ORIGINAL SUBMISSION

GRAS Associates, LLC
27499 Riverview Center Blvd.
Bonita Springs, FL 34134
T: 239.444.1724 | F: 239.444.1723
www.gras-associates.com

5/18/2016

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety (HFS-255)
5100 Paint Branch Parkway
College Park, MD 20740-3835

Attention: Dr. Paulette Gaynor
Re: GRAS Notification – Enzyme Modified Steviol Glycosides

Dear Dr. Gaynor:

On behalf of GLG Life Tech Corporation (Canada), we are submitting for FDA review Form 3667 and the enclosed CD, free of viruses, containing a GRAS notification for *Enzyme Modified Steviol Glycosides*. An Expert Panel of qualified persons was assembled to assess the composite safety information of the subject substance with the intended use as a table top sweetener and as a general purpose non-nutritive sweetener for incorporation into food in general, other than infant formulas and meat and poultry products. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

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

We look forward to your feedback.

Sincerely,
(b) (6)

Cheryl R. Dicks, MS, RAC
Director of Operations
GRAS Associates, LLC
27499 Riverview Center Blvd., Suite 212
Bonita Springs, FL 34134
540-272-3254
dicks@gras-associates.com

Enclosure: GRAS Notification for GLG Life Tech Corporation – *Enzyme Modified Steviol Glycosides*
CC Katrina Emmel, PH.D. emmel@gras-associates.com

GRAS Associates, LLC
27499 Riverview Center Blvd.
Bonita Springs, FL 34134
T: 239.444.1724 | F: 239.444.1723
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GRN 000656

5/18/2016

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety (HFS-255)
5100 Paint Branch Parkway
College Park, MD 20740-3835

Attention: Dr. Paulette Gaynor
Re: GRAS Notification – Enzyme Modified Steviol Glycosides

Dear Dr. Gaynor:

On behalf of GLG Life Tech Corporation (Canada), we are submitting for FDA review Form 3667 and the enclosed CD, free of viruses, containing a GRAS notification for *Enzyme Modified Steviol Glycosides*. An Expert Panel of qualified persons was assembled to assess the composite safety information of the subject substance with the intended use as a table top sweetener and as a general purpose non-nutritive sweetener for incorporation into food in general, other than infant formulas and meat and poultry products. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

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dicks@gras-associates.com

Enclosure: GRAS Notification for GLG Life Tech Corporation – *Enzyme Modified Steviol Glycosides*
CC Katrina Emmel, PH.D. emmel@gras-associates.com

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000656	RECEIVED JUN 1 2016 OFFICE OF FOOD ADDITIVE SAFETY
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*
☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
Brian Meadows	President & CFO

Company *(if applicable)*: GLG Life Tech Corporation

Mailing Address *(number and street)*: Suite 100-10271 Shellbridge Way

City	State or Province	Zip Code/Postal Code	Country
Richmond	British Columbia (BC)	V6X 2W8	Canada

Telephone Number	Fax Number	E-Mail Address
(604) 285-2602		brian.meadows@glglifetech.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position
Katrina Emmel, PH.D.	Senior Scientist/ Associate

Company *(if applicable)*: GRAS Associates, LLC

Mailing Address *(number and street)*: 27499 Riverview Center Blvd.

City	State or Province	Zip Code/Postal Code	Country
Bonita Springs	Florida	34134	United States of America

Telephone Number	Fax Number	E-Mail Address
(239) 444-1724	(239) 444-1723	dicks@gras-associates.com

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

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(604) 285-2602		brian.meadows@glglifetech.com

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Katrina Emmel, PH.D.	Senior Scientist/ Associate

Company *(if applicable)*: GRAS Associates, LLC

Mailing Address *(number and street)*: 27499 Riverview Center Blvd.

City	State or Province	Zip Code/Postal Code	Country
Bonita Springs	Florida	34134	United States of America

Telephone Number	Fax Number	E-Mail Address
(239) 444-1724	(239) 444-1723	dicks@gras-associates.com

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

TasteBoost™ EMS95 (Enzyme Modified Steviol Glycosides; and EMSG)

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Electronic files on physical media with paper signature page

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in FDA's files by reference? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates by reference information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for determination of GRAS status *(Check one)*

☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

☐ Yes *(Proceed to Item 8)*

☒ No *(Proceed to Part IV)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, see attached Designation of Confidential Information

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

PART IV – INTENDED USE OF THE NOTIFIED SUBSTANCE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

Intended to be used as a table top sweetener and as a general purpose non-nutritive sweetener for incorporation into foods in general, other than infant formulas and meat and poultry products, at per serving levels reflecting good manufacturing practices and principles, in that the quantity added to foods should not exceed the amount reasonably required to accomplish its intended technical effect.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☐ Yes ☒ No

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1	Enzyme Modified Steviol Glycosides (TasteBoost™ EMS95; and EMSG)				
2					
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s)),* and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

GLG's Enzyme Modified Steviol Glycosides (EMSG) is manufactured through an enzymatic reaction with *Stevia rebaudiana* Bertoni extract (>95.0% total steviol glycosides) using cyclomaltodextrin glucanotransferase. The resulting preparation is a high-purity enzyme modified steviol glycosides product (≥ 95% total steviol glycosides).

3. Synonyms

Provide as available or relevant:

1	Enzyme modified steviol glycosides
2	EMSG
3	

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☐ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☐ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☒ Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

1. The undersigned is informing FDA that GLG Life Tech Corporation *(name of notifier)*

has concluded that the intended use*(s)* of TasteBoost™ EMS95 (Enzyme Modified Steviol Glycosides; and EMSG) *(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☒ GLG Life Tech Corporation *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

GLG Life Tech Corporation *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

Suite 2168-1050 West Pender St., Vancouver, BC V6E 3S7 Canada *(address of notifier or other location)*

GLG Life Tech Corporation *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ---------- *(GRAS Affirmation Petition No.)*

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
Katrina Emmel, PH.D. Digitally signed by Katrina Emmel, PH.D. DN: cn=Katrina Emmel, PH.D., o=GRAS Associates, LLC, ou=Senior Scientist/ Associate, email=emmel@gras-associates.com, c=US Date: 2016.05.22 15:24:38 -04'00'	Katrina Emmel, PH.D. Senior Scientist / Associate	05/18/2016

PART VIII – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Multiple appendices---Appendices A through M	

OMB Statement: Public reporting burden for this collection of information is estimated to average 150 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

GRAS Assessment

of

Enzyme Modified Steviol Glycosides

Food Usage Conditions for General Recognition of Safety

for

GLG Life Tech Corporation

Suite 2168-1050 West Pender St.
Vancouver, BC V6E 3S7
Canada

Evaluation By
GRAS Expert Panel

Richard C. Kraska, Ph.D., DABT
Robert S. McQuate, Ph.D.
Katrina V. Emmel, Ph.D.

May 17, 2016

TABLE OF CONTENTS

FIGURES

TABLES

I. GRAS EXEMPTION CLAIM

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

GLG Life Tech Corporation (hereinafter "GLG") has determined that its high purity Enzyme Modified Steviol Glycosides product (also known as EMSG and TasteBoost™ EMS95), and which meets the specifications described below, is Generally Recognized As Safe (GRAS) in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. This determination was made in concert with an appropriately convened panel of experts who are qualified by scientific training and experience. The GRAS determination is based on scientific procedures as described in the following sections. The evaluation accurately reflects the intended conditions of food use for the designated stevia-derived sweetener.

Signed:

(b) (6)

Cheryl Dicks, MS, RAC
Director of Regulatory Affairs
GRAS Associates, LLC
27499 Riverview Center Blvd.
Suite 212
Bonita Springs, FL 34134

Date: May 17, 2016

B. Name and Address of Notifier

GLG Life Tech Corporation
Suite 2168-1050 West Pender St.
Vancouver, BC V6E 3S7
Canada

As the notifier, GLG accepts responsibility for the GRAS determination that has been made for its high purity Enzyme Modified Steviol Glycosides product, as described in the subject notification; consequently, the Enzyme Modified Steviol Glycosides preparations having purities no less than 95.0% total steviol glycosides, which meet the conditions described herein, are exempt from premarket approval requirements for food ingredients.

[1] See 62 FR 18938, 17 April 1997. Accessible at: https://www.gpo.gov/fdsys/pkg/FR-1997-04-17/pdf/97-9706.pdf#page=1 (Accessed 2/4/16).

C. Common Name and Identity of Notified Substance

Enzyme Modified Steviol Glycosides, abbreviated as EMSG, is the common name for the notified substance; also see Section III.A.

D. Conditions of Intended Use in Food

The high purity Enzyme Modified Steviol Glycosides preparation (≥ 95.0% total steviol glycosides) is intended to be used as a table top sweetener and as a general purpose non-nutritive sweetener for incorporation into foods in general, other than infant formulas and meat and poultry products, at per serving levels reflecting good manufacturing practices and principles, in that the quantity added to foods should not exceed the amount reasonably required to accomplish its intended technical effect.

E. Basis for GRAS Determination

Pursuant to 21 CFR 170.30, GLG's Enzyme Modified Steviol Glycosides preparation has been determined to be GRAS on the basis of scientific procedures as discussed in the detailed description provided below.

F. Availability of Information

The data and information that serve as the basis for this GRAS notification will be sent to the US Food and Drug Administration (FDA) upon request or will be available for review and copying at reasonable times at the offices of GLG Life Tech Corporation, located at Suite 2168-1050 West Pender St., Vancouver, BC V6E 3S7 Canada.

II. INTRODUCTION

A. Objective

At the request of GLG, GRAS Associates, LLC ("GA") has undertaken an independent safety evaluation of GLG's high purity Enzyme Modified Steviol Glycosides (EMSG) product. The preparation is extracted from the leaves of *Stevia rebaudiana* Bertoni and is purified to yield ≥ 95.0% total steviol glycosides with a minimum of 80.0% α-glucosylated steviol glycosides. The stevia-derived starting material is composed primarily of rebaudioside A, which is glycosylated using the enzyme cyclomaltodextrin glucanotransferase. The purpose of the evaluation is to ascertain whether the intended food uses of Enzyme Modified Steviol Glycosides as a general purpose non-nutritive sweetener, as described in Section IV.A, are generally recognized as safe, i.e., GRAS, under the intended conditions of use.

B. Foreword

GLG provided GA with substantial background information needed to enable the GRAS assessment to be undertaken. In particular, the information provided addressed the safety/toxicity of steviol glycosides; history of use of stevia in food; and compositional details, specifications, and method of preparation of the subject high purity enzyme modified steviol glycosides. GLG was asked to provide adverse reports, as well as those that supported conclusions of safety. Safety/toxicity studies performed with animals were noted to have value, along with available results of an *in vitro* study for metabolism in human fecal homogenate. GLG was also asked to supply past and present human food use information. Knowing how much steviol glycosides have been safely consumed, i.e., the use levels, is critical in extrapolating to safe exposures for highly purified component steviol glycosides when consumed as a food ingredient. The composite safety/toxicity studies, in concert with exposure information, ultimately provide the specific scientific foundation for the GRAS determination.

In addition to the product specifications, chemical properties, manufacturing, and safety related information, GLG also provided some consumption/exposure information, along with other related documentation. This was augmented with an independent search of the scientific and regulatory literature extending through April 19, 2016. A GRAS assessment based primarily on the composite safety information, i.e., based on scientific procedures, was undertaken. Those references that were deemed pertinent to the objective at hand are listed in Section VIII.

C. Summary of Regulatory History of Stevia and Stevia-Derived Sweeteners

Stevia-derived sweeteners are permitted as food additives in South America and in several countries in Asia, including China, Japan, and Korea. In more recent years, these sweeteners have received food usage approvals in Mexico, Australia, New Zealand, Switzerland, France, Peru, Uruguay, Colombia, Senegal, Russia, Malaysia, Turkey, Taiwan, Thailand, Israel, Canada, and Hong Kong (EFSA, 2010; HealthCanada, 2012; Watson, 2010). In the US, steviol glycosides have been used as a dietary supplement since 1995 (J. M. C. Geuns, 2003).

Multiple GRAS notifications for enzyme modified steviol glycosides preparations and/or glucosylated steviol glycosides have been submitted to FDA, which subsequently received “no questions” letters from FDA. The subject material of GRN 337, submitted to FDA by NOW Foods in 2010, was purified steviol glycosides (>95%) composed of natural components of the stevia leaf as well as their 1,4-α-D-glucosylated forms. NOW Foods estimated the material to be 100 times sweeter than sucrose, and they calculated the estimated daily exposure to be 1.9 mg per kg body weight (bw) per day steviol equivalents (FDA, 2011a; NOWFoods, 2010).

The subject material of GRN 375, submitted to FDA by Toyo Sugar Refining Co and Nippon Paper Chemicals Co. in 2011, was enzyme-modified steviol glycosides composed of >95% (w/w) steviol glycosides and α-D-glucosylated steviol glycosides. The material was estimated to be 137 times

sweeter than sucrose, and maximum estimated use would yield 2.12 mg per kg bw per day steviol equivalents (FDA, 2011b; Toyo & Nippon, 2011).

Daepyung Co., Ltd. submitted both GRNs 448 and 452 to FDA regarding two enzyme-modified steviol glycosides products, where the subject materials were composed of ≥95% (w/w) steviol glycosides and α-D-glucosylated steviol glycosides. The subject material in GRN 448 was estimated to be 100 times sweeter than sucrose, and Daepyung estimated a daily intake of 2.17 mg per kg bw per day steviol equivalents. The subject material in GRN 452 was estimated to be 150 times sweeter than sucrose, and the maximum dietary exposure was estimated to be 1.66 mg per kg bw per day in adults and 1.83 mg per kg bw per day in children (Daepyung, 2012a, 2012b; FDA, 2013a, 2013b).

Most recently, PureCircle, Ltd. submitted GRN 607 to FDA regarding a glucosylated steviol glycosides preparation (minimum purity 80%) for use as a flavoring agent and flavor modifier. The notification was filed on November 24, 2015 and is presently under review.

Based on available information from FDA's GRAS Notice Inventory website (FDA, 2015) as of April 19, 2016, the agency has issued 37 "no questions" letters on GRAS notices on rebaudioside A, rebaudioside D, rebaudioside M, or steviol glycosides, including those undergoing enzyme treatment. A summary of these filings is presented in Table 1.

