOQ	n/ap
0.2mg/mL RA in BHI 8h-3	8	2	10.1733	20.3467	0.0639	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RA in BHI 16h-1	16	2	15.9761	31.9521	0.100	<LLOQ	
0.2mg/mL RA in BHI 16h-2	16	2	15.5199	31.0398	0.0975	<LLOQ	n/ap
0.2mg/mL RA in BHI 16h-3	16	2	16.2789	32.5578	0.102	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RA in BHI 24h-1	24	2	12.2283	24.4566	0.0768	<LLOQ	
0.2mg/mL RA in BHI 24h-2	24	2	12.1720	24.3441	0.0764	<LLOQ	n/ap
0.2mg/mL RA in BHI 24h-3	24	2	12.2620	24.5241	0.0770	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	

Rebaudioside X in BHI Data Summary

BRI Sample ID	Time point (hr)	[1] Dilution Factor	Assayed Conc (ng/mL)	Dilution Corrected Conc(ng/mL)	Steviol formed in HFH (µM)	Reported Steviol Conc (µM)	% Reb X Metabolized
0.2mg/mL RX in BHI 0h-1	0	2	10.5056	21.0112	0.0660	<LLOQ	
0.2mg/mL RX in BHI 0h-2	0	2	10.5056	21.0112	0.0660	<LLOQ	n/ap
0.2mg/mL RX in BHI 0h-3	0	2	10.4046	20.8092	0.0653	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RX in BHI 8h-1	8	2	10.5056	21.0112	0.0660	<LLOQ	
0.2mg/mL RX in BHI 8h-2	8	2	10.4046	20.8092	0.0653	<LLOQ	n/ap
0.2mg/mL RX in BHI 8h-3	8	2	10.3036	20.6073	0.0647	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RX in BHI 16h-1	16	2	11.8184	23.6369	0.0742	<LLOQ	
0.2mg/mL RX in BHI 16h-2	16	2	12.3234	24.6467	0.0774	<LLOQ	n/ap
0.2mg/mL RX in BHI 16h-3	16	2	12.4244	24.8487	0.0780	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	
0.2mg/mL RX in BHI 24h-1	24	2	12.3234	24.6467	0.0774	<LLOQ	
0.2mg/mL RX in BHI 24h-2	24	2	12.6263	25.2526	0.0793	<LLOQ	n/ap
0.2mg/mL RX in BHI 24h-3	24	2	12.3234	24.6467	0.0774	<LLOQ	
					Mean	**n/ap**	**n/ap**
					%CV	**n/ap**	

Table 15 Summary of % Molar Equivalent of Steviol observed in the Metabolism of Rebaudioside X, Rebaudioside A, and Steviolbioside at 24 hours

Pooled Human Fecal Homogenate (n=3 donor per pooled sample)	**% Steviolbioside Metabolized at 24 hours (Note 1)**	Inter-Donor Mean %	Inter-Donor CV %	**% Rebaudioside A Metabolized at 24 hours (Note 1)**	Inter-Donor Mean %	Inter-Donor CV %	**% Rebaudioside X Metabolized at 24 hours (Note 1)**	Inter-Donor Mean %	Inter-Donor CV %
Pooled Male-1	80.7	79.8	2.4	97.0	99.1	3.0	115.1	109.6	3.4
Pooled Male-2	79.8			98.0			108.5		
Pooled Female-1	81.6			97.8			107.2		
Pooled Female-2	77.2			103.5			107.6		

Note 1: Not corrected for assay dose concentration.

Table 16 Summary of % Steviol Remaining Over the Incubation Time-Course of 24 Hours

Subjects	% Steviol Observed Following Incubation over 24 Hours			
	0 hr	**8 hr**	**16 hr**	**24 hr**
Male-1	100	99.2	95.7	96.2
Male-2	100	96.7	100.0	91.5
Female-1	100	101.4	106.3	101.6
Female-2	100	98.7	100.0	101.5
Mean	100	99.0	100.5	97.7

Table 17 Summary of % Steviol Observed Following Incubation of Rebaudioside A and Rebaudioside X in Brain-Heart Infusion Buffer for 24 hours

% Steviol Observed Following Incubation of Rebaudioside A in BHI Buffer				
Time-Points	**0 hr**	**8 hr**	**16 hr**	**24 hr**
Mean	**<LLOQ**	**<LLOQ**	**<LLOQ**	**<LLOQ**

% Steviol Observed Following Incubation of Rebaudioside X in BHI Buffer				
Time-Points	**0 hr**	**8 hr**	**16 hr**	**24 hr**
Mean	**<LLOQ**	**<LLOQ**	**<LLOQ**	**<LLOQ**

19 Figures

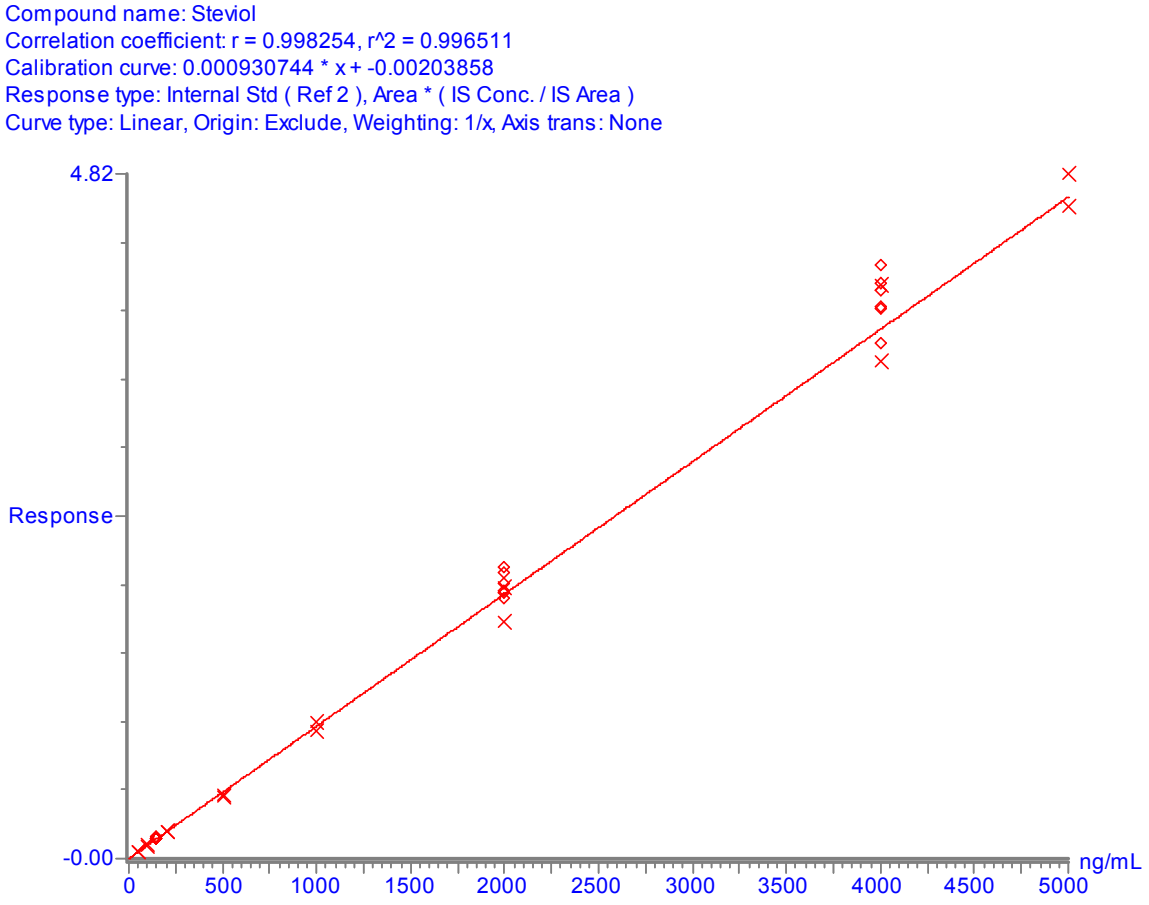

Figure 1 Representative LC/MS Calibration Curve for Quantitation of Steviol in HFH at *Ex Vivo* Spiked Concentrations of 50 ng/mL to 5000 ng/mL (Batch 05Oct2012 for Rebaudioside A)

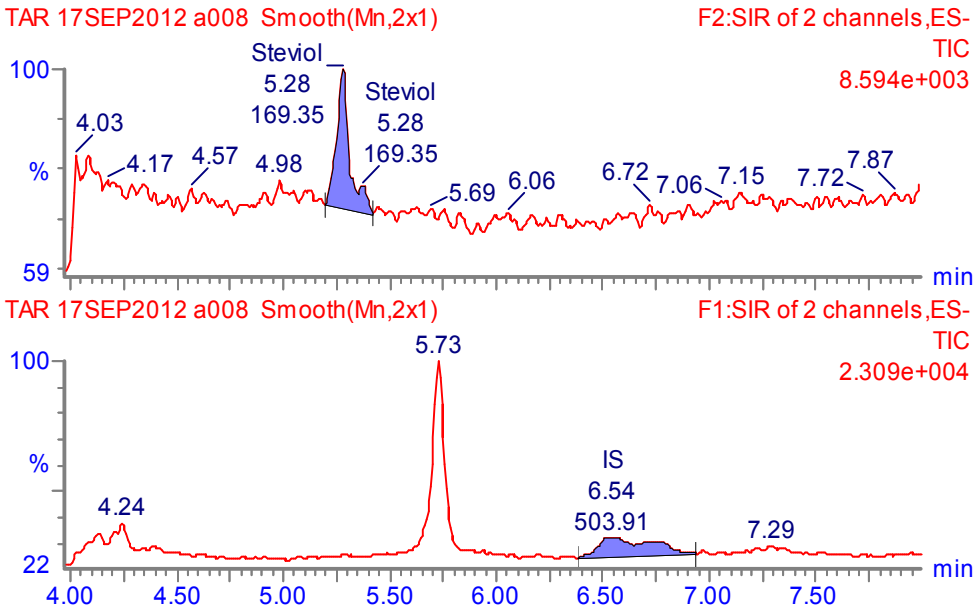

Figure 2 Representative LC/MS Chromatogram of a Blank Sample, Showing Steviol (Top) and Abietic Acid (Bottom) as an Internal Standard (Batch 17SEP2012)