Table 1. FDA's GRAS Notice Inventory on Rebaudioside & Steviol Glycosides Preparations[a]

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
1. Merisant	GRN 252	High-Purity Reb A ≥95%	Variety of food categories & table top sweetener
2. Cargill Inc.	GRN 253	High-Purity Reb A ≥97%	General-purpose sweetener, excluding meat & poultry products
3. McNeil Nutritionals LLC	GRN 275	Purified Steviol Glycosides – Reb A Principal Component	Table top sweetener
4. Blue California	GRN 278	High-Purity Reb A ≥97%	General-purpose & table top sweetener
5. Sweet Green Fields LLC	GRN 282	High-Purity Reb A ≥97%	General-purpose sweetener, excluding meat & poultry products
6. Wisdom Natural Brands	GRN 287	Purified Steviol Glycosides >95% - Reb A and Stevioside Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas
7. Sunwin USA LLC & WILD Flavors	GRN 303	High-Purity Reb A ≥95%/ ≥98%	General-purpose sweetener, excluding meat, poultry products & infant formulas
8. Sunwin USA LLC & WILD Flavors	GRN 304	Purified Steviol Glycosides >95% -	General-purpose sweetener, excluding meat, poultry products & infant formulas

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
		Reb A and Stevioside Principal Component	
9. Pyure Brands, LLC	GRN 318	High-Purity Reb A 95%/ 98%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
10. PureCircle USA Inc	GRN 323	Purified Steviol Glycosides – Reb A Principal Component	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
11. GLG Life Tech Ltd[b]	GRN 329	High-Purity Reb A ≥97%	General-purpose sweetener, excluding meat & poultry products
12. NOW Foods	GRN 337	Enzyme Modified Steviol Glycosides Preparation (EMSGP)	General-purpose sweetener in foods, excluding meat & poultry products, at levels determined by good manufacturing practices
13. GLG Life Tech Ltd[b]	GRN 348	High-Purity Stevioside ≥95%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
14. GLG Life Tech Ltd[b]	GRN 349	High-Purity Steviol Glycosides ≥97%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
15. Guilin Layn Natural Ingredients, Corp.	GRN 354	High-Purity Reb A ≥97%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
16. BrazTek International Inc.	GRN 365	Purified Reb A	General-purpose sweetener, excluding meat & poultry products
17. Sinochem Qingdao Co. Ltd.	GRN 367	High-Purity Steviol Glycosides ≥95%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
18. Shanghai Freemen Americas LLC	GRN 369	Purified Reb A	General-purpose sweetener, excluding meat & poultry products
19. Toyo Sugar Refining Co., Ltd. & Nippon Paper Chemicals Co., Ltd.	GRN 375	Enzyme Modified Steviol Glycosides	General-purpose sweetener in foods, excluding meat and poultry products, at levels determined by good manufacturing practices
20. GLG Life Tech Ltd[b]	GRN 380	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
21. Chengdu Wagott Pharmaceutical	GRN 388	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
22. Chengdu Wagott Pharmaceutical	GRN 389	Steviol Glycosides with Stevioside as the Principal Component	General purpose & table top sweetener, excluding meat & poultry products
23. Daepyung Co., Ltd.	GRN 393	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
24. Daepyung Co., Ltd.	GRN 395	Steviol Glycosides with Reb A and	General purpose & table top sweetener, excluding meat & poultry products

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
		Stevioside as the Principal Components	
25. MiniStar International, Inc.	GRN 418	Purified Reb A	General-purpose sweetener, excluding meat, poultry products & infant formulas.
26. Daepyung Co., Ltd.	GRN 448	Enzyme Modified Steviol Glycosides	General-purpose sweetener, excluding meat, poultry products & infant formulas.
27. Daepyung Co., Ltd.	GRN 452	Enzyme Modified Steviol Glycosides	General-purpose sweetener, excluding meat, poultry products & infant formulas.
28. PureCircle USA, Inc.	GRN 456	High-Purity Reb D ≥95%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
29. Almendra, Ltd.	GRN 461	High-Purity Reb A ≥97%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
30. Qufu Xiangzhou Stevia Products Co., Ltd.	GRN 467	High-Purity Reb A ≥98%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
31. PureCircle USA, Inc.	GRN 473	Purified Steviol Glycosides – Reb M (Reb X) Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas.
32. GLG Life Tech Corp.	GRN 493	High purity steviol glycosides ≥95%	General-purpose sweetener, excluding meat, poultry products.
33. GLG Life Tech Corp.	GRN 512	High purity Reb M ≥95%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
34. Almendra Limited	GRN 516	Steviol Glycosides with Reb A and Stevioside as the Principal Components	General-purpose sweetener, excluding meat, poultry products & infant formulas.
35. GLG Life Tech Corp.	GRN 536	High purity Reb C and Steviol glycosides with Reb C as the Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas.
36. GLG Life Tech Corp.	GRN 548	High purity Reb D	General-purpose sweetener, excluding meat, poultry products & infant formulas.
37. Productora Alysa SpA	GRN 555	Steviol Glycosides with Reb A as the Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas.

[a] This table was derived, in part, from McQuate (2011).

[b] The name of this company is now GLG Life Tech Corporation.

[c] GRN 607, submitted by PureCircle, Ltd. regarding Glucosylated Steviol Glycosides (minimum purity 80%), was filed by FDA on November 24, 2015 and is presently under review. GRN 619, submitted by Pure Circle, Ltd. regarding purified steviol glycosides with rebaudioside A and stevioside as the principal components, was filed by FDA on February 2, 2016 and is presently under review. GRN 626, submitted by Cargill, Inc. regarding steviol glycosides produced in *Saccharomyces cerevisiae,* was filed by FDA on March 1, 2016 and is presently under review. GRN 632, submitted by DSM Nutritional Products, LLC. regarding Rebaudioside A from *Yarrowia lipolytica,* was filed by FDA on March 18, 2016 and is presently under review.

In addition, the Flavor and Extract Manufacturers Association (FEMA) has included several steviol glycoside preparations on their GRAS lists, as shown in Table 2.

Table 2. FEMA GRAS Status for Steviol Glycoside Preparations

Steviol Glycoside Preparation	FEMA Number	Reference
Rebaudioside A	4601	Smith et al. (2009)
Rebaudioside C; dulcoside B	4720	Leffingwell (2011)
Glucosyl steviol glycosides; enzymatically modified stevia extract	4728	Leffingwell and Leffingwell (2014); Marnett et al. (2013)
Stevioside	4763	Leffingwell and Leffingwell (2014); Marnett et al. (2013)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside A 60%	4771	Marnett et al. (2013)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside A 80%	4772	Marnett et al. (2013)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside C 30%	4796	Cohen, Fukushima, Gooderham, Hecht, Marnett, Rietjens, and Smith (2015); Cohen, Fukushima, Gooderham, Hecht, Marnett, Rietjens, Smith, et al. (2015)
Steviol glycoside extract, *Stevia rebaudiana,* Rebaudioside A 22%	4805	Cohen, Fukushima, Gooderham, Hecht, Marnett, Rietjens, and Smith (2015); Cohen, Fukushima, Gooderham, Hecht, Marnett, Rietjens, Smith, et al. (2015)
Steviol glycoside extract, *Stevia rebaudiana* Rebaudioside C 22%	4806	Cohen, Fukushima, Gooderham, Hecht, Marnett, Rietjens, and Smith (2015); Cohen, Fukushima, Gooderham, Hecht, Marnett, Rietjens, Smith, et al. (2015)

The Joint Expert Committee on Food Additives (JECFA) reviewed steviol glycosides at its 51st, 63rd, 68th and 73rd meetings. In 2000, JECFA published the original review on steviol glycosides (WHO, 2000). JECFA established a temporary ADI (acceptable daily intake) of 0-2 mg per kg (on a steviol basis) at its 63rd meeting (WHO, 2006). Additionally, JECFA finalized food grade specifications (FAO, 2007b), although they were subsequently updated in 2008 (FAO, 2008) and 2010 (FAO, 2010) (see below). At the 69th meeting, the temporary status of the ADI was removed, and the ADI was raised to 0-4 mg per kg bw per day (on a steviol basis) as a result of the JECFA review of more recently completed clinical studies with steviol glycosides (WHO, 2008). In 2009, JECFA published a final monograph addendum on steviol glycosides (WHO, 2009).

In early 2009, a number of parties, including the government of Australia and the Calorie Control Council, submitted a request to the Codex Committee on Food Additives in which it was proposed that the JECFA specifications for steviol glycosides should be modified to allow inclusion of rebaudioside D and rebaudioside F as specifically named acceptable glycosides that would be

considered as part of the minimum 95% steviol glycosides composition (CCFA, 2009). This proposed modification was endorsed by the Codex Alimentarius Committee in July 2009; it was on the agenda for discussion at the JECFA Meeting in June, 2010 (FAO/WHO, 2009), and JECFA subsequently took final action in approving the modified steviol glycosides specifications to include rebaudioside D and rebaudioside F (FAO, 2010).

In 2008, Switzerland's Federal Office for Public Health approved the use of stevia as a sweetener citing the favorable actions of JECFA (Switzerland Federal Office of Public Health, 2008). Subsequently, France published its approval for the food uses of rebaudioside A with a purity of 97% (AFSSA, 2009a, 2009b).

Also in 2008, the Food Standards Australia New Zealand (FSANZ) completed its evaluation of an application for use of steviol glycosides in foods. FSANZ recommended that the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) amend the Australia New Zealand Food Standards Code to allow the use of steviol glycosides in food (FSANZ, 2008). In December 2010, FSANZ recommended accepting the increased usage levels as requested since no public health and safety issues were identified (FSANZ, 2010). Subsequently, FSANZ approved an increase in the maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages, and flavored soy beverages up to 200 mg per kg, and in plain soy beverages up to 100 mg per kg (FSANZ, 2011). In a recent risk assessment, FSANZ concluded that the use of Reb M does not pose any "public health and safety issues" (FSANZ, 2015c). In addition, FSANZ proposed to add Reb M to the list of permitted steviol glycosides (FSANZ, 2015b). Reb M was subsequently approved by FSANZ for use as a Steviol Glycoside Intense Sweetener (FSANZ, 2015a). On January 14, 2016, Reb M was approved for use "as a food additive in accordance with the current permissions for steviol glycosides" (FSANZ, 2016).

As of May 2010, the government of Hong Kong amended its food regulations to allow the use of steviol glycosides as a permitted sweetener in foods (Hong Kong Centre for Food Safety, 2010). This action followed in the aftermath of the detailed safety evaluation and favorable findings as reported by JECFA.

On September 18, 2009, based on a review of the international regulation of *Stevia rebaudiana* and the clinical evidence for safety and efficacy, the Natural Health Products Directorate, Health Canada (2009) adopted the following guidelines for the use of stevia and steviol glycosides in Natural Health Products (NHPs) (HealthCanada, 2009). The revised recommendation for the maximum limit for steviol glycosides in NHPs is in accordance with the full ADI of 4 mg steviol per kg bw established by JECFA (WHO, 2008).

In light of JECFA's 2008 findings, and in response to a June 2008 request by the European Commission for European Food Safety Authority (EFSA) to deliver a scientific opinion on the safety of steviol glycosides as a sweetener for use in the food categories specified in the dossiers from

three petitioners, EFSA reexamined the safety of steviol glycosides (EFSA, 2010). After considering all the data on stability, degradation products, metabolism and toxicology, the EFSA Panel established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg per bw per day, which is similar to JECFA's determination.[2] In addition, on May 25, 2011, EFSA published a determination that the daily dietary intake for use of rebaudioside A as a flavoring substance in a variety of foods would be less than the ADI for steviol glycosides (EFSA, 2011a). In 2014, EFSA evaluated extending the use of steviol glycosides as ingredients in food categories including coffee, tea, and herbal and fruit infusions (assessed at 10 mg per L steviol glycosides). Exposure estimates were lower than those determined by the Panel in 2011 due to available data, and remained below the ADI of 4 mg per kg bw per day, with the exception of toddlers from one country at the 95th percentile exposure level of 4.3 mg per kg bw per day (EFSA, 2014). More recently, exposure estimates, based on maximum permitted levels (MPLs) and proposed use levels increased to 29 mg per L steviol glycosides, were found to have a "negligible" impact on dietary intake for all population groups, with the mean exposure estimate below the ADI of 4 mg per kg bw per day, with the exception of toddlers from one country at the 95th percentile exposure level of 4.3 mg per kg bw per day. The EFSA panel concluded that "dietary exposure to steviol glycosides (E 960) is similar to the exposure estimated in 2014 and, therefore, does not change the outcome of the safety assessment" (EFSA, 2015).

The international community continued to exhibit much interest in the food uses of steviol glycosides with additional advances reported in early July 2011. The Codex Alimentarius Commission has adopted proposed maximum use levels for steviol glycosides in all major food and beverage categories, and this action was expected to favorably influence authorizations of stevia uses in India, Indonesia, Thailand, and the Philippines (FoodNavigator, 2011). An article published online by FoodNavigator stated the following: "with approvals now in Vietnam, the Philippines, Malaysia, Singapore and Thailand, Indonesia is the only [Southeast Asian nation] where stevia hasn't been given the rubber stamp" (Whitehead, 2013). Furthermore, the International Alliance of Dietary/Food Supplement Associations (IADSA) reported that the Codex Alimentarius Commission agreed to adopt the use of steviol glycosides for addition to chewable food supplements as had been requested by IADSA (NewHope360, 2011).

The appropriate European regulatory bodies, including the joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA), have now agreed that steviol glycosides are safe for all populations to consume and are a suitable sweetening option for

[2] From a historical perspective, it is noted that the UK's Advisory Committee on Novel Foods and Processes for the Ministry of Agriculture, Fisheries and Food on September 24, 1998 rejected an application for use of steviol glycosides as a sweetener in herbal teas because "the applicant had not provided all of the information necessary to enable an assessment to be made" (MAFF, 1998). In 1999, the Scientific Committee on Food for the European Commission concluded that "there are no satisfactory data to support the safe use of these stevia plants and leaves" (EuropeanCommission, 1999a). In another opinion also dated June 17, 1999, the Committee also reiterated "its earlier opinion that stevioside is not acceptable as a sweetener on the presently available data" (EuropeanCommission, 1999b).

diabetics. Effective December 2, 2011, the EU approved their use as food additives (EU, 2011). In March, 2016, the EU approved the use of steviol glycosides in mustard (Michail, 2016).

On September 10, 2012, the South African Department of Health issued an amendment to labeling regulations indicating: “in the case of the sweetener steviol glycosides, it shall be described as ‘Steviol Glycosides’ or ‘Steviol Extract.’” On the same date, steviol glycosides were added to the List of Permissible Sweeteners (RSADH, 2012a, 2012b).

The Food Safety and Standards Authority of India (FSSAI) convened on September 20, 2012 approved the use of steviol glycosides as a non-nutritive sweetener in a variety of foods. The FSSAI specified that: the steviol glycosides must meet the specifications and purity as established by JECFA; table top sweetener tablets may contain 7 mg of steviol equivalents per 100 mg carrier/filler, as well as established maximum use levels specific to 11 distinct food categories including dairy, beverage, and chewing gum applications (FSSAI, 2012).

On November 30, 2012, Health Canada published its final clearance for use of steviol glycosides as a sweetener in foods (HealthCanada, 2012). In March 2014, Health Canada updated the List of Permitted Sweeteners (Lists of Permitted Food Additives) to include steviol glycosides in applications as a table-top sweetener, and as an ingredient in a variety of foods, beverages, baked goods, meal replacement bars, condiments, and confectionary and gums (HealthCanada, 2014). On January 15, 2016, Health Canada approved the use of Reb M for use as a high-intensity sweetener under the same conditions as the previously approved steviol glycosides (HealthCanada, 2016).

Since December 10, 2012, over thirty registrations have been granted by FDA Philippines to stand-alone steviol glycosides sweeteners or foods containing steviol glycosides as ingredients, including: FR-104390, Steviten Light Brand Steviol Glycosides 95% Sweetener Powder; FR-109427, Del Monte Pineapple Chunks in Extra Light Syrup Reduced Calorie with Steviol Glycosides from Stevia; FR-101120, Diebetamil Zero Calorie Sweetener with Stevia (stick pack); and FR-102127, Sawayaka Stevia Sweetener (1 gram sticks) (Philippines, 2014).

Steviol glycosides are also listed under INS number 960 in the Food Additives Permitted Under the Singapore Food Regulations document prepared by the Agri-Food & Veterinary Authority (AVA) of Singapore (AVA, 2014).

D. FDA Regulatory Framework

In order to be incorporated into conventional foods, food ingredients must undergo premarket approval by FDA as food additives or, alternatively, the ingredients must be determined to be generally recognized as safe (GRAS). The authority to make GRAS determinations is not restricted to FDA. In fact, GRAS determinations may be provided by experts who are qualified by scientific

training and experience to evaluate the safety of food and food ingredients under the intended conditions of use.[3]

In 1997, FDA altered the GRAS determination process by eliminating the formal GRAS petitioning process. At that time, the petitioning process was replaced with a notification procedure.[4] While outlining the necessary content to be considered in making a GRAS determination, FDA encouraged that such determinations should be provided to FDA in the form of a notification. However, notifying FDA of such determinations is strictly voluntary.

III. CHEMISTRY AND MANUFACTURE OF ENZYME MODIFIED STEVIOL GLYCOSIDES

A. Common or Usual Name

Enzyme modified steviol glycosides is the common or usual name of the non-nutritive sweetener derived from the enzymatic glycosylation of a high purity extract of *Stevia rebaudiana* Bertoni. The compositional features of the subject high purity enzyme modified steviol glycosides (≥ 95.0% total steviol glycosides) are described in more detail in this section. EMSG is the term used by GLG in referring to the notified substance. The preparation is also marketed as TasteBoost™ EMS95.

In the scientific literature, steviol glycosides have been referred to as stevia, stevioside, steviol glycosides, and stevia glycoside. JECFA adopted the term, steviol glycosides, for the family of steviol derivatives with sweetness properties that are derived from the stevia plant. Presently, the term, stevia, is used more narrowly to describe the plant or crude extracts of the plant.

B. Chemistry of Enzyme Modified Steviol Glycosides

Enzyme modified steviol glycosides are produced when additional glucose moieties are bonded to the original steviol glycoside structure via α(1→4) linkages, resulting in α-glucosylated steviol glycosides. The product α-glucosylated steviol glycosides consists of a mixture of both α-D-glucosylated steviol glycosides and steviol glycosides, including rebaudioside A, rebaudioside C, dulcoside A, steviolbioside, rubusoside, and rebaudioside B. The enzyme attaches the additional glucose residues by sterio- and regio-specific 1,4-α-D-glycosidic bonds, whereas the glucose is attached by β-glycosidic bonds in naturally occurring steviol glycosides. The primary constituents of enzymatically treated stevia have been identified (E. Koyama et al., 2003) and are described in Table 3, and the chemical structures are shown in

Figure 1.

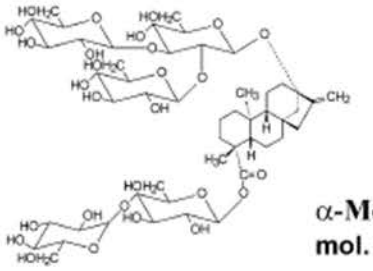
[3] See 21 CFR 170.3(i)(3).
[4] See 62 FR 18938, 17 April 1997. Available at: https://www.gpo.gov/fdsys/pkg/FR-1997-04-17/pdf/97-9706.pdf#page=1 (Accessed 2/4/16).

Table 3. Components Expected to be Present in Glucosylated Steviol Glycosides[a]

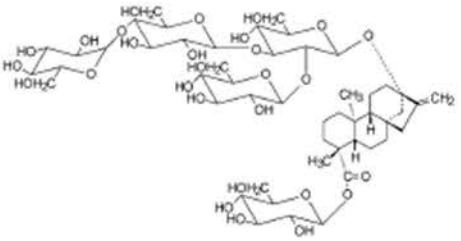
COMPOUND	MOLECULAR WEIGHT	EMPIRICAL FORMULA	LEVEL OF ENZYME GLYCOSYLATION[B]
Steviolbioside	642	$C_{32}H_{50}O_{13}$	--
Dulcoside A	788	$C_{38}H_{60}O_{17}$	--
Stevioside	804	$C_{38}H_{60}O_{18}$	--
Rebaudioside C	950	$C_{44}H_{70}O_{22}$	--
Rebaudioside A	966	$C_{44}H_{70}O_{23}$	--
Monoglucosyl Rebaudioside B	966	$C_{44}H_{70}O_{23}$	+1
Monoglucosyl Stevioside	966	$C_{44}H_{70}O_{23}$	+1
Monoglucosyl Rebaudioside C	1112	$C_{50}H_{80}O_{27}$	+1
Monoglucosyl Rebaudioside A	1128	$C_{50}H_{80}O_{28}$	+1
Diglucosyl Rebaudioside B	1128	$C_{50}H_{80}O_{28}$	+2
Diglucosylstevioside	1128	$C_{50}H_{80}O_{28}$	+2
Diglucosyl Rebaudioside C	1274	$C_{56}H_{90}O_{32}$	+2
Diglucosyl Rebaudioside A	1290	$C_{56}H_{90}O_{33}$	+2
Triglucosyl Rebaudioside B	1290	$C_{56}H_{90}O_{33}$	+3
Triglucosyl Rebaudioside A	1452	$C_{62}H_{100}O_{38}$	+3

[a] Data from E Koyama et al. (2003).
[b] The level of enzymatic glycosylation indicates the number of glucose units that have been added *via* enzyme treatment.

Figure 1. Chemical Structures of Various Steviol Glycosides[a]

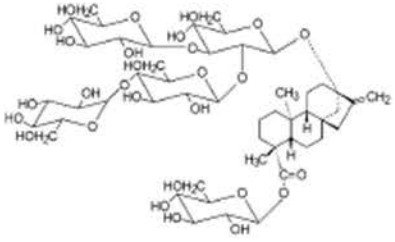


α-Monoglucosylrebaudioside A-1
mol. wt 1129

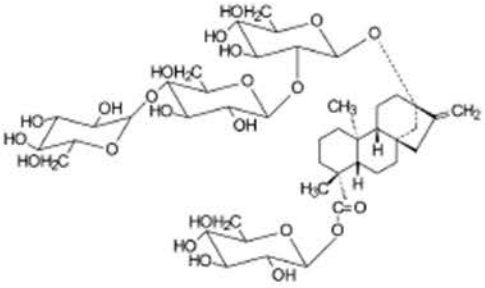


α-Monoglucosylrebaudioside A-3
mol. wt 1129

+

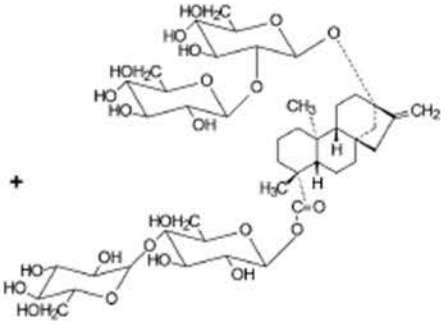


α-Monoglucosylrebaudioside A-2
mol. wt 1129

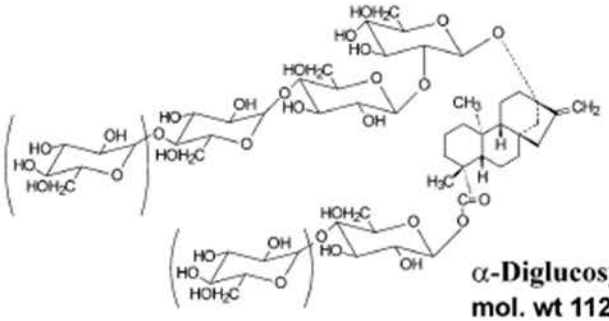


α-Monoglucosylstevioside-1
mol. wt 967

+



α-Monoglucosylstevioside-2
mol. wt 967



α-Diglucosylstevioside
mol. wt 1129

[a] From E Koyama et al. (2003).

In a number of reviews by different authors (J. M. C. Geuns, 2003; Kennelly, 2002; Kinghorn, 2002; Kinghorn & Soejarto, 1989), the structures of the components of steviol glycosides have been described. Through a series of chemical reactions and analyses, the structures, stereochemistry, and absolute configurations of steviol and isosteviol were established over a 20-year period after the seminal work of Bridel and Lavielle (1931) in France. The work by Ogawa et al. [1980, cited in (Brandle, Starratt, & Gijzen, 1998)] on synthetic transformation of steviol into stevioside supported the proposed structures. Two other sweet glycosides, Reb A and Reb B, were obtained from methanol extracts of stevia leaves, along with the major sweet principle constituent, stevioside, and a minor constituent steviolbioside, which was first prepared from stevioside by alkaline hydrolysis by Wood et al. [1955, cited in (Brandle et al., 1998)]. Subsequently, it was suggested that Reb B was an artifact formed from Reb A during isolation (Brandle et al., 1998; Kennelly, 2002). In addition, stevioside can be converted both chemically and enzymatically to Reb A. Further fractionation led to the isolation and identification of three other sweet glycosides, respectively named Reb C, Reb D, and Reb E. It was reported that Reb A and Reb D could be converted to Reb B by alkaline hydrolysis showing that only the ester functionality differed (Brandle et al., 1998). Dulcosides A and B were also described (Kobayashi, Horikawa, Degrandi, Ueno, & Mitsuhashi, 1977). Later, dulcoside B and Reb C were shown to be structurally identical.

More recently, Chaturvedula, Yu, and Mao (2013) reported isolating rebaudioside M, a minor component of total steviol glycosides in commercially available *Stevia rebaudiana* Bertoni extracts.

C. Background Information on Chemistry of Steviol Glycosides

At its 51st meeting, JECFA reviewed the safety related information on steviol glycosides, including the identity and chemistry of these compounds. The following chemistry related description of steviol glycosides is taken from the original JECFA monograph (WHO, 2000).

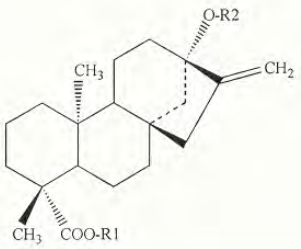
> Stevioside is a glycoside of the diterpene derivative steviol (ent-13-hydroxykaur-16-en-19-oic acid). Steviol glycosides are natural constituents of the plant *Stevia rebaudiana Bertoni*, belonging to the Compositae family. The leaves of *S. rebaudiana Bertoni* contain eight different steviol glycosides,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

In the Chemical and Technical Assessment (FAO, 2007a), JECFA identified the sweetener components. They updated the list of common glycosides and their chemical structures, which are

slightly different from compounds depicted in older publications (Nanayakkara et al., 1987; Suttajit, Vinitketkaumnuen, Meevatee, & Buddhasukh, 1993). They are shown in

Figure 1.

Figure 2. Chemical Structures of Various Steviol Glycosides[a,b]



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

[a] From FAO (2007a).

[b] The indicated C.A.S. No. for Rubusoside as reported in the cited reference is incorrect and should be 64849-39-4.

D. Accepted Identity Specifications for Food Grade Steviol Glycosides

In addition to the manufacturing process, the compositions of *Stevia rebaudiana* Bertoni extracts depend upon the composition of the harvested leaves, which, in turn, is influenced by soil, climate, etc. (FAO, 2007a). JECFA recommended that food grade specifications for steviol glycosides consist of a minimum of 95%, on a dried weight basis, of seven specific steviol glycosides (FAO,

2007b), and this has more recently been expanded to include the original seven specific steviol glycosides plus Reb D and Reb F (FAO, 2010). The predominant glycosides present in stevia leaves with significant sweetness are stevioside and Reb A. In addition to Reb D and Reb F, the other five glycosides are found at substantially lower levels in the preparations of steviol glycosides---and recognized by JECFA---are Reb C, dulcoside A, rubusoside, steviolbioside, and Reb B. Recently, there has been an increased interest in several of these and other steviol glycosides present at diminished levels, including Reb M, for their sweetening and flavor enhancing properties.

E. Manufacturing Processes for Enzyme Modified Steviol Glycosides

Manufacturing processes for stevia-derived sweeteners have been described in the published scientific and patent literature. These processes are summarized below.

1. Scientific and Patent Literature

In general, steviol glycosides are typically obtained by extracting leaves of *Stevia rebaudiana* Bertoni with hot water or alcohols (ethanol or methanol). This extract is a dark particulate solution containing all the active principles, plus leaf pigments, soluble polysaccharides, and other impurities. Some processes remove the “grease” from the leaves before extraction by employing solvents such as chloroform or hexane (Kinghorn, 2002). There are several extraction patents for the isolation of steviol glycosides. Kinghorn (2002) has categorized the extraction patents into those based on solvent, solvent plus a decolorizing agent, adsorption and column chromatography, ion exchange resin, and selective precipitation of individual glycosides. In recent patents, methods such as ultrafiltration, metallic ions, supercritical fluid extraction with CO_2, and extract clarification with zeolite have been employed.

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

The regio- and stereo-specific transglucosylation was applied for the improvement of the quality of sweetness of the steviol glycosides. The enzymatic transglucosylation of rubusoside, a steviol glycoside, yielded an improvement of its sweetness, disclosing some significance for the structure-sweetness relationship of steviolbisglycosides (Darise et al., 1984). Tanaka reviewed the literature on the sweetness of steviol glycosides that was significantly enhanced with modification of sugar moieties by enzymatic transglucosylation (Tanaka, 1997). Cyclomaltodextrin glucanotransferase (CGTase) efficiently catalyzes intermolecular glucosylation to transfer α-glucosyl units from starch

to 4-OH of a glucosyl moiety (trans-α-1,4-glucosylation). Research conducted by Kochikyan, Markosyan, Abelyan, Balayan, and Abelyan (2006) revealed that CGTase produced by mesophilic, thermophilic, alkaliphilic, and halophilic bacilli are effective for the transglucosylation of steviol glycosides when using starch as the donor. These authors also found that the method can be used successfully for direct transglucosylation of stevia extract without purification of its individual components.

2. GLG's Manufacturing Process for Enzyme Modified Steviol Glycosides

GLG's EMSG is manufactured through an enzymatic reaction with *Stevia rebaudiana* Bertoni extract (>95.0% total steviol glycosides, which meets JECFA specifications) using cyclomaltodextrin glucanotransferase. The resulting preparation is a high-purity enzyme modified steviol glycosides product (≥ 95% total steviol glycosides).