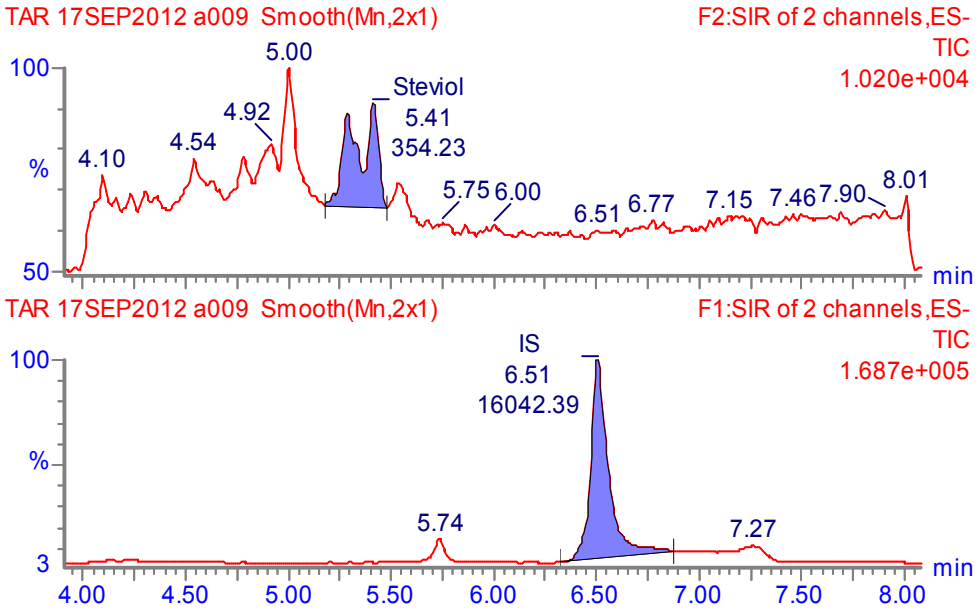

Figure 3 Representative LC/MS Chromatogram of a Blank Sample, Showing Steviol (Top) and Abietic Acid (Bottom) as an Internal Standard (Batch 17SEP2012)

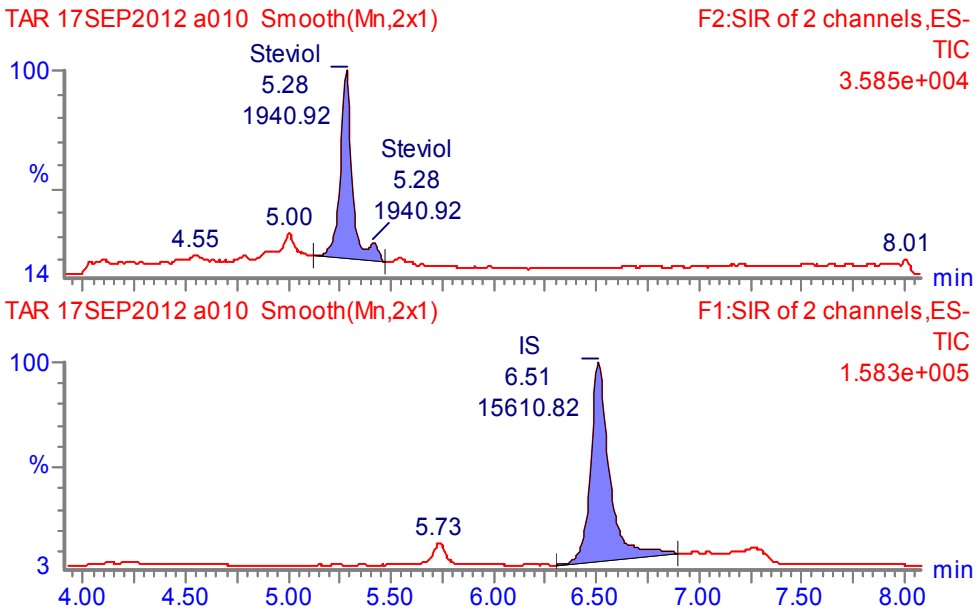

Figure 4 Representative LC/MS Chromatogram of Steviol at Assay LLOQ of 50 ng/mL, Showing Steviol (Top) and Abietic Acid (Bottom) as an Internal Standard (Batch 17SEP2012)

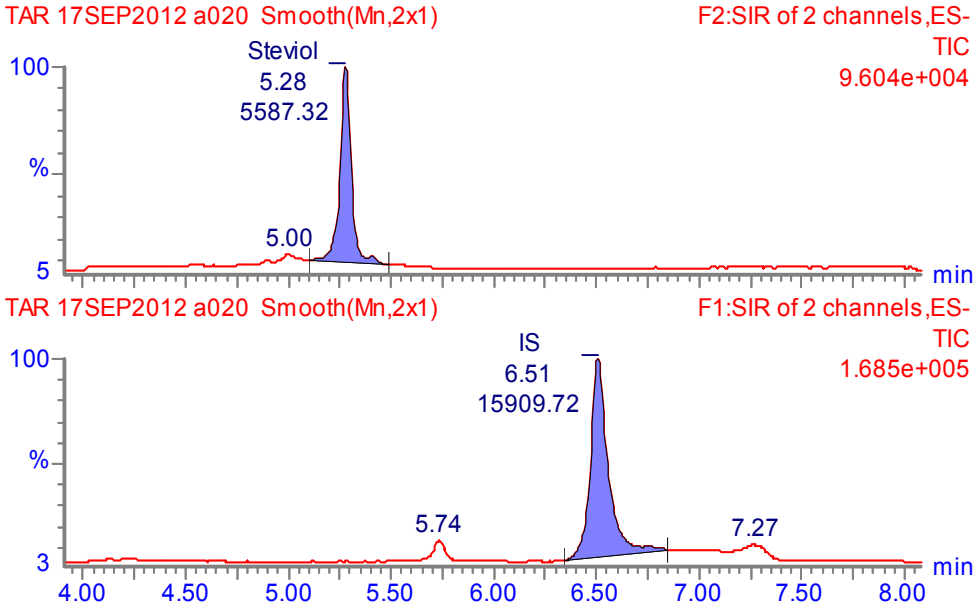

Figure 5 Representative LC/MS Chromatogram of Steviol at Assay Low QC Sample of 150 ng/mL, Showing Steviol (Top) and Abietic Acid (Bottom) as an Internal Standard (Batch 17SEP2012)

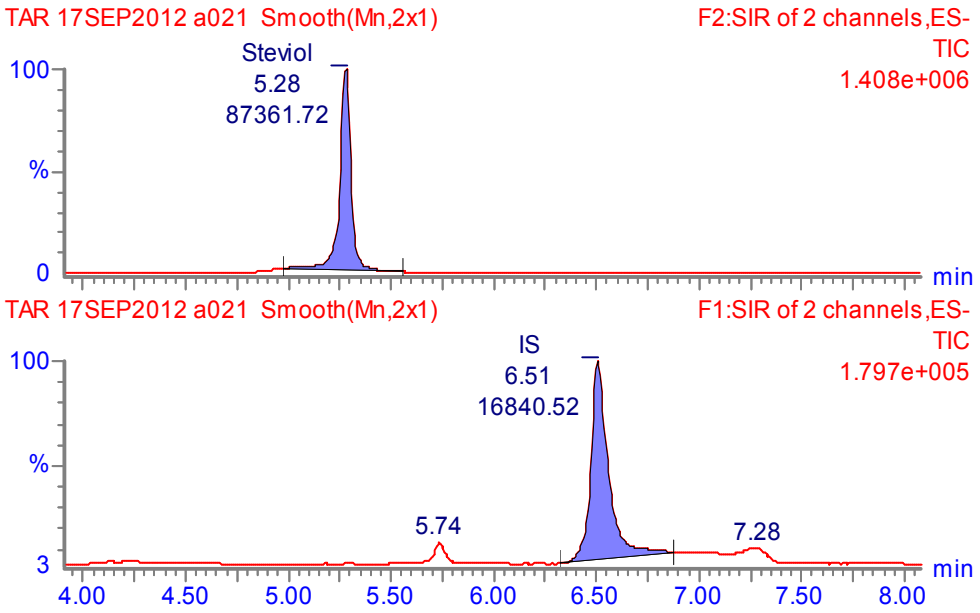

Figure 6 Representative LC/MS Chromatogram of Steviol at Assay Mid QC Sample of 2000 ng/mL, Showing Steviol (Top) and Abietic Acid (Bottom) as an Internal Standard (Batch 17SEP2012)

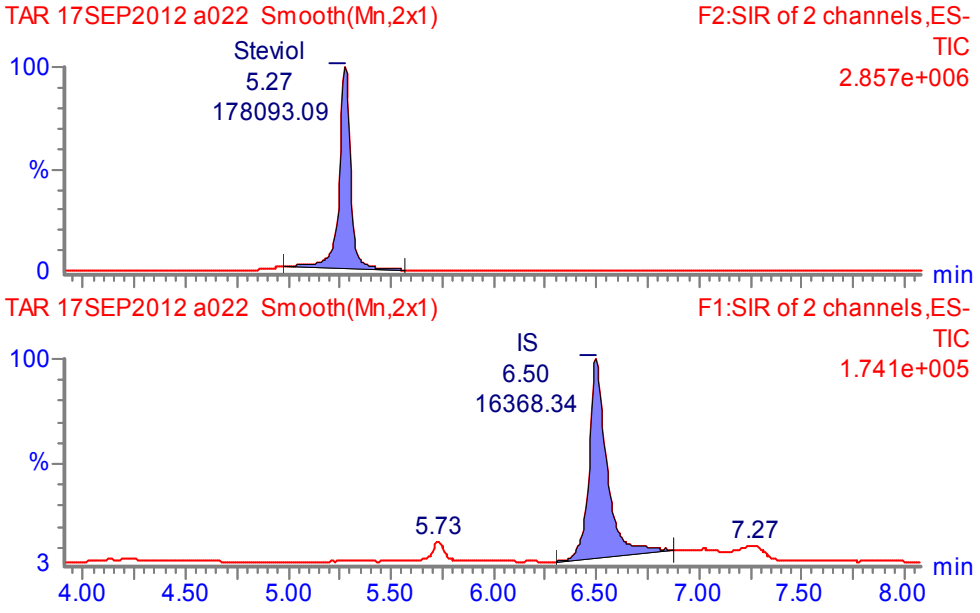

Figure 7 Representative LC/MS Chromatogram of Steviol at Assay High QC Sample of 4000 ng/mL, Showing Steviol (Top) and Abietic Acid (Bottom) as an Internal Standard (Batch 17SEP2012)