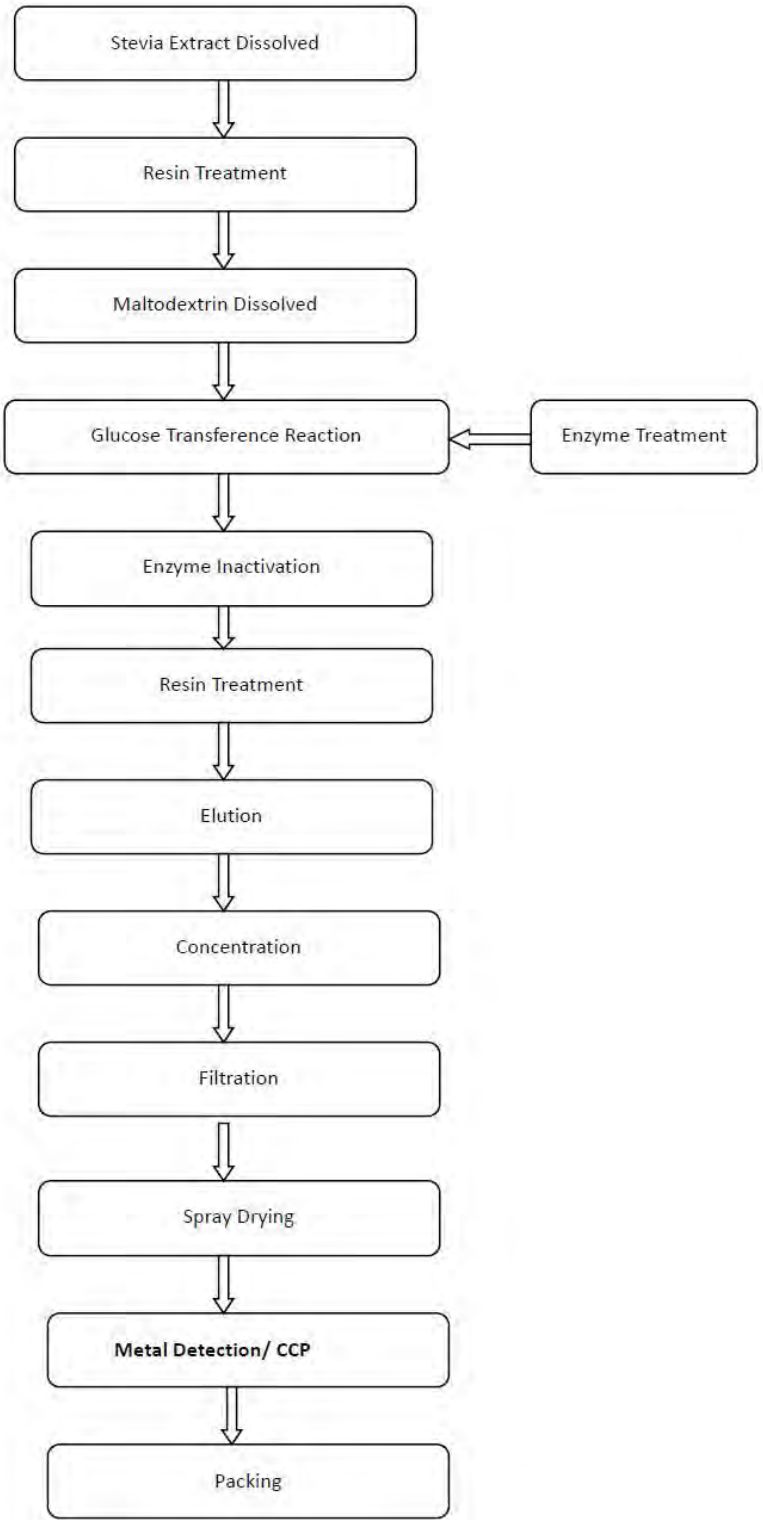
Maltodextrin is used as the glucose source. The ethanol used in the purification process complies with FCC's 9th Edition specifications. The ion exchange resins used in the manufacturing process comply with 21 CFR 173.65. GLG's EMSG is prepared in accordance with current Good Manufacturing Practices (GMP) regulations.

For the manufacturing of the starting steviol glycosides, GLG has developed a state-of-the-art process to extract steviol glycosides from the stevia leaf. In brief, steviol glycosides are obtained through a water extraction process. The extraction solution is passed through plate filtration, followed by two columns packed with anion exchange resin and cation exchange resin, and subsequently several columns packed with macroporous adsorption resin. The steviol glycosides adsorbed on the columns are eluted with food grade ethanol. The adsorbed solution is further purified with anion and cation resins, decolored with activated carbon, and concentrated with film evaporators. The concentrate is spray dried to obtain the primary stevia extract. The primary stevia extract is dissolved in food grade ethanol solution, crystallized, filtered, and spray dried. The total steviol glycosides (TSG) content in the purified stevia extract product is not less than 95%.

GLG uses the purified stevia extract product, maltodextrin, Toruzyme 3.0L[5], and purified water to manufacture its EMSG. After being heated to 80 ±1 °C at pH 5.5-6.0 for 18-24 hours, the mixed starting material solution is deactivated and adsorbed with macroporous adsorption resin, and eluted with food grade ethanol. The eluted solution is then further purified by passing through anion and cation resin columns, concentrating, and then spray drying. The final EMSG product contains more than 95% of total steviol glycosides (including reacted and unreacted steviol glycosides). Specifications for the raw materials and processing aids are provided in Appendix A.

[5] Novozymes has provided information that Toruzyme 3.0L complies with current FAO/WHO, JECFA, and FCC recommended purity specifications for food grade enzymes.

Figure 3. GLG's Manufacturing Process for TasteBoost™ EMS95



F. Product Specifications and Supporting Methods

1. JECFA Specifications for Steviol Glycosides

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

As reported in Section II.C, JECFA has been intimately involved over the past several years in the safety considerations of the steviol glycosides, and their deliberations have explicitly addressed requisite specifications for total steviol glycosides and component steviol glycosides. In summary, JECFA requires a minimum steviol glycosides composition of no less than 95% based on stevioside, Reb A, Reb C, dulcoside A, rubusoside, steviolbioside, Reb B, Reb D, and Reb F.

Furthermore, steviol glycosides are described as a white to yellow powder, odorless to having a slight characteristic odor, and exhibiting a sweetness that is 200-300 times greater than sucrose. The ingredient must consist of a minimum of 95% of nine specific steviol glycosides. The steviol glycosides are freely soluble in water and ethanol, and the 1 in 100 solutions exhibit pH values between 4.5 and 7.0. The product should not have more than 1% ash, with no more than a 6% loss on drying at 105^{o}C for 2 hours. Any residual methanol levels should not exceed 200 ppm, while ethanol residues should not exceed 5,000 ppm. Arsenic levels should not exceed 1 ppm as determined by the atomic absorption hydride technique. Lead levels should not exceed 1 ppm.

GLG has adopted product specifications for its purified steviol glycosides extract starting material that meet or exceed current JECFA specifications, as demonstrated in Table 4. Typical glycosides content of production batches is provided in Table 5.

Table 4. Specifications for Steviol Glycosides Starting Material

Physical & Chemical Parameters	JECFA[a] Specifications Steviol Glycosides	GLG Specifications Steviol Glycosides Starting Material
Appearance Form	Powder	Powder
Appearance Color	White to light Yellow	White/off-white
Solubility	Freely soluble in water	NS
Assay	Not less than 95% total steviol glycosides[b]	≥ 95.0%
Residual Ethanol	NMT 5,000 mg/kg	≤ 5,000 mg/kg
Residual Methanol	NMT 200 mg/kg	≤ 200 mg/kg

Physical & Chemical Parameters	JECFA[a] Specifications Steviol Glycosides	GLG Specifications Steviol Glycosides Starting Material
Loss on Drying	NMT 6.0%	≤ 4.0%
pH, 1% Solution	4.5-7.0	4.5-7.0
Total Ash	NMT 1%	≤ 1.0%
Arsenic	NMT 1 mg/kg	≤ 1.0 mg/kg
Lead	NMT 1 mg/kg	≤ 1.0 mg/kg

NS = not specified; NMT = not more than.

[a] Prepared at 73rd JECFA, 2010.

[b] Total steviol glycosides as the sum of stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside A, rubusoside, and steviolbioside.

Table 5. Levels of Steviol Glycosides in Untreated Stevia Extract & Enzyme Modified Stevia Extract

Component	Untreated Stevia Extract[A]	Enzyme Modified Stevia Extract[b]
Stevioside	24.5-25.0%	5.25-5.51%
Rebaudioside C	10.8-11.6%	0.48-0.67%
Rebaudioside A	60.5-61.1%	6.82-7.31%
Glucosylstevioside m/z 966	ND	35.1-36.2%
Glucosylstevioside m/z 1111	ND	2.3-2.8%
Glucosylstevioside m/z 1128	ND	22.4-23.1%
Glucosylstevioside m/z 1273	ND	1.7-1.9%
Glucosylstevioside m/z 1290	ND	10.6-11.2%
Glucosylstevioside m/z 1435	ND	1.0-1.2%
Glucosylstevioside m/z 1452	ND	5.2-6.1%
Glucosylstevioside m/z 1776	ND	1.2-1.7%

COMPONENT	UNTREATED STEVIA EXTRACT[A]	Enzyme Modified Stevia Extract[b]
Glucosylstevioside m/z 1614	ND	1.5-1.9%

ND = Not detected

2. Specifications for Enzyme Modified Steviol Glycosides With Supporting Methods

No established regulatory specifications were identified for food grade enzyme-modified steviol glycosides. However, multiple GRNs have based their specifications for their subject enzyme-modified steviol glycosides products on those determined by JECFA and the FCC. The specifications established by NOW Foods (GRN 337), Toyo and Nippon (GRN 375), and Daepyung (GRNs 448 and 452) are also based on JECFA and FCC specifications. A “no questions” letter was issued by FDA for each of these four notifications (FDA, 2011a, 2011b, 2013a, 2013b).

GLG has likewise adopted similar product specifications for its high purity EMSG preparation that meets or exceeds JECFA recommendations (FAO, 2010), while also complying with Food Chemicals Codex (FCC, 2010) specifications for rebaudioside A as a consumable human food substance. Analytical results for five product batches of TasteBoost™ EMS95 provided by GLG are compared in Table 6 to the specifications provided by JECFA and FCC. Results of analyses performed by GLG demonstrate that the five production batches of ESMG meet the designated specifications.

Details of the analytical methodology employed to determine steviol glycosides, including sample calculations, are provided in Appendix B, the chromatograms for representative EMSG preparations (pre- and post-deglycosylation) are provided in Appendix C, and certificates of analysis for five representative lots of EMSG are provided in Appendix D. Test reports for analysis of pesticide residues in a representative lot is located in Appendix E. The collection of these reports demonstrates that the substance is well characterized and meets the established purity criteria.

The typical nutritional content of GLG’s TasteBoost™ EMS95 is detailed in Table 7. The nutritional analysis report is provided in Appendix F.

Table 6. Specifications for Enzyme Modified Steviol Glycosides Preparations

Physical & Chemical Parameters	JECFA[a] Specifications Steviol Glycosides	FCC[b] Specifications Rebaudioside A	GLG Specifications Enzyme Modified Steviol Glycosides EMSG	Results of Batch Numbers				
				GLG-EMS95-20151201	GLG-EMS95-20151203	GLG-EMS95-20151206	GLG-EMS95-20151210	GLG-EMS95-20151212
Appearance Form	Powder	Crystal, granule or powder	Powder	Powder	Powder	Powder	Powder	Powder
Appearance Color	White to light Yellow	White to off-white	White/off-white	White	White	White	White	White
Solubility	Freely soluble in water	Freely soluble in water:ethanol (50:50)	Freely soluble in water, colorless and clear	PASS	PASS	PASS	PASS	PASS
Assay	≥ 95%	≥ 95%	≥ 95.0% Total Steviol glycosides ≥ 80% α-glucosylated steviol glycosides ≤ 15.0% unreacted steviol glycosides	95.2% 82.1% 13.1%	95.2% 82.4% 12.8%	95.3% 81.5% 13.8%	95.2% 82.1% 13.1%	95.3% 82.5% 12.8%
Residual Ethanol	NMT 5,000 mg/kg	NMT 0.5%	≤ 0.5%	PASS	PASS	PASS	PASS	PASS
Residual Methanol	NMT 200 mg/kg	NMT 0.02%	≤ 0.02%	PASS	PASS	PASS	PASS	PASS
Loss on Drying	NMT 6.0%	NMT 6.0%	≤ 4.0%	3.68%	3.32%	3.29%	3.38%	3.41%
pH, 1% Solution	4.5-7.0	4.5-7.0	4.5-7.0	5.25	5.38	5.30	5.19	5.36
Total Ash	NMT 1%	NMT 1%	≤ 1.0	0.08%	0.09%	0.09%	0.06%	0.08%
Arsenic	NMT 1 mg/kg	NMT 1 mg/kg	≤ 1.0 ppm	0.03 ppm	0.04 ppm	0.02 ppm	0.03 ppm	0.05 ppm
Lead	NMT 1 mg/kg	NMT 1 mg/kg	≤ 1.0 ppm	0.06 ppm	0.02 ppm	0.04 ppm	0.05 ppm	0.06 ppm
Total Plate Count (cfu/g, max)	NA	NA	< 1,000	< 10	< 10	< 10	< 10	< 10
Yeast & Mold (cfu/g, max)	NA	NA	< 100	< 10	< 10	< 10	< 10	< 10
Salmonella spp (in 25 g)	NA	NA	Negative	Negative	Negative	Negative	Negative	Negative
Staphylococus aureus (cfu/g)	NA	NA	< 10	< 10	< 10	< 10	< 10	< 10
E. coli (mpn/g)	NA	NA	< 3	< 3	< 3	< 3	< 3	< 3

[a] Prepared at 73rd JECFA, 2010.

[b] Rebaudioside A monograph. Food Chemicals Codex (7th Ed.). (FCC, 2010).

NS = not specified; NA = not applicable; NLT = not less than; NMT = not more than; ND = not detected.

Table 7. Typical Nutritional Content of GLG's TasteBoost™ EMS95[a]

COMPONENT	REPRESENTATIVE RESULTS
Protein	<0.1 g/100 g
Ash	0.15 g/100 g
Potassium	49.15 mg/kg
Sodium	225.83 mg/kg
Calcium	391.39 mg/kg
Iron	3.01 mg/kg
Phosphorus	<50 mg/100 g
Total fat	ND
Saturated fatty acid	ND
Saturated fat	ND
Mono-unsaturated fatty acid	ND
Mono-unsaturated fat	ND
Multi-unsaturated fatty acid	ND
Multi-unsaturated fat	ND
Trans fatty acid	ND
Cholesterol	ND
Fructose	ND
Glucose	ND
Sucrose	ND
Maltose	ND
Total Sugars	ND
Vitamin C	ND
Moisture	4.83 g/100g
Carbohydrate	95.0 g/100g
Energy	1,615 kJ/100 g

[a] Based on results reported for TasteBoost™ EMS95 Lot GLG-EMS95-20151201.

G. Stability Documentation

1. Stability Data on Steviol Glycosides

Steviol glycosides have been reported to be stable over the pH range 3-9 and can be heated at 100°C for 1 hour, but, at pH levels greater than 9, they rapidly decompose (Kinghorn, 2002). At pH 10, steviolbioside would be the major decomposition product produced from stevioside by alkaline hydrolysis (Wood, Allerton, DIEHL, & FLETCHER JR, 1955). Chang and Cook (1983) investigated the stability of pure stevioside and Reb A in carbonated phosphoric and citric acidified beverages. Some degradation of each sweetening component after 2 months of storage at 37°C was noted. However, no significant change at room temperature or below, following 5 months of storage of stevioside and 3 months of storage of Reb A, was noted. Exposure to one week of sunlight did not effect stevioside but did result in approximately 20% loss of rebaudioside A. Heating at 60°C for 6 days resulted in 0-6% loss of rebaudioside A (Chang & Cook, 1983).