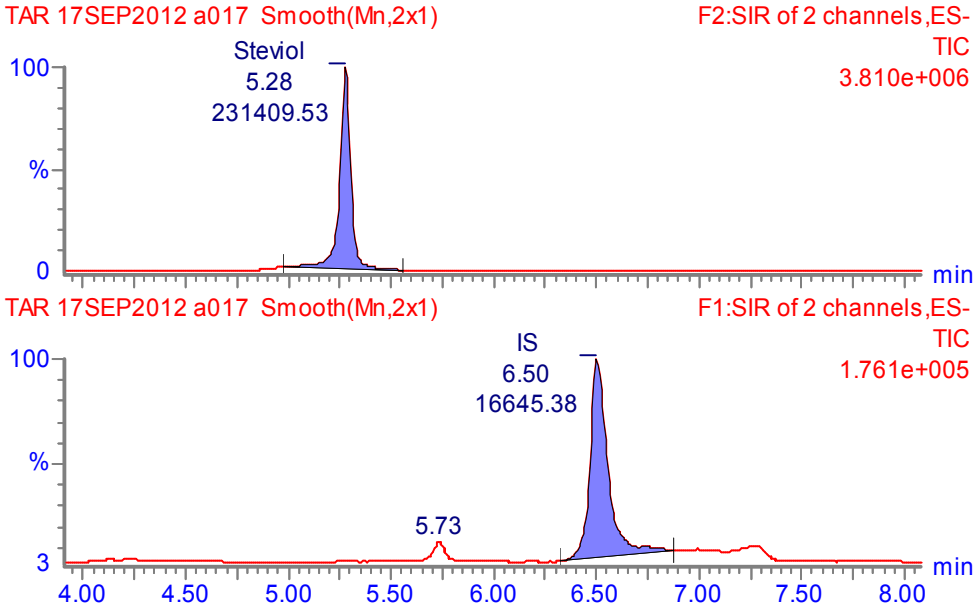

Figure 8 Representative LC/MS Chromatogram of Steviol at Assay ULOQ Sample of 5000 ng/mL, Showing Steviol (Top) and Abietic Acid (Bottom) as an Internal Standard (Batch 17SEP2012)

20 Appendices

Appendix 1 Certificate of Analysis of Rebaudioside X

RFS-1294

Reviewed by: XT Date: 07/9/12
Comments: Okay, FW is calculate to be 1291.29 based on the chemical structure in Protocol-TAR-2012-001. XT 07/9/12

PureCircle

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Negeri Sembilan, Malaysia
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E-mail: office@purecircle.com

CERTIFICATE OF ANALYSIS

NAME OF PRODUCT:	Rebaudioside X
BATCH #:	PT140312
MANUFACTURING DATE:	14th MARCH 2012
EXPIRY DATE:	2 Years from Manufacturing Date
MANUFACTURING CONDITIONS:	GMP (ISO 22,000:2005 / 22,002-1:2009)
STORAGE CONDITIONS:	STORE REFRIGERATED AT 4°C

PARAMETER	METHODS	SPECIFICATION	RESULT
Appearance	Sensory Evaluation	White to off-white powder	Approved
Sweetness, fold sweetness to sucrose	" "	>200	Approved
Rebaudioside X, % (wt/wt) on anhydrous basis	JECFA, 2010*	≥95.0	97.33
Other Steviol Glycosides, % (wt/wt)	" "	≤5.0	3.34
Loss on Drying, % (wt/wt)	JECFA Vol.4	≤6.0	5.56
pH, 1% in water	" "	4.5 - 7.0	5.11
Residual Ethanol, % (wt/wt)	USP<467>	<0.30	0.013
Residual Methanol, % (wt/wt)	" "	<0.02	0.002
Ash, % (wt/wt)	JECFA Vol.4	<1.0	0.01
Lead (as Pb), ppm	AOAC 993.14	<1.0	<0.005
Arsenic (as As), ppm	" "	<1.0	<0.005
Cadmium (as Cd), ppm	" "	<1.0	<0.005
Total Plate Count, CFU/g	Chapter 3, FDA	<1000	10
Yeast and Mold, CFU/g	Chapter 18, FDA	<200	Not Detected
Total Coliforms, MPN/g	Chapter 4, FDA	Not Detected	Not Detected
E.coli count, MPN/g	" "	Not Detected	Not Detected

* "Steviol Glycosides" specification of FAO JECFA Monographs 10 (2010)

WARNING! THIS MATERIAL IS FOR R&D USE ONLY. NOT FOR DRUG, HOUSEHOLD AND OTHER USES

(b) (6)

Prepared by : _____
Noorwaeda Muslan
Quality Assurance Department
Date: 6th July 2012

(b) (6)

Reviewed by : _____
Alina Dmitrieva
Quality Assurance Department
Date: 6th July 2012

Appendix 2 Certificate of Analysis of Rebaudioside A

PureCircle

(RFS 1217)

Reviewed by: XT Date: 07/03/12
Comments: ok, FW=967.01
XT 07/03/12

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E-mail: office@purecircle.com

CERTIFICATE OF ANALYSIS

NAME OF PRODUCT: **Rebaudioside A**
BATCH #: **DR0311329**
MANUFACTURING DATE: **8th MARCH 2011**
EXPIRY DATE: **2 Years from Manufacturing Date**
MANUFACTURING CONDITION **GMP (ISO 22,000:2005 / 22,002-1:2009)**
STORAGE CONDITIONS: **STORE REFRIGERATED AT 4°C**

PARAMETER	METHODS	SPECIFICATION	RESULT
Appearance	Sensory Evaluation	White to off white powder	Approved
Rebaudioside A, % (wt/wt) on anhydrous basis	JECFA, 2010*	>97.0	99.30
Other Steviol Glycosides, % (wt/wt)	" "	<3.0	0.97
Residual Ethanol, % (wt/wt)	USP <467>	<0.30	0.075
Residual Methanol, % (wt/wt)	USP <467>	<0.02	Not Detected
Ash, % (wt/wt)	AOAC 945.46	<0.2	<0.1
Loss on drying, % (wt/wt)	JECFA vol.4	<5.0	0.29
pH, (1% solution)	JECFA vol.4	4.5 to 7.0	5.6
Lead (as Pb), ppm	AOAC 993.14	<1.0	<0.005
Cadmium (as Cd), ppm	AOAC 993.14	<0.1	<0.005
Arsenic (as As), ppm	AOAC 993.14	<0.1	<0.005
Total Coliforms, MPN/g	Chapter 4, FDA	<3	Not Detected
Escherichia coli count, CFU/g	AOAC 991.14	<10	Not Detected
Standard Plate Count, CFU/g	AOAC 990.12	<1000	Not Detected
Yeast and Mold, CFU/g	AOAC 997.02	<100	Not Detected

* "Steviol Glycosides" specification of FAO JECFA Monographs 10 (2010)

WARNING! THIS MATERIAL IS FOR R&D USE ONLY. NOT FOR DRUG, HOUSEHOLD AND OTHER USES

(b) (6)

Prepared by:
Noorwaeda Muslan
Quality Assurance Department
Date: 17th May 2012

(b) (6)

Reviewed by:
Choung Chia Mei
Quality Assurance Department
Date: 17th May 2012

Appendix 3 Certificate of Analysis of Steviolbioside

RFS-1295

Reviewed by: XT Date: 07/13/12
Comments: okay, FW = 642.734
XT 07/13/12

PureCircle

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CERTIFICATE OF ANALYSIS

NAME OF PRODUCT: Steviolbioside
BATCH #: PT081111
MANUFACTURING DATE: 8th NOVEMBER 2011
EXPIRY DATE: 2 Years from Manufacturing Date
MANUFACTURING CONDITIONS: GMP (ISO 22,000:2005 / 22,002-1:2009)
STORAGE CONDITIONS: STORE REFRIGERATED AT 4°C

PARAMETER	METHODS	SPECIFICATION	RESULT
Appearance	Sensory Evaluation	White to off-white powder	Approved
Sweetness, fold sweetness to sucrose	" "	>200	Approved
Steviolbioside, % (wt/wt) on anhydrous basis	JECFA, 2010*	≥95.0	96.81
Other Steviol Glycosides, % (wt/wt)	" "	≤5.0	2.33
Loss on Drying, % (wt/wt)	JECFA Vol.4	≤6.0	5.28
pH, 1% in water	" "	4.5 - 7.0	6.38
Residual Ethanol, % (wt/wt)	USP<467>	<0.30	0.013
Residual Methanol, % (wt/wt)	" "	<0.02	0.001
Ash, % (wt/wt)	JECFA Vol.4	<1.0	<0.01
Lead (as Pb), ppm	AOAC 993.14	<1.0	<0.005
Arsenic (as As), ppm	" "	<1.0	<0.005
Cadmium (as Cd), ppm	" "	<1.0	<0.005
Total Plate Count, CFU/g	Chapter 3, FDA	<1000	Not Detected
Yeast and Mold, CFU/g	Chapter 18, FDA	<200	Not Detected
Total Coliforms, MPN/g	Chapter 4, FDA	Not Detected	Not Detected
E.coli count, MPN/g	" "	Not Detected	Not Detected

* "Steviol Glycosides" specification of FAO JECFA Monographs 10 (2010)