Merisant (2008) conducted stability testing on rebaudioside A (1) as a powder, (2) as a pure sweetener in solution, and (3) on both cola-type and citrus carbonated beverages. In these investigations, no degradation was detected when the powder was stored at 105˚C for 96 hours. It was concluded that the powder was stable when stored for 26 weeks at 40 ± 2˚C with relative humidity of 75 ± 5%. Both published and unpublished testing results from Merisant revealed that rebaudioside A in carbonated citric acid beverages and phosphoric acid beverages did not significantly degrade during prolonged storage at refrigeration, normal ambient, or elevated ambient temperatures. Minimal loss of rebaudioside A was detected after storage at 60˚C, with considerable degradation noted after 13 hours at 100˚C for carbonated beverage solutions and pure sweetener solutions.

Cargill (2008) also conducted extensive stability testing on rebaudioside A as a powder under various storage conditions and under a range of pHs and temperatures. Additionally, Cargill also investigated rebaudioside A stability in several representative food matrices at room temperature and elevated temperatures. Stability profiles were created for table top sweetener applications, mock beverages including cola, root beer and lemon-lime, thermally processed beverages, yogurt, and white cake. The results of stability testing revealed some degradation products that had not been detected in bulk rebaudioside A. These degradation products were structurally related to the steviol glycosides that are extracted from the leaves of *Stevia rebaudiana* Bertoni*.* All the degradation products were found to share the same steviol aglycone backbone structure as found in stevioside and rebaudioside A, but they differ by virtue of the glucose moieties present. The results of stability testing revealed that rebaudioside A is stable in various food matrices following several days or weeks of storage. The extent and rate of degradation is dependent on pH, temperature, and time. When placed in beverages, rebaudioside A is more stable in the pH range 4 to 6, and at temperatures from 5˚C to 25˚C (Cargill, 2008). Photostability studies of the dry powder and mock beverages were performed to ascertain rebaudioside A behavior under defined conditions of fluorescent and near UV light exposure. Rebaudioside A was found to be photostable under the defined conditions of analysis (Clos, DuBois, & Prakash, 2008).

In addition to the above-described stability reports for purified rebaudioside A, in a GRAS notification by Sunwin and WILD Flavors (2010)---regarding purified steviol glycosides with rebaudioside A and stevioside as the principal components---stability was investigated using a 0.04% solution of Reb A 80% in acidic solutions between pH 2.81 and 4.18. In this study, the solutions were stored at 32°C for 4 weeks, and the Reb A content was determined at 1, 2, and 4 weeks. Reb A 80% was found to be very stable at pH 3.17 and above. At pH 2.81, after 4 weeks of storage under accelerated conditions, only a 7% loss of Reb A was noted. Sunwin and WILD Flavors also studied the stability of Reb A 80% in simulated beverages using 0.1% citric acid (pH 3.2). The solutions were pasteurized and stored for 8 weeks at 4°C and 32°C, and little difference in sweetness perception was observed under these conditions (Sunwin/WILD, 2010).

2. Stability Data on Enzyme Modified Steviol Glycosides

GLG conducted a shelf-stability test study on a representative lot of EMSG. Over the course of 24 months, samples were stored in original packaging at 25°C ± 5°C and a relative humidity of 60% ± 5%. The stability samples were tested for total steviol glycosides and microbial parameters. A summary of the shelf-stability results is presented in Table 8, and a detailed stability report is provided in Appendix G.

Table 8. GLG's EMSG Storage Stability Data[a]

Duration	Total Steviol Glycosides (%dry weight)	Total Plate Count (cfu/g)	Salmonella	*E. coli*	Staphylococcus
t = 0	95.9	<10	Negative	Negative	Negative
3 months	95.9	<10	Negative	Negative	Negative
6 months	95.8	<10	Negative	Negative	Negative
12 months	95.6	<10	Negative	Negative	Negative
18 Months	95.4	<10	Negative	Negative	Negative
24 months	95.2	10	Negative	Negative	Negative

[a] Results obtained for lot GLG-EMS95-20131201.

H. Sweetness Equivalence Enzyme Modified Steviol Glycosides

GLG conducted a sweetness equivalence evaluation to compare EMSG to sucrose at various concentrations. The results of this comparison show that EMSG, at a concentration of 0.039%, is estimated to be equivalent to a 5.0% sucrose solution. This suggests a sweetness intensity of approximately 130 times the sweetness of sucrose, which is consistent with previously reported values ranging from 100-150 times the sweetness of sucrose for other enzyme-modified steviol glycosides preparations (Daepyung, 2012a, 2012b; NOWFoods, 2010; Toyo & Nippon, 2011). The sweetness equivalence report for GLG's EMSG is provided in Appendix H.

I. Calculation of Steviol Equivalents

The steviol glycosides content of GLG's stevia extract starting material was analyzed by high pressure liquid chromatography (HPLC) to ensure that the stevia extract composition is compatible and consistent with JECFA specifications of greater than 95% steviol glycosides (FAO, 2010). The most abundant steviol glycosides were rebaudioside A, stevioside, and rebaudioside C, and the typical ranges of steviol glycosides in the starting material are provided in Table 9.

Table 9. Steviol Glycosides in Stevia Extract Starting Material

Component Steviol Glycoside	% Occurrence in Starting Material
Rebusoside	0.56-1.12
Dulcoside A	0.78-1.36
Stevioside	24.5-25.0
Rebaudioside C	10.8-11.6
Rebaudioside A	60.5-61.1

For comparative purposes, the content of steviol glycosides is often expressed as steviol or steviol equivalents. Each component steviol glycoside has a steviol equivalence factor that is calculated based upon the ratio of the molecular weights of steviol and a particular steviol glycoside, as shown in Table 10.

Table 10. Steviol Equivalency Factors for Various Steviol Glycosides

Component Steviol Glycoside	Molecular Weight	Steviol Equivalency Factor[a]
Rebusoside	643	0.495
Dulcoside A	788	0.404
Stevioside	804	0.395
Rebaudioside C	950	0.335
Rebaudioside A	966	0.329

[a] Calculated by dividing the molecular weight of steviol (MW=318) by the molecular weight of each glycoside.

Using these steviol equivalency factors, along with the percent composition of the stevia extract starting material, it is possible to determine the steviol equivalency of the stevia extract. Both the low and high levels of the components were used to determine the steviol equivalency values presented in Table 11. To summarize, the starting material stevia extract has a steviol equivalency of 33.791-34.966%.

Table 11. Steviol Equivalency of Stevia Extract Starting Material

Component Steviol Glycoside	Low-Level Steviol Equivalence	High-Level Steviol Equivalence
Rebusoside	0.277%	0.554%
Dulcoside A	0.315%	0.549%
Stevioside	9.677%	9.875%
Rebaudioside C	3.618%	3.886%
Rebaudioside A	19.904%	20.102%
Total Steviol Equivalence	33.791%	34.966%

The stevia extract starting material is then enzymatically glucosylated as discussed in Section III.E.2. The glucosylation process uses a glucosyltransferase enzyme to add glucose moieties, obtained from a maltodextrin source, to the steviol glycosides present in the stevia extract starting material. It is reasonable to assume that all steviol glycosides, and their subsequently glucosylated steviol glycosides, will maintain the same level of steviol equivalence described in Table 11, since no other reactions are known to occur with the glucosyltransferase enzyme. While the glucosylated steviol glycosides will have reduced steviol equivalents per mole due to the addition of glucose residues to the molecule, the steviol equivalence of the EMSG preparation can be calculated by simple dilution of maltodextrin. Thus, the addition of the stevia extract to the enzyme reactor containing maltodextrin and enzyme will reduce the steviol equivalency of the processed raw material through simple dilution. The enzyme modified steviol glycosides finished product will therefore have a steviol equivalency ranging from 16.896 and 17.483 grams steviol per 100 grams EMSG, as shown in Table 12.

Table 12. Steviol Equivalency of GLG's Enzyme Modified Steviol Glycoside Preparation

	Percent Steviol Equivalence in Starting Extract	Dilution Factor[a]	Percent Steviol Equivalence EMSG
Low-Level Steviol Equivalence	33.791%	0.500	16.896%
High-Level Steviol Equivalence	34.966%	0.500	17.483%

[a] Based on the ratio of stevia extract added to the enzyme reactor

IV. INTENDED FOOD USES AND ESTIMATED DIETARY INTAKE

A. Intended Uses

The subject GLG high purity enzyme modified steviol glycosides (≥ 95% total steviol glycosides) is intended to be used as a table top sweetener and general purpose non-nutritive sweetener in various foods other than infant formulas and meat and poultry products. The intended use as a non-nutritive sweetener is defined in 21 CFR 170.3(o)(19).[6] The intended use levels will vary by actual food category, but the actual levels are self-limiting due to organoleptic factors and consumer taste considerations. However, the amounts of GLG's EMSG preparation to be added to foods will not exceed the amounts reasonably required to accomplish its intended technical effect in foods as required by FDA regulation.[7]

B. Estimated Daily Intake of Enzyme Modified Steviol Glycosides

There have been many scholarly estimates of potential dietary intake replacement of sweeteners, including steviol glycosides, that have been published (FSANZ, 2008; A. G. Renwick, 2008; WHO,

[6] Non-nutritive sweeteners: Substances having less than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.
[7] See 21 CFR 182.1(b)(1).

2003) or submitted to FDA (Merisant, 2008). These are summarized in Appendix I. In GRAS notification 301, a simplified estimate was proposed to, and accepted by FDA, based on the estimates of exposure in "sucrose equivalents" (A. G. Renwick, 2008) and the sweetness intensity of any particular sweetener (BioVittoria, 2009). As summarized in GRN 301, the 90th percentile consumer of a sweetener which is 100 times as sweet as sucrose when used as a total sugar replacement would be a maximum of 9.9 mg per kg bw per day for any population subgroup.

The estimated sweetness intensity for GLG's EMSG is 130-fold that of sucrose (Appendix H). Therefore, the highest 90th percentile consumption by any population subgroup of GLG's EMSG preparation would consume approximately 7.62 mg per kg bw per day. Based on an estimate that EMSG preparations consist of approximately 16.896-17.483% steviol equivalents, the consumption would be less than 1.33 mg per kg bw per day on a steviol equivalents basis for any population group. These calculations are summarized in Table 13.

Table 13. Daily Intake of Sweeteners (In Sucrose Equivalents) & Estimated Daily Intakes of Enzyme Modified Steviol Glycosides

Population Group	Intakes of Sweeteners (mg sucrose/kg bw/day)[a]		Calculated Intake of EMSG (mg/kg bw/day)[b]		Calculated Intake of EMSG as Steviol Equivalents (mg/kg bw/day)[c]	
	Low	High	Low	High	Low	High
Healthy Population	255	675	1.96	5.19	0.34	0.91
Diabetic Adults	280	897	2.15	6.90	0.38	1.21
Healthy Children	425	990	3.27	7.62	0.57	1.33
Diabetic Children	672	908	5.17	6.98	0.90	1.22

[a] From A. G. Renwick (2008).

[b] Calculated by dividing the sucrose intake by the average relative sweetness value of 130 for EMSG.

[c] Calculated using maximum steviol equivalency for EMSG of 0.175, as determined in Table 12.

The values in Table 13 assume that EMSG constitutes the entire sweetener market, which makes these estimates extremely conservative since the likelihood of that occurrence is minimal. For the general healthy adult population, the estimated maximum intake of EMSG is 5.19 mg per kg bw per day, or 0.91 mg per kg steviol equivalents. For healthy children, the estimated maximal intake is 7.62 mg per kg bw per day, or 1.33 mg per kg as steviol equivalents. In all population groups, the estimate daily intake of EMSG, expressed as steviol equivalents, is well below the JECFA-established ADI of 4.0 mg per kg bw per day steviol equivalents.

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

For about 25 years, consumers in Japan and Brazil, where stevia has long been approved as a food additive, have been using stevia extracts as non-caloric sweeteners (Raintree, 2012). It was previously reported that 40% of the artificial sweetener market in Japan is stevia-based and that

stevia is commonly used in processed foods in Japan (Lester, 1999). Although there are no reported uses of rebaudioside A as a dietary supplement, use of steviol glycosides as a dietary supplement is presently permitted in the US, Canada, Australia, and New Zealand, and as a natural health product in Canada. It has wide use in China and Japan in food and in dietary supplements. In 2005, it was estimated that sales of stevia in the US reached $45 million (Newsday, 2006).

More recent reports of consumption figures for stevia reveal pronounced increases in global consumption. Worldwide, Zenith International estimates stevia sales of 3,500 metric tons in 2010, which represents a 27% increase over 2009 figures. The market value is estimated to have increased to $285 million (Zenith, 2011). In 2013, worldwide sales of stevia was reported to reach 4,100 tons which represents a 6.5% increase over 2011 figures, and this corresponds to an overall market value of $304 million (Zenith, 2013).

In October 2014, Zenith International reported that worldwide stevia sales were on course to increase 14% to 4,670 tons, associated with a market value of $336 million. Furthermore, it has been projected that the total market for stevia in 2017 will be 7,150 tons with an associated market value of $578 million (Zenith, 2014).

More recently, NewHope360 reported that the global market for stevia in 2014 was $347 million, and that is expected to increase to $565.2 million by 2020. In addition, consumption is expected to increase from 2014 levels of 5,100.6 tons to 8,506.9 tons by 2020 (NewHope360, 2015).

Hawke (2003) reported that stevia is commonly used as a treatment for type 2 diabetes in South America**.** However, for its therapeutic effects, elevated doses in the range of 1 gram per person per day or more were reported to be necessary (Gregersen, Jeppesen, Holst, & Hermansen, 2004).

V. SAFETY INVESTIGATIONS FOR STEVIOL GLYCOSIDES AND ENZYME MODIFIED STEVIOL GLYCOSIDES

A. Safety Studies on Steviol Glycosides: Recent Reports & Reviews by Expert Bodies & Other Scientists

The biological, toxicological, and clinical effects of stevia and steviol glycosides have been extensively reviewed (M. C. Carakostas, Curry, Boileau, & Brusick, 2008; J. M. C. Geuns, 2003; Huxtable, 2002). Additionally---and as noted earlier---the national and international regulatory agencies have thoroughly reviewed the safety of stevia and its glycosides. Most notably, over the years, JECFA has evaluated purified steviol glycosides multiple times (WHO, 2000, 2006, 2007, 2008), and their findings have been summarized in Section II.C. FSANZ (2008) also evaluated steviol glycosides for use in food. The JECFA reviews, as well as the other reviews completed before 2008, primarily focused on mixtures of steviol glycosides. These studies are summarized in Appendix J. Since the JECFA evaluation (WHO, 2008), more than thirty GRAS notifications for steviol glycosides or enzyme modified steviol glycosides were submitted to FDA, all of which were

determined to be GRAS based largely on the ADI established by JECFA. Among these notifications, several assessed purified preparations of rebaudioside A, which were supported by additional toxicology and clinical studies that are summarized in Appendix L. To date, 37 of the submitted notifications have had "no questions" letters of response from FDA (see Table 1).

GLG's enzyme modified steviol glycosides (≥ 95% total steviol glycosides) preparation contains not less than 80% α-glucosylated steviol glycosides. Given the structural similarities with rebaudioside A, stevioside, and other steviol glycosides, and considering analogous metabolic pathways for all these substances, the safety data on stevia and its other components have a direct bearing on the present safety assessment for TasteBoost™ EMS95. This is further supported by a decade and a half of scientific studies on the safety of these substances along with fact that the major regulatory bodies views the results of toxicology studies on either stevioside or rebaudioside A as applicable to the safety assessment of all known steviol glycosides, since all are metabolized and excreted by similar pathways, with steviol being the common metabolite for each.