WARNING! THIS MATERIAL IS FOR R&D USE ONLY, NOT FOR DRUG, HOUSEHOLD AND OTHER USES

(b) (6)

Prepared by : _____
Noorwaeda Muslan
Quality Assurance Department
Date: 6th July 2012

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Reviewed by : _____
Alina Dmitrieva
Quality Assurance Department
Date: 6th July 2012

Appendix 4 Certificate of Analysis of Steviol

PureCircle

RFS 1296

Reviewed by: XT Date: 07/03/12
Comments: okay, use Adjusted Purity, FW = 318.45
XT 07/03/12

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CERTIFICATE OF ANALYSIS

NAME OF PRODUCT: Steviol
BATCH #: PT270312
MANUFACTURING DATE: 27th MARCH 2012
EXPIRY DATE: 2 Years from Manufacturing Date
MANUFACTURING CONDITIONS: GMP (ISO 22,000:2005 / 22,002-1:2009)
STORAGE CONDITIONS: STORE REFRIGERATED AT 4°C

PARAMETER	METHODS	SPECIFICATION	RESULT
Appearance	Sensory Evaluation	White to off-white powder	Approved
Chromatographic Purity, %	JECFA, 2010	≥95.0	99.18
Adjusted Purity, %	Adjusted for water and solvent	≥90.0	97.37
Water Content, %	JECFA Vol.4	≤6.0	1.79
pH, 1% in water	" "	4.5 - 7.0	5.29
Residual Ethanol, %	USP<467>	<0.30	0.022
Residual Methanol, %	" "	<0.02	0.001
Ash, %	JECFA Vol.4	<1.0	0.004
Lead (as Pb), ppm	AOAC 993.14	<1.0	<0.005
Arsenic (as As), ppm	" "	<1.0	<0.005
Cadmium (as Cd), ppm	" "	<1.0	<0.005
Total Plate Count, CFU/g	Chapter 3, FDA	<1000	Not Detected
Yeast and Mold, CFU/g	Chapter 18, FDA	<200	Not Detected
Total Coliforms, MPN/g	Chapter 4, FDA	Not Detected	Not Detected
E.coli count, MPN/g	" "	Not Detected	Not Detected

WARNING! THIS MATERIAL IS FOR R&D USE ONLY. NOT FOR DRUG, HOUSEHOLD AND OTHER USES.

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Prepared by : _____
Noorwaeda Musian
Quality Assurance Department
Date: 17th May 2012

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Reviewed by : _____
Choung Chia Mei
Quality Assurance Department
Date: 17th May 2012

Appendix 5 BR Study Protocol-TAR-2012-001

BRI Biopharmaceutical Research Inc.

Study Protocol

Study Title:	***In vitro* metabolism of Rebaudioside X in Pooled Human Intestinal Fecal Homogenate under Physiological Anaerobic Conditions**
BRI Study No:	**TAR-2012-001**
Sponsor Reference No:	**Not available**
BRI Study Protocol No:	**Protocol-TAR-2012-001**

Study Protocol Approval:

(b) (6)

Xiaowei Teng, Ph.D.
Study Director
BRI Biopharmaceutical Research Inc.

06/19/12
Date

(b) (6)

Clara Faan, B.Sc.
VP Business Development
BRI Biopharmaceutical Research Inc.

06/19/2012
Date

(b) (6)

Stanley M. Tarka, Jr., Ph.D.
Sponsor Representative
The Tarka Group, Inc.

18 June, 2012
Date

Study Protocol

Study Protocol No: Protocol-TAR-2012-001

Analytical Facility:
BRI Biopharmaceutical Research Inc.
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Vancouver, BC, V6P 3S8
Phone: +1 (604) 432-9237
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Participating Personnel

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Study Sponsor:
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Sponsor Representative:
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1. INTRODUCTION

In vitro studies have confirmed that steviol glycosides including stevioside and rebaudioside A are metabolized by the gut microflora to the aglycone steviol (figure 1) prior to absorption. Steviol (Figure 1) is the glycoside terpene aglycone of the principle and secondary steviol glycoside sweetener components of stevia extract, derived from *Stevia rebaudiana [ref 1]*.

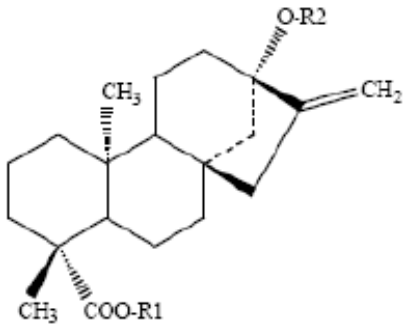

Figure 1. Steviol

Steviol glycosides are being investigated in this study, where R1 and R2 are detailed in Table 1 below.

Test Article Compound Name	R1	R2
Steviol	H	H
Rebaudioside A (CAS 58543-16-1)	ß-Glc-	ß-Glc- ß-Glc (2→1)- ß-Glc (3→1)
Rebaudioside X	β-Glc-β-Glc- β-Glc	β-Glc-β-Glc- β-Glc
Steviolbioside	H	β-Glc- β-Glc

In recent published articles [ref 2 and 3], *in vitro* metabolism of selected steviol glycosides has provided kinetic information on the time-course of anaerobic metabolic degradation of steviol glycosides by intestinal bacterial flora. In contrast, aerobic metabolism of steviol glycosides by intestinal bacterial floral has led to the observation of steviol 16,17α-epoxide, a potentially harmful metabolite [ref 2]. Hence, *in vitro* metabolism of steviol glycosides should be investigated under strict anaerobic conditions.

Published *in vitro* metabolism data indicated that steviol glycosides were initially hydrolyzed by intestinal bacterial flora to steviolbioside as an intermediate followed by subsequent rapidmetabolism to steviol as a final end metabolite which was stable in human fecal homogenate for at least 72 hours [ref 2].

In this current study, *in vitro* anaerobic metabolism of Rebaudioside X by human intestinal bacterial flora will be investigated based on a published literature procedure [ref 3] involving the use of human fecal homogenate under anaerobic conditions. The metabolism of Rebaudioside A will be evaluated in parallel as a metabolic activity positive control. Since this proposed study is designed to assess the formation of steviol from hydrolysis of rebaudioside A and X; steviol and steviolbioside, a hydrolysis intermediate, will also be evaluated in parallel to serve as metabolic stability controls.

2. STUDY OBJECTIVES

This objective of this study is to investigate the *in vitro* metabolism and hydrolysis of Rebaudioside X in a pooled human fecal homogenate preparation at 37°C under anaerobic conditions to provide metabolism kinetic time-course data.

3. ABBREVIATIONS

COA certificate of analysis
DMSO dimethylsulfoxide
Hrs hours
LLOQ lower limit of quantitation
ULOQ upper limit of quantitation
LQC low quality control
Min minutes
MQC mid quality control
n/ap not applicable
HQC high quality control
RT room temperatures
RF refrigeration
SOP standard operating procedures
OD Optical Density
CV coefficient of variation
NMT not more than

4. STUDY MATERIALS AND TEST ARTICLE

The following test articles and their Certificates of Analysis are required from the Study Sponsor. Chemical characterization information from the Certificate of Analysis including manufacture lot number, identification, purity and storage conditions for each of the Controls and the Test Article will be observed upon receipt of the materials and during the course of this study:

Identification of Test Articles	Quantity	Storage
Steviol	500 mg	Room temperature
Rebaudioside A (CAS 58543-16-1)	2g	Room temperature
Rebaudioside X (CAS 1220616-44-3)	2g	Room temperature
Steviolbioside	2g	Room temperature

The following human fecal study materials are required:

Study Materials	Source	Storage
Pooled human fecal homogenate (n=6 male and 6 female subjects, note 1)	BRI volunteers or commercial vendor	Refrigerated

Note 1: Human fecal homogenate will be obtained from six healthy male and six healthy female volunteers of Caucasian and Asian ethnicities without known gastrointestinal disease and without previous ingestion of stevia-based natural sweeteners, laxatives and antimicrobial drugs at least 7 days prior to the morning of fecal sample collection.

For anaerobic metabolism incubation, the fecal homogenates will be pooled from 3 subjects of each sex to yield a total of 4 test samples (2 males and 2 females). All critical reagents and materials used for the study will be documented in the Study Report.

5. HANDLING AND SAFETY PROCEDURES

The precautions necessary for safe handling of the analytical reference standard are based on information available from public literature or provided by the Study Sponsor. The Test Articles being evaluated in this study are considered safe, however, all study materials and analytical reference standards and all of the related stock solutions must be handled following universal safety precautions. All personnel involved in this study will wear safety protective equipment appropriate for this type of study and for the function(s) performed. Refer to the BRI Safety and Policy Manual.

All personnel must review the relevant and available Material Safety Data Sheets for chemicals required for this study prior to any experimentation.

6. EQUIPMENTS, ANALYTICAL INSTRUMENTS, AND COMPUTER SYSTEMS

All major computer systems and electronic equipment operating in support of this study are listed below:

Equipment/Computer System	Name	Model / Versions	Compliance Status
Automatic Water-Jacketed Over/Under CO2 Incubators (Thermal Conductivity) or equivalent	VWR CO2 Incubator	2350	Qualified
Anaerobic Chamber with 99.9% high purity nitrogen and O2 Sensor/Monitor	oxygen monitor	Drager Pac III	n/ap
MS Excel (for data analysis)	Microsoft Excel	2007	Validation in progress [1]
LIMS (Laboratory Integrated Management System) (for sample logistics and data handling and processing)	Thermo Watson LIMS	7.4.0.0	Validated for bioanalytical data handling required for this study
HPLC System	Agilent HPLC System	Model	Qualified

		1100	
Mass Spectrometer	Micromass triple quadrupole tandem mass spectrometer	Quattro®-LC	Qualified
LC/MS/MS Operation Software	Waters	Masslynx ver 4.0	Qualified

[1]A second study team individual will 100% verify all Excel calculations by manually reviewing all calculation formulas.

7. EXPERIMENTAL

The rationale, experimental design and procedures used in this study for evaluating the metabolic stability of rebaudioside in freshly collected human fecal homogenate have been previously described in published literature [1, 2, and 3].

7.1 Preparation and Storage of Test Article Stock Solutions

The controls, Steviol, Steviolbioside and Rebaudioside A, as well as the Test Article, Rebaudioside X, will each be dissolved in a vehicle consisting of 0.2M potassium phosphate buffer (pH 7.0) containing 10% DMSO to provide a stock solution at a nominal concentration of 200 mg/mL to provide a 100X spiking stock solution. An aliquot of 10 µL of this 200 mg/mL spiking stock solution will be used to spike into 1 mL anaerobic human fecal homogenate to provide a final test concentration at 2 mg/mL.

Similarly for each control and the Test Article, a stock solution at a nominal concentration of 20 mg/mL will be prepared to provide a 100X spiking stock solution. An aliquot of 10 µL of this spiking stock solution will be used to spike into 1 mL anaerobic human fecal homogenate to provide a final test concentration at 0.2 mg/mL.

Stock solutions will be prepared and stored at room temperature until use for spiking to human fecal homogenate within 24 hours.

7.2 Assay Verification of Stock Solution Concentrations

The labelled concentrations of each of the controls and Test Article dose solutions will be verified by the same LC/MS assay qualified for steviol in support of this study. The assay performance for steviolbioside, rebaudioside A and rebaudioside X concentrations in the stock solutions will be demonstrated by evaluation of stock solution reference standards and QC samples during the quantitation assay batch. Data acceptance criteria for stock solution assay will be identical to those as described in Section 8 (Data Evaluation and Statistical Analysis).

7.3 *In Vitro* Metabolism of Rebaudioside X in a Pooled Human Fecal Homogenate Preparation at 37°C Under Anaerobic Conditions

7.3.1 Anaerobic Potassium Phosphate Buffer (pH 7.0)

Based on the following ratio of a 1.0 M potassium phosphate buffers, a 0.2 M potassium phosphate buffer at pH 7.0 is prepared and stored screw-capped and refrigerated for use within one week.

pH (Verified by pH meter)	1 M Potassium Dibasic Phosphate (K_2HPO_4), 87.1g per 500 mL water	1 M Potassium Monobasic Phosphate (KH_2PO_4), 68.1g per 500 mL water	De-ionized HPLC water (sterile filtered by 0.2 µm)
7.0	61.5 mL	38.5 mL	400 mL

7.3.2 Human Fecal Homogenate

The preparation of human fecal homogenate will follow in principle the procedure as previously described [2]. Upon voiding followed by immediate collection of fecal material in a pre-labeled 30 mL screw-capped polypropylene specimen container from each subject with the use of the spoon attachment on the screw-cap, a pre-aliquoted 10-mL anaerobic buffer will be immediately added to the fecal material to maintain an anaerobic sample environment. An initial stool-to-buffer ratio of the collected sample will be determined based on the tare-weight of the screw-capped stool sample container with the initial volume of anaerobic buffer used. A final fecal homogenate with a stool-to-buffer ratio of 1:1 (stool/buffer) will be achieved by adding the balance of an anaerobic buffer volume required.

Following preparation of the fecal homogenate, the fecal homogenate materials from each of twelve subjects will be transferred to an anaerobic nitrogen anaerobic chamber via an air lock. Commercially available 99.9% high purity dry nitrogen (Airgas) connected on-line to the anaerobic chamber will be used to flush the chamber and air lock continuously for 60 minutes following the transfer of samples and test materials inside the anaerobic chamber for handling of sample materials for each experiment. This procedure allows a commercial Certification of Analysis of the high purity nitrogen to support anaerobic conditions used for this study. Atmospheric oxygen level inside the chamber is being monitored using an oxygen monitor at a detection limit of 1% as a fail-safe readout to alert of air leakage throughout the fecal homogenate handling procedures.

Within the anaerobic chamber, the 1:1 (stool/buffer) fecal materials will be pooled by equal volume based on 3 subjects per sex and then homogenized with 25 volumes (%v/v) of 0.2M potassium phosphate buffer (pH 7.0) by vigorous vortex-mixing in a clean 10 mL screw-capped glass test tube to provide a 50-fold diluted pooled human fecal homogenate for incubation. The screw-capped homogenate centrifuge tubes will be removed from the chamber and centrifuged at 500 *xg* for 1 min at 37°C and returned to the anaerobic chamber for transfer of the fecal homogenate supernatant to a clean 10 mL screw-capped glass tube for incubation at 37°C. A total of 2 male and 2 female pooled human fecal homogenate supernatant samples will be similarly prepared from n=3 subjects for each pool fecal sample.

All dilutions and sample transfer steps will be carried out using 0.2M potassium phosphate buffer (pH 7.0) previously sparged with high purity nitrogen for 60 minutes in an anaerobic chamber to provide an anaerobic buffer. Metabolic incubations will be carried out in triplicates a sealed glass screw-capped test tube incubated at 37°C to maintain an anaerobic condition during incubation.

The controls, Steviol, Steviolbioside and Rebaudioside A, as well as the Test Article, Rebaudioside X, will each be incubated independently at test concentrations of 0.2 mg/mL and 2 mg/mL under an anaerobic environment for 0, 8, 24, and 48 hours at 37°C±5°C in a calibrated heating block. At the end of each incubation time point, an equal volume of 1 mL acetonitrile will be added to the fecal homogenate sample to stop the enzyme reaction with gentle vortex mixing. The samples will be centrifuged at 12,000 *xg* for 10 minutes to remove protein components. Triplicate samples (n=3) will be aliquoted from each incubation time point and then stored at nominal –80°C (-72°C to -88°C) until LC/MS assay.

The experimental design is summarized below:

Sample Type	Test materials	Concentrations	Incubation Time Points
Assay Stability Control	Steviol	0.2 mg/mL and 2 mg/mL of the test and control samples	37°C for 0, 8, 24 and 48 hours (n=3) replicates
Hydrolysis intermediate	Steviolbioside		
Metabolism Positive Control	Rebaudioside A (CAS 58543-16-1)		
Test Article	Rebaudioside X		

7.4 LC/MS Assay for Quantitation of Steviol in Human Fecal Homogenate Samples

LC/MS conditions for suitable chromatography of steviol as the metabolite end product will be verified to confirm necessary chromatographic conditions for resolution of steviol from any sample matrix components. A reversed-phase HPLC column operating with acetonitrile and water gradient mobile-phase conditions containing 2 mM ammonium acetate or ammonium formate will be evaluated with reference to assay batch acceptance criteria and general method qualification procedures as specified in BRI SOP and in accordance with FDA Bioanalytical Method Validation Guidelines ("Guidance for Industry: Bioanalytical Method Validation" U.S. Department of Health and Human Services, FDA, CDER and CVM, May 2001).

Quantification of steviol in the test samples will be based on an internal standard approach with abietic acid added to the samples as the internal standard.

Due to the investigative nature of this study, LC/MS quantitation of steviol in the test samples will not be required to undergo a full validation of the assay. The assay quantitation performance will be verified based on evaluating assay precision, accuracy, quantitation range, lower limit of quantitation, specificity, recovery, and dilution integrity. Method verification parameters and data acceptance criteria are summarized in the table below.

Table 1: LC/MS Assay Assessment Parameters for Quantitation of Steviol in Human Fecal Homogenate Metabolism Test Samples

Assay Parameters	Assay & Data acceptance Criteria
Calibration & QC samples	All calibration standards and QC samples will be independently prepared *ex-vivo* by spiking analyte stock solutions to a blank human fecal homogenate matrix.
Quantitation Range (Calibration Standards)	Matrix calibration standards will be assayed over 8-levels (plus blank control) in duplicate (n=2 sets of calibration samples) over a concentration range from 50 ng/mL to 5000 ng/mL. A single assay batch will be evaluated for calibration standard precision at each concentration.

Assay Parameters	Assay & Data acceptance Criteria
Calibration Regression Model Selection	Regression analysis of calibration standard data against peak area response ratio to their corresponding internal standards will be evaluated. The coefficient of determination (r^2) of the regression line will be calculated and should be equal to or greater than 0.99. Weighting of the calibration data will be performed to determine the best fitted regression function and the regression line will be used to calculate back-interpolated observed concentrations. The observed concentrations should be within a ±15% bias and 15% CV at each concentration of the regression line, except within ±20% bias and 20% CV at the LLOQ.
Assay Accuracy	Assay accuracy will be evaluated based on a single assay batch of QC samples prepared from a common set of stock solutions used for calibration standards. Within-batch accuracy will be evaluated based on observed QC at concentrations from samples prepared in six replicates (n=6) at the LLOQ of 50 ng/mL and at the corresponding low, mid and high QC samples at 150, 2000 and 4000 ng/mL, respectively. The observed concentrations should be within bias of ±15% of expected concentrations, except within ±20% at the LLOQ
Assay Precision	Assay precision will be evaluated based on a single assay batch of QC samples prepared from a common set of stock solutions used for calibration standards. Within-batch precision will be evaluated based on observed QC concentrations from samples prepared in six replicates (n=6) at the LLOQ of 50 ng/mL and at the corresponding low, mid and high QC samples at 150, 2000 and 4000 ng/mL, respectively. The observed concentrations should be within a precision of 15% CV, except within 20% CV at the LLOQ.
Assay Recovery	Assay recovery of the test article from sample matrix will be evaluated based on peak area response ratio in QC samples (extracted samples) prepared in replicates of six (n=6) at the corresponding low and high QC samples at 150 and 4000 ng/mL, respectively, compared against peak area response ratio from recovery control samples (non-extracted samples) prepared in replicates of six (n=6) at comparable concentrations by spiking into extracted blank sample matrix. Verification of recovery for abietic acid as the internal standard at the assay nominal concentration will also be performed. Assay recovery for each test article and the internal standard should be sufficient over the QC concentrations to provide a reproducible assay with %CV of NMT 15% for replicates at each concentration level.
Lower Limit of Quantitation (LLOQ)	The assay lower limit of quantitation (LLOQ) at 50 ng/mL (or lower if attainable) will be determined based on six replicates (n=6) from a single assay. The observed values are expected within ±20% bias and 15% CV (see assay accuracy and precision), and additionally, with a signal-to-noise ratio greater than 5:1.