B. Safety Studies on Enzyme Modified Steviol Glycosides

The safety of GLG's enzyme modified steviol glycosides can be supported by both the structural and metabolic similarities of its mixture of naturally occurring steviol glycosides and α-glucosylated analogs. Due to these similarities, the use of both published and unpublished studies on the metabolic and scientific data for steviol glycosides, α-glucosylated steviol glycosides and non-enzymatically modified steviol glycosides, is relevant to the present safety assessment. The primary documentation for EMSG safety consists of a combination of several studies on α-glucosylated steviol glycosides and evidence supporting the safety of rebaudioside A and other steviol glycosides. The foundational safety of Reb A, other steviol glycosides and steviol has been summarized in Section V.A, with key studies detailed in Appendices J-M.

It is important to note that steviol glycosides are not readily absorbed from the upper small intestine (Gardana, Simonetti, Canzi, Zanchi, & Pietta, 2003; E Koyama et al., 2003). A. M. Hutapea, Toskulkao, Buddhasukh, Wilairat, and Glinsukon (1997) and J. M. Geuns, Buyse, Vankeirsbilck, and Temme (2007) reported that human digestive enzymes are not capable of hydrolyzing β-glycosidic bonds.

E Koyama et al. (2003) published an *in vitro* study in which α-glucosylated steviol glycosides were degraded by fecal microflora to steviol glycosides. These are subsequently hydrolyzed to the aglycone steviol, demonstrating that the metabolic fate of α-glucosylated steviol glycosides follows that of non-modified steviol glycosides. This was confirmed by NOW Foods in an unpublished *in vitro* study using human fecal homogenate, which was submitted to FDA, as found in GRN 337. Due to the similarities in metabolic fate, the safety of α-glucosylated steviol glycosides can be established based on studies conducted with non-modified steviol glycosides. Furthermore, as individual steviol glycosides show similar pharmacokinetics and metabolic pathways in humans,

the results of toxicology studies on individual steviol glycosides are applicable to the safety for steviol glycosides in general.

Specific unpublished toxicology results on α-glucosylated steviol glycosides data were presented in GRN 375 (Toyo & Nippon, 2011) for a 13-week dietary study in rats receiving 1.25, 2.5, or 5.0% α-glucosylated steviol glycosides (0, 253, 519, or 1,059 mg steviol equivalents per kg bw per day for males and 1, 289, 601, or 1,153 mg steviol equivalents per kg bw per day for females, respectively). It was concluded that, under the conditions of the study, the NOAEL of dietary exposure to α-glucosylated steviol glycosides for 13 weeks was 1,059 and 1,153 mg per kg bw per day for males and females, respectively. This was consistent with the findings of the subchronic toxicity studies conducted with non-modified steviol glycosides. GRN 375 also reports a lack of genotoxic activity in both *in vitro* and *in vivo* studies (Toyo & Nippon, 2011).

Based on these data, four glucosylated steviol glycosides preparations have been determined to be GRAS by independent expert panels and have received "no questions" responses from FDA: GRN 337 (NOWFoods, 2010), GRN 375 (Toyo & Nippon, 2011), GRN 448 (Daepyung, 2012a), and GRN 452 (Daepyung, 2012b).

VI. GRAS CRITERIA AND PANEL SAFETY FINDINGS

A. GRAS Criteria

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

> "…reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use. It is impossible in the present state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any substance."[8]

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

Furthermore, in discussing GRAS criteria, FDA notes that:

> "…General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food."

[8] See 21 CFR 170.3(i)

> "General recognition of safety through experience based on common use in food prior to January 1, 1958, shall be based solely on food use of the substance prior to January 1, 1958, and shall ordinarily be based upon generally available data and information."[9]

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following component elements:[10]

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and,

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

The apparent imprecision of the terms "appreciable," "at the time," and "reasonable certainty" demonstrates that the FDA recognizes the impossibility of providing absolute safety in this or any other area (Lu, 1988; A. G. Renwick, 1990; Rulis & Levitt, 2009).

As noted below, this safety assessment to ascertain GRAS status for enzyme modified steviol glycosides for the specified food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

B. Discussion on Safety Studies of High Purity Enzyme Modified Steviol Glycosides

Based on fundamental toxicological principles, there is a presumption that EMSG is safe under the anticipated food use conditions. The natural steviol glycosides, which are enzymatically altered in the manufacturing process for EMSG, are deemed to be safe as discussed elsewhere in this dossier. The enzymatic process merely adds additional glucose residues to the naturally occurring steviol glycosides that serve as the JECFA-compliant starting material. The larger molecular weight glucosides are not expected to be absorbed from the GI tract *per se,* since there is convincing evidence that the smaller glycoside molecules are not absorbed. These additional glucose residues are not expected to be non-enzymatically cleaved in the GI tract because enzyme modified steviol glycosides have been shown to be stable under acidic and basic conditions. If these residues were cleaved in the small intestine, the end products would likely be the familiar naturally occurring steviol glycosides encountered from the stevia plant. These naturally occurring steviol glycosides would subsequently be converted to steviol in the large intestine.

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

E. Koyama et al. (2003) has previously demonstrated that any higher molecular weight glycosides that reach the large intestine will slowly be converted to steviol. FDA GRAS submissions GRN 337 (NOWFoods, 2010) and GRN 375 (Toyo & Nippon, 2011) both extensively examined the safety of very similar enzyme modified steviol glycosides to be used as general purpose non-nutritive sweeteners where the starting material included a high percentage of rebaudioside A. In both of these submissions, the enzyme modified steviol glycosides were found to be GRAS by their respective panels and were followed by FDA "no questions" letters (FDA, 2011a, 2011b). The Panel concludes that EMSG molecules are safe for their intended food uses based on the established metabolic pathway in the GI tract and the numerous safety reviews discussed more fully in Appendix J that show that the intended consumption of naturally occurring steviol glycosides is safe.

The GLG EMSG product identified in the subject notification meets the purity standard equivalence of 95% compared to the JECFA specifications for purity. In particular, the Panel recognizes that the steviol glycosides mixture that serves as the starting material in the production of EMSG meets the steviol glycosides JECFA specifications in that the nine specific glycosides constitute 95% or more of the dry weight. Appropriate safety documentation has been supplied regarding pre- and post-enzymatic conversion processes, as described in Sections III.E and III.F. Furthermore, EMSG is manufactured by a process that complies with FDA Good Manufacturing Practices regulations, and GLG maintains a rigorous set of chemical and microbiological specifications to assure that safe products are generated. The Panel concludes that the EMSG finished preparation is a carefully manufactured food grade product.

C. Discussion of Safety Data on High Purity Steviol Glycosides

Because of their sweetness characteristics, steviol glycosides have viable uses as a non-nutritive sweetener in foods.[11] Periodic reviews by JECFA over the years indicate the progression of knowledge on the safety of steviol glycosides. Several early safety-related studies on these compounds were performed on crude extracts of stevia. As noted earlier, these studies also included multiple investigations with *in vivo* and *in vitro* models, which explored the biological activity of stevia extracts at high doses or high concentrations. These early investigations raised several concerns, including impairment of fertility, renal effects, interference with glucose metabolism, and inhibition of mitochondrial enzymes. In recent years, as more and more studies were performed on purified glycosides, the toxicology profile of steviol glycosides eventually

[11] It has also been reported that steviol glycosides may have pharmacological properties, which can be used to treat certain disease conditions such as hypertension and type 2 diabetes. Chatsudthipong and Muanprasat (2009), as well as others, have published reviews where they note that such therapeutic applications have not been firmly established as being due to steviol glycosides. The reviewers point out that the effects occur at higher doses than would be used for sweetening purposes. Furthermore, many effects noted in older studies may have been due to impurities in preparations that do not meet the contemporary purity specifications established by JECFA for use as a sweetener. If oral doses of steviol glycosides impart pharmacological effects, such effects would undoubtedly occur due to actions of the principal metabolite, steviol, but the pharmacological effects of steviol have not been comprehensively investigated. For more a more comprehensive discussion of this subject, see Section 7 of Appendix K.

proved to be rather unremarkable. A number of subchronic, chronic, and reproductive studies have been conducted in laboratory animals. These studies were well designed with appropriate dosing regimens and adequate numbers of animals to maximize the probability of detection of important effects. Notably, the initially reported concerns related to the effects of stevia leaves or crude extracts on fertility were refuted by the well-designed reproductive studies with purified steviol glycosides. All other concerns failed to manifest themselves at the doses employed in the long-term rat studies.

As discussed in Appendix J and elsewhere, at its 51st meeting, JECFA determined that there were adequate chronic studies in rats---particularly the study by Toyoda et al. (1997)---that demonstrated an adequate NOAEL and no evidence of any carcinogenic activity to establish a temporary ADI of 0 - 2 mg per kg bw per day with an adequate margin of safety. The Committee also critically reviewed the lack of carcinogenic response in other well-conducted studies (Xili et al., 1992; Yamada, Ohgaki, Noda, & Shimizu, 1985). These studies justified the Committee conclusion that the *in vitro* mutagenic activity of steviol did not present a risk of carcinogenic effects *in vivo* and, therefore, all common steviol glycosides that likely share the same basic metabolic and excretory pathway and that use high purity preparations of various steviol glycosides, are safe as a sugar substitute. Subsequently, the additional clinical data reviewed by JECFA, and later published by L. A. Barriocanal et al. (2008), eliminated concerns for effects on blood pressure and blood glucose levels to allow the Committee to establish a permanent ADI of 0 - 4 mg per kg bw per day (based on steviol equivalents). The GRAS Expert Panel critically reviewed the JECFA assessment and agrees with the calculation of the ADI for steviol glycosides.

Several published and unpublished studies (summarized in Appendix L) on purified preparations of rebaudioside A showed an absence of toxicological effects in rats (Curry & Roberts, 2008; Nikiforov & Eapen, 2008) and dogs (Eapen, 2008) in subchronic studies, and an absence of reproductive (Curry, Roberts, & Brown, 2008; Sloter, 2008a) and developmental effects (Sloter, 2008b) in rats. Clinical studies on purified rebaudioside A showed an absence of effects on blood pressure (Maki, Curry, Carakostas, et al., 2008) and blood glucose levels (Maki, Curry, Reeves, et al., 2008) at doses comparable to the exposures expected in food. Most notably, pharmacokinetic studies in rats (A Roberts & Renwick, 2008) and humans (Wheeler et al., 2008) on purified rebaudioside A follow the same pathway of being degraded to steviol by intestinal bacteria with subsequent rapid glucosylation and elimination in urine and feces. The Panel concludes that these studies on rebaudioside A strengthen the argument that all steviol glycosides that follow the same metabolic pathway are safe at the JECFA established ADI.

The Panel has reviewed the findings from human clinical studies. The Panel noted that, regarding the clinical effects reported in humans, in order to corroborate the observations in these studies that these effects of steviol glycosides only occur in patients with either elevated blood glucose or blood pressure (or both), JECFA called for studies in individuals that are neither hypertensive nor diabetic (WHO, 2006). The supplemental data presented to JECFA and also published by L. Barriocanal et al. (2006) demonstrate the lack of pharmacological effects of steviol glycosides at

11 mg per kg bw per day in normal individuals, or approximately slightly more than 4 mg per kg bw on the basis of steviol equivalents. It is possible that JECFA may also have reviewed the preliminary results associated with the published clinical studies on rebaudioside A (Maki, Curry, Carakostas, et al., 2008; Maki, Curry, Reeves, et al., 2008). The Panel concludes that there will be no effects on blood pressure and glucose metabolism in humans at the doses of rebaudioside A or related steviol glycosides, including enzyme-modified preparations, expected from their use in food as a non-nutritive sweetener.

Two recent studies summarized in Appendix K raised a potential concern regarding the toxicological effects of steviol glycosides. In one study, DNA damage was seen in a variety of organs as assessed by Comet assay in rats given drinking water containing 4 mg per mL steviol glycosides for up to 45 days (A. Nunes et al., 2007). Several experts in the field have since questioned the methodology used in this study (Brusick, 2008; J. M. Geuns, 2007a; G. M. Williams, 2007). The Panel has reviewed the cited publications, along with the responses made by the authors (A. P. M. Nunes et al., 2007a, 2007b), and concurs with the challenges to the methodology utilized by A. Nunes et al. (2007), thereby discounting the validity and importance of this study.

In another study with stevioside in rats, tartrate-resistant alkaline phosphatase (TRAP) levels were measured and found to be significantly decreased at doses as low as 15 mg per kg bw (Awney, Massoud, & El-Maghrabi, 2011). TRAP is an enzyme that is expressed by bone-resorbing osteoclasts, inflammatory macrophages, and dendritic cells. This enzyme was not measured in any previous toxicology studies on steviol glycosides, nor has it been adequately vetted for application in toxicological studies. Critical reviews of this study by M. Carakostas (2012) and (Waddell, 2011) revealed a poor study design that included: insufficient numbers of animals; group-housing with the potential for stress-related changes; unreliable access to steviol *via* drinking water resulting in suspect dosing calculations in group-housed cages; no indication of fasting prior to blood collection (which affects many chemistry and hematological values); no urine collection; and no histopathological evaluations for confirmation of findings beyond the controls. Additionally, the report did not adequately describe mean or individual organ weight data, and it lacked comparison of study findings against laboratory historical control data.

In a recent review, J. Urban, Carakostas, and Brusick (2013) examined the extensive genotoxicity database on steviol glycosides because some concern has been expressed in two recent publications (Brahmachari, Mandal, Roy, Mondal, & Brahmachari, 2011; Tandel, 2011) in which the authors concluded that additional testing is necessary to adequately address the genotoxicity profile (J. Urban et al., 2013). The review aimed to address this matter by evaluating the specific genotoxicity studies of concern, while evaluating the adequacy of the database that includes more recent genotoxicity data not noted in these publications. The results of this literature review showed that the current database of *in vitro* and *in vivo* studies for steviol glycosides is robust, and does not indicate that either stevioside or rebaudioside A are genotoxic. This finding, combined with a paucity of evidence for neoplasm development in rat bioassays, establishes the safety of all steviol glycosides with respect to their genotoxic/carcinogenic potential.

The Expert Panel agrees with the safety conclusions of the 37 GRAS Expert Panels in the notifications for steviol glycosides previously submitted to FDA that resulted in "no questions" responses from FDA (as summarized in Table 1), JECFA (WHO, 2006, 2008), and A. G. Renwick (2008) that a sufficient number of good quality health and safety studies exist to support the determination that purified preparations of steviol glycosides when added to food at levels up to full replacement of sucrose on a sweetness equivalency basis meet FDA's definition of safe.

D. Panel Findings on Safety of Enzyme Modified Steviol Glycosides

Based on fundamental toxicological principles, in concert with the supporting safety data on structurally similar steviol glycosides and the safety studies on the EMSGs reported herein, along with a thorough review of GLG's manufacturing process, food grade specifications, and Certificates of Analysis that support reproducibility and quality of subject evaluation, GLG's high purity EMSG is considered to be safe under the anticipated food use conditions. The major naturally-occurring steviol glycosides, due to their structural and metabolic similarities to the studies discussed more fully in Appendices J-M are deemed to be safe based in large measure on the fact that they are metabolized to steviol.

Based on the discussions in Sections IV.B and IV.C, and the summarized studies found in Appendices J through M, the Panel agrees that GLG's EMSG preparation is sufficiently similar to those used in all key studies conducted on enzyme modified steviol glycosides, and those on other enzyme modified steviol glycosides preparations previously reviewed by FDA, and there is no need for further safety studies to be conducted on the GLG EMS95 product. The Panel has also reviewed the expected levels of dietary intake and agrees that there is sufficient information to conclude that the subject EMSG preparation can be safely used as a table top sweetener and as a general purpose non-nutritive sweetener in various foods other than infant formulas and meat and poultry products.

E. Acceptable Daily Intake for GLG's Enzyme Modified Steviol Glycosides

The Panel concludes that it is reasonable to apply the JECFA ADI of 4 mg per kg bw per day for steviol glycosides (expressed on a steviol basis) to EMSG. Therefore, with the steviol equivalence values shown in Table 13, the Panel concludes that, for the general population, the estimated maximum daily intake of EMSG is 5.19 mg per kg bw or 0.91 mg per kg expressed as steviol equivalents. Based upon these calculations, the intake of EMSG safely aligns with the 4 mg per kg bw per day ADI expressed as steviol equivalents as determined by JECFA.

F. Common Knowledge Elements for GRAS Determinations

The first common knowledge element for a GRAS determination requires that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing studies published in peer-reviewed scientific journals. The second common knowledge

element for a GRAS determination requires that consensus exists within the broader scientific community.

1. Generally Available Information

The majority of the studies reviewed on steviol glycosides and steviol have been published in the scientific literature as summarized in Appendices K, L, and M. Most of the literature relied upon by JECFA has also been published, most importantly the chronic rat studies on steviol glycosides. JECFA did make limited use of unpublished studies, and they were summarized in the two JECFA monographs. Moreover, JECFA publicly releases the results of their safety reviews, and their meeting summaries and monographs are readily available on their website.

With regard to the safety documentation, the key pharmacokinetic data establish that EMSG is metabolized similarly to natural steviol glycosides; EMSG are not absorbed through the GI tract, *per se*; they are converted to steviol by bacteria normally present in the large intestine, and the steviol is absorbed but rapidly metabolized and excreted. It has been well-established experimentally from various published studies that EMSG and steviol glycoside molecules are not absorbed from the GI tract (Gardana et al., 2003; E. Koyama et al., 2003). The action of bacteria in the large intestine is directly supported by the published study that steviol glycosides can be converted to steviol in the large intestine by normal anaerobic GI flora as demonstrated by an *in vitro* study in fecal homogenates (E Koyama et al., 2003; A. Renwick & Tarka, 2008). The ADI for steviol glycosides has been set largely based on published chronic study in rats (Toyoda et al., 1997) and several published clinical studies that there are no pharmacological effects in humans at doses several fold higher than the ADI (L. Barriocanal et al., 2006; L. A. Barriocanal et al., 2008; Wheeler et al., 2008). The toxicity of the metabolite steviol has been well reviewed in the published literature (J. M. C. Geuns, 2003; J. Urban et al., 2013; WHO, 2006). Unpublished studies on EMSG for toxicity in rats and metabolic conversion in human fecal homogenates are corroborative to the published information.

2. Scientific Consensus

The second common knowledge element for a GRAS determination requires that there must be a basis to conclude that consensus exists among qualified scientists about the safety of the substance for its intended use. The Panel maintains that well-qualified scientists would conclude that EMSG is not absorbed from the GI tract, *per se.* By virtue of fundamental principles of pharmacokinetics, the majority of scientists would support this determination, and they would likewise concur that EMSG ultimately undergoes a conversion to steviol as is known to be the case with the other naturally occurring steviol glycosides.

A number of well-respected regulatory agencies, including JECFA, EFSA, FSANZ, the Switzerland Office of Public Health, and HealthCanada have indicated that steviol glycosides are safe for human consumption at doses in the range of the JECFA ADI (EFSA, 2010; FAO, 2010; FSANZ,

2008; J. M. C. Geuns, 2003; HealthCanada, 2012; Switzerland Federal Office of Public Health, 2008; Toyoda et al., 1997; G. M. Williams, 2007; Xili et al., 1992). We also note that, since December 2008, more than thirty GRAS notifications have been submitted to FDA for stevia-derived sweetener products, and FDA detailed reviews have consistently yielded "no questions" letters.

In summary, a compelling case can be made that scientific consensus exists regarding the safety of EMSG, as well as the other steviol glycosides, when of sufficiently high purity. The central role of conversion to steviol and subsequent elimination with these naturally occurring steviol glycosides extends to the manner in which EMSG molecules are metabolized and eliminated from the body. Due to the similarities in metabolic fate, the safety of EMSG can be established based on studies conducted with non-modified steviol glycosides. While the scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, the Panel believes that a wide consensus does exist in the scientific community to support a GRAS conclusion as evidenced by several publications (Brusick, 2008; M. Carakostas, 2012; J. M. Geuns, 2007a; J. Urban et al., 2013; Waddell, 2011; G. M. Williams, 2007) that refute safety concerns expressed by a minority of scientists. Furthermore, FDA has reviewed four notifications regarding enzyme modified stevia preparations which yielded "no questions" letters, which further supports a scientific consensus of safety for EMSG.

VII. CONCLUSIONS[12]

In consideration of the aggregate safety information available on EMSG and the naturally occurring steviol glycosides, the Panel concludes that EMSG is safe for use as a general purpose non-nutritive sweetener in foods other than infant formulas and meat and poultry products. Based on the information that EMSG exhibits similar pharmacokinetics to the other naturally occurring steviol glycosides, the JECFA ADI for steviol glycosides of 4 mg per kg bw per day (as steviol equivalents) can be applied to EMSG. In light of published dietary exposure data for other approved sweeteners and adjusting for relative sweetness intensity, the intake of enzyme modified steviol glycosides was estimated for healthy non-diabetic children and adults, and diabetic children and adults with the following findings.

The estimated intakes of EMSG for several population groups summarized in Table 13 are no greater than 1.33 mg per kg bw per day expressed as steviol equivalents, which is below the ADI of 4 mg per kg bw expressed as steviol equivalents as established by JECFA. The Panel finds that

[12] The detailed educational and professional credentials for two of the individuals serving on the Expert Panel can be found on the GRAS Associates website at www.gras-associates.com. Drs. Kraska and McQuate worked on GRAS and food additive safety issues within FDA's GRAS Review Branch earlier in their careers and subsequently continued working within this area in the private sector. Dr. Emmel has substantial food safety experiences in addressing steviol glycosides and other food ingredients. All three panelists have extensive technical backgrounds in the evaluation of food ingredient safety and in participating in the deliberations of GRAS Expert Panels. Dr. Kraska served as Chair of the Panel.

the dietary levels from anticipated food consumption will not exceed the ADI when EMSG is used as a general non-nutritive sweetener.

The Panel also finds that the 95% total steviol glycosides purity specification for EMSG is sufficient in view of the accepted JECFA specification for 95% purity for other naturally occurring steviol glycosides. The Panel concludes that EMSG, as manufactured by GLG, is an appropriate food grade ingredient and that adverse pharmacological effects are not likely to occur at this designated ADI level. Furthermore, even high consumers of steviol glycosides are not likely to exceed this specified ADI. Therefore, the Panel concludes that EMSG, when consumed in foods as described within this GRAS notification, is generally recognized as safe (GRAS) within the meaning of the Food, Drug, and Cosmetic Act.

GLG Life Tech Corporation's Enzyme Modified Steviol Glycosides, referred to as EMSG and TasteBoost™ EMS95, when produced in accordance with FDA Good Manufacturing Practices requirements and when meeting at a minimum the JECFA purity specifications for steviol glycosides, is Generally Recognized As Safe when consumed as a non-nutritive sweetener in foods other than infant formulas and meat and poultry products within the JECFA ADI of 4 mg per kg bw per day on a steviol equivalent basis. In order to remain within the designated ADI, it is important to observe good manufacturing practices principles in that the quantity of a substance added to food should not exceed the amount reasonably required to accomplish its intended technical effect.

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

(b) (6)

Richard Kraska, Ph.D., DABT

(b) (6)

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(b) (6)

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Appendix A Specifications & Certificates of Analysis for Production Processing Aids

A-1 GLG Specifications for Ethanol
A-2 GLG Specifications for Maltodextrin
A-3 GLG Specifications for Toruzyme 3.0L
A-4 GLG Specifications for Macroporous Resin
A-5 GLG Specifications for Steviol Glycosides Starting Material

A-1 GLG Specifications for Ethanol

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质量管理文件
Quality Management File

Title	The Quality Standard for the Food Grade Ethanol			No.	RD-SMP-QA-12
				Version No./Revision No.	A/2
				Page	1/2
Prepared by	Ma faxing	Checked by	Zhang Lei	Approved by	Kevin Li
Date of Preparation	01/08/2015	Date of Checking	05/08/2015	Date of Approval	05/08/2015
Dept. of Issue	QA	Effective Date	10/08/2015	Draft Reservation	QA

1. Purpose: to establish the quality standard of food grade ethanol and provide the basis for determining the product.

2. Scope: used food grade ethanol in the production process.

3. The responsible person: QA Dept., QC Dept., Production Dept., supply and storage Dept. in the park.

4. Content:

Name: Food Grade Ethanol		Specification: Superior	
Use: raw materials for production			
GB10343-2008 Standard Basis: National Standard GB10343-2008			
Storage conditions: dry, low-temperature.			
Item	Statutory standards	Internal standards	Test methods
Appearance	Colorless and transparent	Colorless and transparent	Q/GLG-01-2014-05
Smell	With the inherent smell of the alcohol and no peculiar smell	With the inherent smell of the alcohol and no peculiar smell	Q/GLG-01-2014-05
The ethanol volume fraction %	≥95.5	≥95.5	Q/GLG-01-2014-05
Methanol mg/l	≤50	≤50	Q/GLG-01-2014-05
Normal alcohol mg/l	≤15	≤15	Q/GLG-01-2014-05
Iso-butyl alcohol mg/l	≤2	≤2	Q/GLG-01-2014-05
Non-volatile mg/l	≤15	≤15	Q/GLG-01-2014-05
Heavy-metal (counted in PB) mg/l	≤1	≤1	Q/GLG-01-2014-05

[键入文字]

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质量管理文件
Quality Management File

6. Packaging

6.1 The loading of food grade ethanol should adopt special tanks, tank vehicles and iron tubs, and is prohibited from using aluminum tubs or galvanized barrels for packaging. Before packaging, manufacturers should strictly confirm the health and safety inspections of vessels.

6.2 The tankers or tank vehicles as manufacturers' shipments should add seals. After the receipt of goods, it is required to confirm the seal intact, and then confirm the quality and quantity of the products.

7. Transportation

7.1 Transportation tools should be clean and sanitary, and can not be mixed with toxic, harmful and corrosive or smelly items.

7.2 During handling, products are required to load and unload lightly, and to be kept away from fire and heat.

8. Storage

8.1 Products can not be mixed with toxic, harmful, corrosive or toxic substances during the storage.

8.2 Products should be stored in a cool, dry and ventilated environment, and there should be anti-high temperature, fire, electricity and lightning, facilities. In the storage area there should be a warning mark with noticeable "no fire" signs.

Dept.of Distribution	Production Dept.; QC Dept. Comprehensive Office; Warehouse

A-2 GLG Specifications for Maltodextrin

Product Specification Sheet
Maltodextrin

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File NO: GLG-QA-STD-3/07
Reviewed by: Zhang lei . QA Manager
Approved by: Kevin Li. Vice President
Date: 23/07/2013

Product Description:

Maltodextrin occurs as a white, slightly hygroscopic powder, as granules of similar description, or as a clear to hazy solution in water. It is a purified, concentrated, nutritive mixture of saccharide polymers obtained by the partial hydrolysis of edible starch. Powders or granules are freely soluble or readily dispersible in water.

Shelf Life: 24 months

Physical and Organoleptic Standards

CHARACTERISTIC	SPECIFICATION	METHOD
Appearance	White or light yellow hygroscopic powder	Organoleptic AS IS
Odor	With special odour of maltodextrin	Organoleptic AS IS
Identification	Confirms	FCC V

Specification

CHARACTERISTIC	SPECIFICATION	METHOD
DE (dextrose equivalent) value	≤11,11-16,16-20	GB/T 20884-2007
Sulfated Ash	≤1.0%	GB/T 20884-2007
Moisture	≤6.0%	GB/T 20884-2007
Solubility	≤98%	GB/T 20884-2007
PH	4.5-6.5	GB/T 20884-2007
Protein	≤0.5%	FCC V
Sulfur Dioxide	≤0.0025%	FCC V
Lead	≤0.5mg/kg	FCC V
Arsenic	≤0.5 mg/kg	GB/T5009.11

Microbiological Standards

CHARACTERISTIC	LIMIT	UNITS	METHOD
Total Plate Count	<1000	CFU/g	FDA-BAM chapter 3
Yeast & Mold	<100	CFU/g	FDA-BAM chapter 18
E.coli	Negative	MPN/g	FDA-BAM chapter 4
Staphylococcus aureus	Negative	CFU/g	FDA-BAM chapter 12
Salmonella (/25g)	Negative	CFU/25g	FDA-BAM chapter 5

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A-3 GLG Specifications for Toruzyme 3.0L

Enzyme Specification

Prepared by GLG QA Department
File No. GLG-QA-EM2016

Product name: Enzyme

Brand Name: Toruzyme® 3.0 L, Manufactured by Novozymes®

Description: Cyclomaltodextrin glucanotransferase

Source organism: *Bacillus licheniformis.*

Composition:

Component	CAS No.	Approximate % (wt/wt)
Water	7732-18-5	73
Propylene glycol	57-55-6	23
Cyclomaltodextrin glucanotransferase	9030-09-5	4

Physical and Organoleptic Standards

Characteristics	Specifications
Appearance	Brown liquid
Approx. Density (g/ml)	1.04
Viscosity (cPs)	1 - 25
Declared activity	3 KNU-CP/g

Physical and Chemical Standards

Characteristics	Specifications
Alpha-amylase unit KNU-CP	≥ 3/g
pH at 25 °C	6 - 7
Heavy Metal	≤ 30 ppm
Lead (as Pb)	≤ 5 ppm
Arsenic (as As)	≤ 3 ppm
Cadmium (as Cd)	≤ 0.5 ppm
Mercury (as Hg)	≤ 0.5 ppm

Microbiological Standards

Characteristics	Specifications
Total Plate Count	≤ 50,000 CFU/g
Coliform Bacteria	≤ 30 MPN/g
E. Coli	Negative
Salmonella	Negative

Storage conditions: Product should be stored at 0 - 10 °C/32 – 50 °F with complete package in a cool and dry place.