8. DATA EVALUATION AND STATISTICAL ANALYSIS

A description of all statistical methods to be employed and the calculations to be performed on the data to evaluate the performance of the assay data are summarized in **Appendix 1**. Additional methods may be used and will be specified in the study report.

Steviol concentrations in the following samples will be used to evaluate the metabolic hydrolysis stability of the controls and the Test Article during incubation in human fecal homogenate over 0, 8, 24 and 48 hours.

Sample Type	Test materials	Concentrations	Incubation Time Points	Data Acceptance
Assay Stability Control	Steviol	0.2 mg/mL and 2 mg/mL of the test and control samples	37°C for 0, 8, 24 and 48 hours (triplicate)	Steviol concentrations within ±15% of original spiked concentration
Hydrolysis intermediate	Steviolbioside			Steviol concentrations equivalent to 50±10% of steviolbioside hydrolysis
Metabolism Positive Control	Rebaudioside A (CAS 58543-16-1)			Steviol concentrations equivalent to 50±10% of rebaudioside A hydrolysis
Test Article	Rebaudioside X			Steviol concentrations equivalent to 50±10% of rebaudioside X hydrolysis

9. STUDY REPORT

After completion of the experimental work for this study, a comprehensive draft report containing the results of all tests, analysis, observations, and measurement required by this protocol will be submitted to the Study Sponsor. Revisions to the initial draft report will be provided to the Sponsor as a final report released by the QAU of BRI following receipt of revision comments from the Sponsor. An electronic copy (PDF) will be provided with the final report.

The study report will include, but be not limited to, the report components listed below:

- GLP Compliance Statement
- BRI Quality Assurance Statement
- Table of Contents
- Lists of Abbreviations, Figures, Tables and Appendices
- Introduction & Study Objectives
- Experimental Methods and Procedures
- Instrumentation and Equipment
- Results and Discussion
- Conclusion
- Appendices (Study Protocol, Representative Plots, Protocol Amendment and Deviation Forms)

Three months after issuance of the draft report, if no requests for revision or instructions to finalize have been communicated by the Sponsor, the draft report will be issued as the final report, signed by the Study Director and submitted to the Sponsor. Any modifications or changes

to the draft report requested three months after issuance of the draft will be performed at additional cost to the Sponsors.

10. GLP COMPLIANCE

The experimental work described in this study protocol will be carried out in conformance with the following applicable Good Laboratory Practice (GLP) Regulations/Standards/Guidelines:

- United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, current.
- Organization for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Series on Principles of Good Laboratory Practice and Compliance Monitoring, Monograph No.1 to 15,1997
- Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997

The Quality Assurance Unit, in accordance with BRI Biopharmaceutical Research Inc. Standard Operating Procedures (SOPs), will audit the study protocol, the assay performance (in-phase audit), all experimental records, and the study report. Copies of all QA audit findings will be provided to the Study Director and BRI Management.

All protocol amendments will be documented and approved by the Study Director prior to execution of the changes. In addition, approval by the Study Sponsor will be obtained for any major change to the study protocol. All study deviations will be documented and signed by the Study Director and the Sponsor Representative will be notified when impact to the study is considered significant as per BRI procedures in SOP-QA-034. Copies of plan amendments will be maintained with the study protocol.

11. STUDY ARCHIVES

All experimental raw data, related documentation, and the study report will be archived at BRI's archives (103-8898 Heather St., Vancouver, BC, Canada) following procedures described in BRI Standard Operating Procedures for a period of at least 5 years following completion of the study (final report issue date). BRI agrees to give the Study Sponsor sufficient advance notification of any relocation of the archived materials after the 5-year holding period to the Study Sponsor, or to an alternative storage facility.

12. MATERIALS AND SAMPLE DISPOSITION

Sponsor provided materials remaining at the end of the study will be kept at BRI unless specifically instructed otherwise in writing by the Sponsor. In house prepared samples will be

13. STUDY TIMELINES

Proposed Experimental Start Date:	An experimental start date defined as the first date on which the first experiment for the study is conducted. A tentative start date is proposed to be in July 2012
Proposed Experimental Completion Date:	Defined as the last date on which data are collected directly from the study at BRI. The completion date is pending on the actual start date and it is proposed to be within the month of July 2012
Proposed Unaudited Data Summary Submission Date:	Within approximately 5 working days after the experimental completion date
Proposed Audited Draft Report Submission Date:	Within approximately 10 working days after the experimental completion date

14. REFERENCES

[1] Harriet Wallin. Steviol glycosides. Chemical and Technical Assessment. 63rd JECFA. 2004

[2] Koyama E, Kitazawa K, Ohori Y, et.al. In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. Food Chem Toxicol. 2003 Mar;41(3):359-74.

[3] C Gardana, P Simonetti, and E Canzi, etal. Metabolism of Stevioside and Rebaudioside A from Stevia rebaudiana extracts by human microflora. J Agri. Food Chem. 2003, 51, 6618-6622.

Appendix 1 – Data Evaluation

Value	Calculation Formula
%Bias Measurement of individual sampling accuracy	$\%Bias = \frac{(\text{experimental value} - \text{expected value})}{\text{expected value}} \times 100$
Mean %Bias Measurement of assay accuracy	Mean %Bias = arithmetic average of %Bias
%CV Measurement of assay precision	$\%CV = \frac{\text{standard deviation [observed values]}}{\text{mean [observed values]}} \times 100$
%Difference Measurement of variability between two values	$\%Difference = \frac{\text{absolute difference [value A and B]}}{\text{mean [value A and B]}} \times 100$
Calibration Model Logistic regression analysis for calibration standard curves	$y = mx + b$ *Where:* *y = response* *x = analyte concentration* *m* = slope *b* = y-intercept
Outlier T-Test Identification of a value that deviates significantly from other replicates with which it occurs	$T = \frac{\lvert Xi - Xavg \rvert}{SD}$ *Where, for n number of observations:* *Xi = Largest or smallest individual observation* *Xavg = Mean of n observations* *SD = Standard deviation calculated from n observations* *Xi is an outlier when T is greater than critical values for T with n observations*

END of STUDY PROTOCOL

Appendix 6 Study Protocol Amendment No. 1 (ADF-252)

BRI Biopharmaceutical Research Inc. ADF-252

SOP Form-QA-034-1
Amendment/Deviation Form

☒Amendment(s) ☐Deviation(s)

BRI Study/Project Number:	TAR-2012-001
BRI Study/Project Title:	*In vitro* metabolism of Rebaudioside X in Pooled Human Intestinal Fecal Homogenate under Physiological Anaerobic Conditions
Testing Facility Study Number:	N/ap
Sponsor Reference Number:	N/ap
Sponsor Name:	Stanley M. Tarka, Jr., Ph.D.

I. Description

With reference to experimental data generated prior to this amendment, the following two observations were made:

A) A lower than expected anaerobic metabolic turnover of rebaudioside A as a positive control at 8 and 24 hours of incubation with fecal homogenate;

B) Variability of steviol metabolite formation between triplicate spiking of samples following 48 hours of anaerobic metabolism incubation.

II. Reason/Rationale/Justification

Following a review of the experimental data and procedures used, an amendment to the following relevant sections of the study protocol is warranted:

A) Section 4. Pooled Human Fecal Homogenate
It has been suspected that the use of commercially obtained fecal materials collected under aerobic conditions over an extended period of time, their shipping and handling, as well as the total time delay (~72 hrs) prior to study initiation may have all contributed to the reduced the anaerobic metabolic turnover rate of rebaudioside A.

Therefore, during a repeat of the experiment, human fecal homogenate will be obtained from six healthy male and six healthy female donor volunteers from personnel within BRI to allow the use of an anaerobic buffer to maintain anaerobic conditions of the fecal materials immediate following collection. The fecal materials will be collected with the use of an anaerobic buffer and donors declared free of known gastrointestinal disease and without previous ingestion of stevia-based natural sweeteners, laxatives and antimicrobial drugs at least 7 days prior to the morning of fecal sample collection will be included in this study. The fecal homogenates will be pooled from 3 subjects of each sex to yield a total of 4 test samples (2 males and 2 females).

B) Section 7.3. *In Vitro* Metabolism of Rebaudioside X in a Pooled Human Fecal Homogenate Preparation at 37°C Under Anaerobic Conditions
The observed variability of steviol metabolite concentrations between the triplicate metabolism samples was suspected to have resulted from the lack of homogeneity during spiking of rebaudioside X and rebaudioside A to the pooled fecal homogenate, particularly when solubility of the two rebaudiosides in their spiking vehicles at 100X the incubation concentrations is limited.

Therefore, during a repeat of the experiment, each of the pooled human fecal homogenate materials will be spiked in a single bulk volume with both 0.2 mg/ml and 2.0 mg/ml of each of the test substances followed by homogenization by vortex mixing prior to aliquotting the fecal homogenate into triplicate samples for metabolic incubation over the time course of 0, 8, 24 and 48 hours.

III. Corrective Actions

Corrective actions are not relevant since the proposed amendment changes are necessary adjustments in the

BRI Biopharmaceutical Research Inc. ADF-252

experimental procedures and not unintentional deviations to the experimental procedures.

IV. Assessment of Impact

This amendment to the in vitro metabolism experimental procedures is expected to provide improvements to the conditions used and the data obtained are expected to be representative of in vivo metabolism of rebaudioside X in human fecal materials under anaerobic conditions.

V. Recommendations

No further recommendation is required.

Approval Signatures:

(b) (6)

Xiaowei Teng, Ph.D. — 22Aug2012
Study Director — Date

N/Ap — N/ap
Principal Investigator / Analytical Investigator — Date

(b) (6)

Clara Faan — 22Aug2012
BRI Management — Date

(b) (6)

Stanley M. Tarka, Jr., Ph.D. — 21 August 2012
Sponsor Representative — Date

Appendix 7 Study Protocol Amendment No. 2 (ADF-256)

BRI Biopharmaceutical Research Inc. ADF-256

SOP Form-QA-034-1
Amendment/Deviation Form

☒Amendment(s) ☐Deviation(s)

BRI Study/Project Number:	TAR-2012-001 (Protocol Amendment #2)
BRI Study/Project Title:	*In vitro* metabolism of Rebaudioside X in Pooled Human Intestinal Fecal Homogenate under Physiological Anaerobic Conditions
Testing Facility Study Number:	N/ap
Sponsor Reference Number:	N/ap
Sponsor Name:	Stanley M. Tarka, Jr., Ph.D.

I. Description

Please note that text to be removed is identified by a ~~strikethrough~~ and new text is presented in **bold**.
With reference to the approved Protocol-TAR-2012-001 and AD-252, the following amendments are made:

A) Section 4

The following human fecal study materials are required:

Study Materials	Source	Storage
Pooled human fecal homogenate (n=6 male and 6 female subjects, note 1)	BRI volunteers ~~or commercial vendor~~	Refrigerated

Note 1: Human fecal homogenate will be obtained from six healthy male and six healthy female volunteers ~~of Caucasian and Asian ethnicities~~ without known gastrointestinal disease and without previous ingestion of stevia-based natural sweeteners, laxatives and antimicrobial drugs at least 7 days prior to the morning of fecal sample collection.

B) Section 7.1 Preparation and Storage of Test Aritcle Stock Solutions

The controls, Steviol, Steviolbioside and Rebaudioside A, as well as the Test Article, Rebaudioside X, will each be dissolved in a vehicle ~~consisting of 0.2M potassium phosphate buffer (pH 7.0) containing 10% DMSO~~ to provide a stock solution at a nominal concentration of **2 mg/mL or** 200 mg/mL to provide a 100X spiking stock solution. Each of the test substances except for steviol will be spiked in a single bulk volume of pooled human fecal homogenate materials for 0.2 mg/mL and 2.0 mg/mL followed by homogenization by vortex mixing prior to aliquotting the fecal homogenate into triplicate samples for metabolic incubation over the time course of 0, 8, 16 and 24 hours. **For steviol**, an aliquot of 10 µL of ~~this~~ **20 mg/mL or** 200 mg/mL ~~spiking~~ stock solution will be ~~used to spike~~ **spiked** into 1 mL anaerobic human fecal homogenate to provide a final test concentration at **0.2 mg/mL or** 2 mg/mL, **respectively**.

~~Similarly for each control and the Test Article, a stock solution at a nominal concentration of 20 mg/mL will be prepared to provide a 100X spiking stock solution. An aliquot of 10 µL of this spiking stock solution will be used to spike into 1 mL anaerobic human fecal homogenate to provide a final test concentration at 0.2 mg/mL.~~

Stock solutions will be prepared and stored at room temperature until use for spiking to human fecal homogenate within 24 hours.

C) Section 7.3.2 Human Fecal Homogenate

The preparation of human fecal homogenate will follow in principle the procedure as previously described [2]. Upon voiding followed by immediate collection of **10 - 20 g** fecal material in a pre-labeled ~~30~~ **100** mL screw-capped polypropylene specimen container **pre-filled with 50 mL anaerobic buffer** from each subject ~~with the use of the spoon attachment on the screw cap, a pre-aliquoted 10 mL anaerobic buffer will be immediately added to the fecal material to maintain an anaerobic sample environment~~. An initial stool-to-buffer ratio of the collected sample will be determined based on the tare-weight of the screw-capped stool sample container with the initial volume of anaerobic buffer used. ~~A final fecal homogenate with a stool-to-buffer ratio of 1:1 (stool/buffer) will be achieved by adding the balance of an anaerobic buffer volume required.~~

BRI Biopharmaceutical Research Inc. ADF-256

Following preparation of the fecal homogenate, the fecal homogenate materials from each of twelve subjects will be transferred to an anaerobic nitrogen chamber via an air lock. Commercially available 99.9% high purity dry nitrogen (Airgas) connected on-line to the anaerobic chamber will be used to flush the chamber and air lock continuously for 60 minutes following the transfer of samples and test materials inside the anaerobic chamber for handling of sample materials for each experiment. This procedure allows a commercial Certification of Analysis of the high purity nitrogen to support anaerobic conditions used for this study. Atmospheric oxygen level inside the chamber is being monitored using an oxygen monitor at a detection limit of 1% as a fail-safe readout to alert of air leakage throughout the fecal homogenate handling procedures.

Within the anaerobic chamber, the ~~1:1 (stool/buffer)~~ fecal materials will be pooled by ~~equal~~ **appropriate** volume based on 3 subjects per sex and then homogenized with ~~25~~ **appropriate** volumes (%v/v) of 0.2M potassium phosphate buffer (pH 7.0) by vigorous vortex-mixing in a clean ~~10~~ **50** mL screw-capped ~~glass~~ test tube to provide a ~~50~~ **12.5**-fold diluted pooled human fecal homogenate ~~for incubation~~. The screw-capped homogenate centrifuge tubes will be removed from the chamber and centrifuged at 500 xg for 1 min at 37°C and returned to the anaerobic chamber for transfer of the fecal homogenate supernatant to a clean ~~10 mL screw-capped glass tube~~ **container, dilution with 3 volumes of Brain Heart Infusion Broth (BHI) and addition of Oxyrase for Broth (2%. v/v)** for **pre-**incubation at 37°C **overnight**. A total of 2 male and 2 female pooled human fecal homogenate supernatant samples will be similarly prepared from n=3 subjects for each pool fecal sample.

All dilutions and sample transfer steps will be carried out using 0.2M potassium phosphate buffer (pH 7.0) previously sparged with high purity nitrogen for 60 minutes **followed by using BHI** in an anaerobic chamber to provide an anaerobic buffer. Metabolic incubations will be carried out in triplicates a sealed glass screw-capped test tube incubated at 37°C to maintain an anaerobic condition during incubation.

The controls, Steviol, Steviolbioside and Rebaudioside A, as well as the Test Article, Rebaudioside X, will each be incubated independently at test concentrations of 0.2 mg/mL and 2 mg/mL under an anaerobic environment for 0, 8, **16, and** 24~~, and 48~~ hours at 37°C±5°C in a calibrated ~~heating block~~ **environmental chamber**. At the end of each incubation time point, an equal volume of ~~1 mL acetonitrile~~ **methanol containing 5% ammonium hydroxide** will be added to the fecal homogenate sample to stop the enzyme reaction with gentle vortex mixing **and sonication for 5 min**. The samples will be centrifuged at ~~12,000~~ **3270** *xg* for 10 minutes to remove protein components. Triplicate samples (n=3) will be aliquoted from each incubation time point and then stored at nominal –80°C (-72°C to -88°C) until LC/MS assay.

The experimental design is summarized below:

Sample Type	Test materials	Concentrations	Incubation Time Points
Assay Stability Control	Steviol	0.2 mg/mL and 2 mg/mL of the test and control samples	37°C for 0, 8, **16, and** 24 ~~and 48~~ hours (n=3) replicates
Hydrolysis intermediate	Steviolbioside		
Metabolism Positive Control	Rebaudioside A (CAS 58543-16-1)		
Test Article	Rebaudioside X		

C) During a repeat of the experiment (the third experiment), rebaudioside A and rebaudioside X at 0.2 mg/mL and 2.0 mg/mL will be incubated with BHI in the presence of Oxyrase for Broth over the time course of 0, 8, 16 and 24 hours in parallel.

II. Reason/Rationale/Justification

Following a review of the experimental data and procedures used in the initial, second and a pilot experiment, an amendment to the relevant sections of the study protocol is warranted:

A) It has been suspected that the use of commercially obtained fecal materials collected under aerobic conditions over an extended period of time, their shipping and handling, as well as the total time delay (~72 hrs) prior to study initiation may have contributed to the reduced anaerobic metabolic turnover rate of rebaudioside A in the initial experiment. Therefore, during a repeat of the experiment, human fecal homogenate will be obtained from six healthy male and six healthy female donor volunteers from personnel within BRI to allow the use of an anaerobic buffer to maintain anaerobic conditions of the fecal materials immediate following collection.

B) A single bulk spiking of the rebaudioside X, rebaudioside A, and steviobioside in the second experiment reduced the variability of steviol metabolite concentrations between the triplicate metabolism samples observed in first experiment performed using individual spiking of test articles to the 50x pooled fecal homogenate.

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Precipitation of steviol in the pooled fecal homogenate was observed during the first and the second experiment. Therefore, steviol will be spiked individually into triplicate samples for metabolic incubation over the time course.

C) The 100 mL specimen container has a larger opening to allow easy collection of the fecal material.

The oxygen level in the nitrogen anaerobic chamber was sometimes greater than 1% (up to 4.7%) as shown on the oxygen monitor during the experiments, due to door opening for transfer of experimental materials. This could have also contributed to the reduced anaerobic metabolic turnover rate of rebaudioside A in the initial and second experiments. In the pilot study prior to this amendment, an optimal human fecal anaerobic culture for investigating the metabolism of Rebaudioside A and Rebaudioside X was determined to be the use of 12.5x pooled fecal homogenate diluted with 3 volumes of Brain Heart Infusion Broth (BHI) to achieve 50x pooled fecal homogenate with Oxyrase added at the start of incubation, as compared to those samples without the use of BHI and Oxyrase for Broth to remove oxygen in the pilot experiment. BHI is a pre-reduced anaerobically sterilized nutritionally enriched medium that is reduced with cysteine and is used for isolating and growing anaerobes. Secondly, the addition of Oxyrase for Broth is critical to remove oxygen in the sample to establish and maintain an anaerobic environment.

In the pilot study prior to this amendment, 100% conversion of rebaudioside A to steviol was observed in 24 hours. Therefore, the 48 hour time point will not be required and a 16 hour time point will be added.

The change of heating block to environmental chamber is due to that the latter but not the former is calibrated.

The change of reaction terminating reagent is to keep steviol soluble and avoid salt precipitation from buffer. Sonication of the samples is necessary to facilitate the dissolving of steviol in the sample.

Reduced centrifugation force is necessary for this type of the test tube.

D) Incubation of rebaudioside A and rebaudioside X with BHI in the presence of Oxyrase for Broth is to demonstrate the lack of conversion of these two substances in this matrix.

III. Corrective Actions

Corrective actions are not relevant since the proposed amendment changes are necessary adjustments in the experimental procedures and not unintentional deviations to the experimental procedures.

IV. Assessment of Impact

This amendment to the in vitro metabolism experimental procedures is expected to provide improvements to the anaerobic conditions and the data obtained are expected to be representative of in vivo metabolism of rebaudioside X in human fecal materials under anaerobic conditions.

V. Recommendations

No further recommendation is required.

Approval Signatures:

(b) (6)

Xiaowei Teng, Ph.D. — 27 Sep 2012
Study Director — Date

N/Ap — N/ap
Principal Investigator / Analytical Investigator — Date

(b) (6)

Clara Faan — 27 Sep 2012
BRI Management — Date

(b) (6)

27 September 2012
Sponsor Representative — Date

Appendix 8 Representative LC/MS Method Parameters for Steviol Analysis

Acquisition Experiment Report
File:w:\raw data (v4.0)\tar\2012-001\tar12-01 hf17sep2012stv-hl2).pro\data\tar 17sep2012 a008.raw

Header
Acquired File Name: TAR 17SEP2012 a008
Acquired Date: 17-Sep-2012
Acquired Time: 15:32:10
Job code: TAR STV 17SEP 2012
Task code:
User Name:
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID: Blank-1
Bottle Number: 3
Description: Blank-1

Instrument Calibration
Parameters
MS1 Static:
Mass 122 Da to 1930 Da.
Resolution : 15.0/13.0
Ion Energy : 0.1
Reference File : Naineg
Acquisition File : STATMS1
MS1 Scanning:
Mass 125 Da to 1950 Da.
Resolution : 15.0/13.0
Ion Energy : 0.1
Reference File : Naineg
Acquisition File : SCNMS1
MS1 Scan Speed:
Scan up to 1 amu/sec.
Resolution : 15.0/13.0
Ion Energy : 0.1
Reference File : Naineg
Acquisition File : FASTMS1
MS2 Static: None
MS2 Scanning: None
MS2 Scan Speed: None
Calibration Time: 17:37
Calibration Date: 07/20/12
Coefficients
MS1 Static: -0.000000263728*x^2 + 1.000790245238*x +-0.344520284825

MS2 Static: -0.000000135841*x^2 + 1.000484764131*x +-0.278184537600

Function 1: None
Function 2: None

Parameters for D:\Masslynx\Projects\TAR12-01 HF17SEP2012STV-HL2).PRO\ACQUDB\TAR STV 17SEP 2012.EXP

Prescan Statistics:

Initial Average Intensity	64.0462
Initial Average Std Dev	2.9334
Bunch Zero Level	1.2192
Bunch Std Dev	0.2199
Bunch Threshold	1.7591
Spike Removal Std Dev	2.7562

Instrument Parameters - Function 1:

Polarity	ES-		
Calibration	Static 1		
Capillary (kV)	2.5	-2.41	
Cone (V)	60	-64	
Extractor (V)	3	-4	
RF Lens (V)	0.5		
Source Temperature (°C)	95	95	
Desolvation Temperature (°C)	280	279	
Cone Gas Flow (L/Hr)	25		
Desolvation Gas Flow (L/Hr)	499		
LM 1 Resolution	14		
HM 1 Resolution	14		
Ion Energy 1	0.3		
Entrance	50	50	
Collision	1	1	
Exit	50	50	
LM 2 Resolution	15		
HM 2 Resolution	15		
Ion Energy 2	1.5		
Multiplier (V)	650	-647	
Collision Cell Pressure(mbar)	<1.00e-4		
Analyser Pressure (mbar)	OFF		

Instrument Parameters - Function 2:

Polarity	ES-		
Calibration	Static 1		
Capillary (kV)	2.5	-2.41	
Cone (V)	60	-64	
Extractor (V)	3	-4	
RF Lens (V)	0.5		
Source Temperature (°C)	95	95	
Desolvation Temperature (°C)	280	279	
Cone Gas Flow (L/Hr)	25		
Desolvation Gas Flow (L/Hr)	499		
LM 1 Resolution	14		
HM 1 Resolution	14		
Ion Energy 1	0.3		
Entrance	50	50	
Collision	1	1	
Exit	50	50	
LM 2 Resolution	15		
HM 2 Resolution	15		
Ion Energy 2	1.5		
Multiplier (V)	650	-647	

Collision Cell Pressure(mbar) <1.00e-4
Analyser Pressure (mbar) OFF

ACE Experimental Record
HP1100 LC Pump Initial Conditions
Solvents
A% 95
B% 5
C% 0
D% 0
Valve A set to channel 1
Valve B set to channel 1
Flow (ml/min) 0.4
Stop Time (mins) 12
Min Pressure (bar) 0
Max Pressure (bar) 400
Oven Temperature Left(°C) 25
Oven Temperature Right(°C) 25

HP1100 LC Pump Gradient Timetable
The gradient Timetable contains 7 entries which are :

Time	A%	B%	C%	D%	Flow (ml/min)	Pressure
0.00	95.0	5	0	0	0.4	400
0.50	95.0	5	0	0	0.4	400
2.50	40.0	60	0	0	0.4	400
5.00	0.0	100	0	0	0.4	400
6.90	0.0	100	0	0	0.4	400
7.00	95.0	5	0	0	0.4	400
12.00	95.0	5	0	0	0.4	400

HP1100 LC Pump External Event Timetable

The Timetable contains 6 entries which are :

Time	Column Switch	Contact 1	Contact 2	Contact 3	Contact 4
Initial	On	Off	Off	Off	Off
0.00	On	Off	Off	Off	On
0.01	On	Off	On	Off	Off
0.10	Off	Off	On	Off	Off
4.00	On	Off	On	Off	Off
8.00	Off	Off	On	Off	Off

HP1100 Autosampler Initial Conditions
Injection Volume(µl) 5
Draw Speed 200
Eject Speed (µl/min) 200
Draw Position (mm) 3
Stop Time (mins) 12
Vial Number
Thermostat On
Thermostat Temperature(°C) 23

Function 1
Scans in function: 1789
Cycle time (secs): 0.550

Inter Scan Delay (secs): 0.05
Retention window (mins): 0.000 to 12.000
Ionization mode: ES-
Data type: SIR or MRM data
Function type: SIR of 2 channels

Chan	Mass	Dwell(secs)	Cone Volt.
1 :	301.20	0.05	60.0
2 :	301.30	0.05	60.0

Function 2
Scans in function: 1789
Cycle time (secs): 0.550
Inter Scan Delay (secs): 0.05
Retention window (mins): 0.000 to 12.000
Ionization mode: ES-
Data type: SIR or MRM data
Function type: SIR of 2 channels

Chan	Mass	Dwell(secs)	Cone Volt.
1 :	317.20	0.05	60.0
2 :	317.30	0.05	60.0

Shepherd, Lillian

From: Ramos-Valle, Moraima
Sent: Friday, May 10, 2013 3:08 PM
To: ARoberts@cantox.com
Cc: Shepherd, Lillian
Subject: GRAS submission for Rebaudioside X

Hi Ashley,

To follow up with our phone conversation about the information labeled confidential (Appendix B and reference list) in the GRAS submission for Rebaudioside X. My email is below.

Thanks and have a great weekend,

Moraima

Moraima J. Ramos Valle, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Phone: (240) 402-1248
Email: Moraima.Ramos-Valle@fda.hhs.gov

Shepherd, Lillian

From: Ashley Roberts Intertek <ashley.roberts@intertek.com>
Sent: Friday, May 10, 2013 5:00 PM
To: Ramos-Valle, Moraima
Cc: Shepherd, Lillian
Subject: RE: GRAS submission for Rebaudioside X
Attachments: Response to FDA re clarifications.docx

Dear Moraima,

Please find attached the letter indicating that the attached report should not have been labelled as being "confidential".

I hope this is all that is needed. If not, please let me know.

Best Wishes

Asahley

From: Ramos-Valle, Moraima [mailto:Moraima.Ramos-Valle@fda.hhs.gov]
Sent: May-10-13 3:08 PM
To: Ashley Roberts Intertek
Cc: Shepherd, Lillian
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Moraima

Moraima J. Ramos Valle, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Phone: (240) 402-1248
Email: Moraima.Ramos-Valle@fda.hhs.gov

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May 10, 2013

Moraima J. Ramos Valle, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration

Dear Ms. Ramos Valle:

In follow-up to our discussion regarding the term "confidentiality" that was used to reference the *in vitro* metabolism report conducted by Biopharmaceutical Research Inc. (BRI) that was attached to the rebaudioside X GRAS notification, I am writing to confirm that this report is indeed non-confidential. As such, this report can be considered for review along with the other information submitted to the FDA to support PureCircle's self-GRAS determination of rebaudioside X for use as a general purpose sweetening agent and can be included as part of the GRAS documentation on FDA's website.

Sincerely,

(b) (6)

Ashley Roberts, Ph.D
Senior Vice President
Intertek Cantox

cc:

RECEIVED
MAY 10 2013
OFFICE OF
FOOD ADDITIVE SAFETY

Shepherd, Lillian

From: Ramos-Valle, Moraima
Sent: Monday, May 13, 2013 1:41 PM
To: Shepherd, Lillian
Subject: FW: GRAS submission for Rebaudioside X
Attachments: Response to FDA re clarifications.docx

Hi Lillian,

If everything else is good to go with this one then it can be filed.

Thanks,
Moraima

From: Ashley Roberts Intertek [mailto:ashley.roberts@intertek.com]
Sent: Friday, May 10, 2013 5:00 PM
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Cc: Shepherd, Lillian
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Thanks and have a great weekend,

Moraima

Moraima J. Ramos Valle, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review

Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
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Phone: (240) 402-1248
Email: Moraima.Ramos-Valle@fda.hhs.gov

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