A-4 GLG Specifications for Macroporous Resin

Macroporous Resin Specification

1. **Anion Exchange Resin** - SQD913 macroporous acrylic weak basic anion exchange resin (D941)

Description: Poly (St-DVB) based gel- and microporous- type ion exchange resins

Prepared by GLG QA Department
File No. GLG-QA-MR2016

Specifications

Characteristics	**Specifications**
Appearance	Pale white or pale yellow opaque sphere
Moisture (%)	54 - 64
Mass full exchange capacity (mmol/g) (dry basis)	7.5
Bulk density in wet state (g/ml)	0.70 - 0.80
True density in wet state (g/ml)	1.07 - 1.12
Particle size range (0.315-1.25) (%)	≥ 95
Sphere rate after abrasion（%）	≥ 95
Degree of swelling upon complete conversion (%) $OH^- \rightarrow Cl^-$	≤ 25
Final product form	$-N(CH_3)_2$

2. **Cation Exchange Resin** – 001X16 polystyrene strong cation exchange resin

Description: Poly (St-DVB) based gel- and microporous- type ion exchange resins

Specifications

Characteristics	**Specifications**
Moisture %	28 - 36
Mass full exchange capacity (mmol/g)	≥ 4.0
Volume exchange capacity (mmol/ml)	≥ 2.4
Bulk density in wet state (g/ml)	0.83 - 0.89
Ture density in wet state (g/ml)	1.30 - 1.35
Particle size %	(0.40-1.25mm) ≥ 95
Effective pore size (mm)	0.45 - 0.70
Homogeneous Coefficient	≤ 1.60
Sphere rate after abrasion %	≥ 95
Final product form	Na

3. **Macroporous Adsorption Resin** – DA201-H macroporous adsorption resin

Description: Polymer with ethenylbenzene and ethenylethylbenzene resin

Specifications:

Characteristics	**Specifications**
Appearance	Milk white opaque spherical
Moisture %	55 - 65
True density in wet state (g/ml)	1.00 - 1.08
Bulk density in wet state (g/ml)	0.65 - 0.75
Particle size %	(0.315-1.20mm) ≥ 95
Specific surface area (m^2/g)	≥ 800
Average Pore Size (nm)	6 - 8
Pore volume (ml/g)	≥ 1.5
Polarity	Non-polarity

A-5 GLG Specifications for Steviol Glycosides Starting Material

Product Specification Sheet

GLG
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BETTER STEVIA, MONK FRUIT AND MORE.

File No.: GLG-QA-STD-041-1
Reviewed by: Zhang Lei, QA Manager
Approved by: Kevin Li, Vice President
Date: 30/06/2015

Product Name: Anysweet™ RA60 plus

Product Description:

Anysweet™ RA60 plus is extract containing primarily rebaudioside A from Stevia rebaudiana Bertoni leaf. It is a white hygroscopic powder that is used as a high potency sweetener for food and beverages.

Brand: Anysweet™

Shelf Life: 2 years

Physical and Organoleptic Standards

CHARACTERISTIC	SPECIFICATION	METHOD
Appearance	White/off-white hygroscopic powder	GB/T 5492-2008
Flavor	Sweet	GB/T 5492-2008
Aroma	Sweet	GB/T 5492-2008
Particle Size	80-100 mesh	Ro Tap 25g for 5 minutes

Specification

CHARACTERISTIC	SPECIFICATION	LABEL CLAIM	METHOD
Rebaudioside A (wt/wt)	≥60.0% (on dry basis)	≥60.0%	JECFA 2010
Stevioside (wt/wt)	≥20.0% (on dry basis)	≥60.0%	JECFA 2010
Total Steviol Glycosides (wt/wt)	≥95.0% (on dry basis)	≥95.0%	JECFA 2010
Total Metals	≤10 ppm	None	USP<231>
-Arsenic	≤1.0 ppm	None	JECFA Vol.4
-Lead	≤1.0 ppm	None	JECFA Vol.4
Loss on Drying	≤4.0%	None	JECFA Vol.4
PH	4.5-7.0	None	JECFA Vol.4
Residue on Ignition	≤1.0%	None	JECFA Vol.4
Solvents, total	≤5200 ppm	None	JECFA Vol.4
-Ethanol	≤5000 ppm	None	JECFA Vol.4
-Methanol	≤200 ppm	None	JECFA Vol.4

Microbiological Standards:

CHARACTERISTIC	LIMIT	UNITS	METHOD
Total Plate Count	＜1000	cfu/g	FDA-BAM chapter 3
Yeast & Mold	＜100	cfu/g	FDA-BAM chapter 18
E.coli	Negative	****	FDA-BAM chapter 4
Staphylococcus aureus	Negative	****	FDA-BAM chapter 12
Salmonella (/25g)	Negative	(/25g)	FDA-BAM chapter 5

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Page 1of 2

Product Specification Sheet (Countinued)

GLG
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BETTER STEVIA, MONK FRUIT AND MORE.

File No.: GLG-QA-STD-041-1
Reviewed by: Zhang Lei, QA Manager
Approved by: Kevin Li, Vice President
Date: 30/06/2015

Storage and Handling

Transport of the product shall be under such conditions that will prevent contamination. The product shall be stored in a sealed container in a cool, dry place.

Packaging

The product shall be shipped in packaging that is suitable for inland and ocean transportation. It shall be contained in a suitable inner bag (e.g. plastic). The inner bag shall be contained in an appropriate outer container (e.g. suitable cardboard box) and the outer container should have a conspicuous label on the side of the outer container. The outer container label shall be legible, indelible and permanent and indicate the proper name of the product, the lot number, purchaser name and country of origin.

Product Guarantee

This product was produced in a plant that conforms to Good Manufacturing Practices and meets state and federal regulations. This product has critical control points to protect against the inclusion of metal or other extraneous material in the product. GLG Life Tech Corporation warrants that the lead contained in the product occurs naturally and is ≤1ppm. The product meets the requirements listed in this specification sheet unless otherwise stated by GLG Life Tech Corporation. A certificate of analysis is supplied with each lot of Anysweet™ RA60 plus and shall include the name and location of the production facility.

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Appendix B Analytical Methodology

B-1 HPLC Assay for Steviol Glycosides

B-2 HPLC-MS Analysis Method for Enzyme Modified Stevia

B-1 HPLC Assay for Steviol Glycosides

GLG
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GLG HPLC Assay of Rebaudioside A and Related Steviol Glycosides

File No.: GLG-QA-STD-HPLC

Prepared by: Zhang Lei, QA/QC Manager

Issued by: Kevin Li, VP of Technology

Date of Issue: 08/08/2015

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GLG HPLC Assay of Rebaudioside A and Related Steviol Glycosides

PRINCIPLE:

This assay is capable of determining the concentrations of rebaudioside A and related steviol glycosides using an isocratic LC system.

SCOPE:

The assay is capable of determining the content of rebaudioside A and related steviol glycosides in final product by LC analysis. The assay is applicable for rebaudioside A samples that have rebaudioside A concentration in the range of 50%-102% and related steviol glycosides in the range of 5.0%- 0.05%.

EQUIPMENT AND REAGENTS:

1. Agilent1200 HPLC system equipped with binary pump, auto sampler), thermostatted column compartment and UV detector, (Agilent Technologies, USA);
2. LC Amine Column; Zorbax NH2,4.6x250mm, 5um particle
3. Analytical balance capable of 0.00001g
4. Vacuum system
5. Sonicator
6. Volumetric flasks (10ml, 25ml, 50mL and 100ml)
7. Class A pipettes (1ml and 5 mL)
8. Acetonitrile, LC grade, suitable for analysis at 210nm
9. Ultra high purity water suitable for LC analysis (NanoPure, E-Pure or equivalent)
10. Ammonium acetate, reagent grade (VWR or equivalent)
11. Glacial acetic acid, reagent grade (VWR or equivalent)

STANDARDS

1. Rebaudioside A Standard; (Chromadex Inc. Irvine, CA USA);
2. Stevioside Standard; (Chromadex Inc. Irvine, CA USA);
3. Rebaudioside B Standard; (Chromadex Inc. Irvine, CA USA);
4. Rebaudioside C Standard; (Chromadex Inc. Irvine, CA USA);
5. Rebaudioside D Standard; (Chromadex Inc. Irvine, CA USA);
6. Steviolbioside Standard; (Chromadex Inc. Irvine, CA USA);
7. Dulcoside A Standard; (Chromadex Inc. Irvine, CA USA);

SAFETY NOTES:

1. Always follow the Chemical Hygiene Plan and established safety procedures for handling materials, cleaning up spills and disposing of wastes.
2. Read and observe all precautionary measures and hazards noted in the Material Safety Data Sheets for all chemicals used in this procedure.

3. Steviol glycoside materials are typically powders that can become airborne if shaken, dropped or otherwise agitated. Once airborne they can be tasted and smelled by the analyst. Utilize caution to prevent material from becoming airborne.

PROCEDURE:

A. Standard and Sample Equilibration to Moisture

1. Rebaudioside A and the related steviol glycosides are hygroscopic compounds. Standards and samples require moisture equilibration before analysis. The standards and samples should be left out, in the same room as the analytical balance, for no less than 24 hours before weighing. Intermittent stirring of the dry powder will insure uniform sample absorption.
2. At time of weighing, a moisture value should be determined for all standards and samples.

B. Mobile Phase Preparation

1. Prepare the aqueous buffer solution (0.0125% acetic acid, 0.0125% ammonium acetate) by dissolving 0.125 g ammonium acetate (NH_4OAc) and 125 µL glacial acetic acid (HOAc) in one liter of water. The aqueous buffer may be scaled up as needed. See Table 1 for appropriate amounts of ammonium acetate and glacial acetic acid.

Table 1: Amounts of NH_4OAc, HOAc and water needed for buffer solution scale up

Volume of buffer Solution to be made (mL)	Amount of NH_4OAc (mg)	Amount of HOAc (µL)	LC grade Water (mL)
1500	187.5	187.5	1500
2000	250.0	250.0	2000
2500	312.5	312.5	2500
3000	375.0	375.0	3000
4000	500.0	500.0	4000

2. Prepare the Mobile Phase (acetonitrile: buffer) at a ratio of 87:13 as to ensure sufficient separation of analytes.
    a. Combine the appropriate volumes of acetonitrile and buffer.

Table 2: Volumes of acetonitrile and aqueous buffer for preparation of mobile phase

Acetonitrile: Aqueous Buffer	Volume of mobile phase to be made (mL)	Volume of ACN (mL)	Volume of buffer solution (mL)
87:13	4000	3480	520

b. Combine the volumes, given above in Table 2, of acetonitrile and aqueous buffer, allow the solution to reach ambient temperature and degas the solution using a vacuum system and the sonicator.

3. Prepare the diluent solution (25% buffer in acetonitrile) by combing 750 mL of acetonitrile and 250 mL of aqueous buffer and mix thoroughly. Allow diluent to come to room temperature. The diluent solution may be scaled up as needed.

C. Standard Preparation

1. Prepare the rebaudioside A standard curve
   a. The rebaudioside A curve consists of 6 points varying in concentration from 2.5 mg/mL to 5.5 mg/mL.
   b. Mass individual samples of rebaudioside A standard (equilibrated for moisture) at 62.5, 87.5, 100, 112.5, 125 and 137.5 mg (+/- 2 mg).
   c. Dissolve in 25 ml volumetric flasks with diluent solution.

2. Prepare the stevioside standard curve
   a. The stevioside calibration curve consists of seven points designed to span a concentration of 0.0005 to 0.25 mg/mL.
   b. Weigh 125 mg of a stevioside standard (equilibrated for moisture) into a 50 ml volumetric flask, creating the 2.5 mg/mL stock A solution.
   c. Prepare the stock B solution by diluting 1 mL of stock A in a 10 mL volumetric flask with diluent. The remaining dilutions can found in the Table 3.

Table 3: Serial dilutions for the stevioside calibration curve

Standard	Stock Solution	Stock Std (mL)	Volumetric Flask (mL)	Std Conc. (mg/mL)
Stock A			50	2.5
Stock B	A	1	10	0.25
1	A	1	50	0.05
2	B	1	10	0.025
3	B	5	100	0.0125
4	B	1	50	0.005
5	B	1	100	0.0025
6	2	1	50	0.0005

3. Sample Preparation
    a. Weigh 125 ± 5 mg, recorded to the nearest 0.01 mg, of sample (equilibrated for moisture) in a 25 mL volumetric flask.
    b. Dilute to volume with the diluent solution.
    c. If necessary, stir, shake or sonicate the solution until completely dissolved. This will make an approximately 5.0 mg/mL sample.
    d. Samples are run in triplicate.

INSTURMENT CONDITIONS

Table 4: Instrument conditions for LC

Column:	Zorbax NH2,4.6x250mm, 5um
Temperature	30°C
Isocratic Mobile Phase:	13% buffer, 87% acetonitrile
Flow Rate:	1.5 mL/min
Injection:	15 µL
Detection:	UV at 210 nm (4 nm bandwidth), Reference: 260 nm (100 nm bandwidth)
Run Time:	75 min
Autosampler Temperature:	Ambient
Sample Concentration	5000 mg/L

ANALYSIS PROCEDURE

A. System Suitability

1. Perform a Detector Sensitivity Check by injecting stevioside standard 6.
2. Verify that the peak to noise ratio of the stevioside peak is ≥ 3. If not investigate the instrument and take corrective action before continuing. Record any necessary corrective actions.

B. Assay Sequence

1. Inject the system suitability detector sensitivity check.
2. Inject all rebaudioside A standards in increasing concentrations.
3. Inject stevioside standards 5 through B in increasing concentrations.
4. Inject the samples.
5. Bracket the samples with standards by re-injecting the 5.0 mg/mL standard after a maximum of 12 sample injections and once at the end of the sequence.

C. Integration Parameters

The integration is done using the software tools. An example chromatogram is provided in appendix C for reference on how the integration should occur.

D. Standard Curve Calculation and Acceptance Criteria

Prepare full fit linear regression standard curve by plotting rebaudioside A or stevioside concentration in mg/L on the ordinate scale versus its respective area counts on the abscissa scale. Alternatively, the data acquisition software may be used to prepare the calibration curve.

Acceptance Criteria for Rebaudioside A

1. For all rebaudioside A concentration levels to be acceptable for use in the calibration curve, the standard recoveries (see appendix A) must be within 100.0 ± 3%.
2. The correlation coefficient for the standard curve is acceptable if it is greater than 0.9900.
3. Calculate the tailing factor, T, using the rebaudioside A peak of the 5.0 mg/mL injection of the rebaudioside A standard. Tailing factor should be $0.8 \geq T \geq 2$ (see appendix B).
4. Calculate the System Drift (see appendix B).
    a. Determining the area counts for all of the 5.0 mg/mL rebaudioside A standard that was used to bracket the sample injections.

    b. Calculate the % RSD (see appendix B) for the rebaudioside A standard that was used to bracket the sample injections. The %RSD must be ≤ 2.0%.

5. If the standard curve fails the acceptance criteria notify your supervisor and investigate the problem.

Acceptance Criteria for Stevioside

1. The stevioside curve is comprised of stevioside standards 5 through B. For all stevioside concentration levels to be acceptable for use in the calibration curve, the standard recoveries must be within 100.0 ± 10%.
2. The correlation coefficient for the standard curve is acceptable if it is ≥ 0.9900.
3. Calculate signal to noise for the stevioside curve. The Limit of Quantitation (LOQ) is 0.05%. The signal to noise must be ≥ 10 for this concentration. The Limit of Detection (LOD) is 0.01%. The signal to noise must be ≥ 3.
4. If the standard curve fails the acceptance criteria notify your supervisor and investigate the problem

Analyte Calculation and Acceptance Criteria

1. Identify analytes of interest by matching retention time with standards if available. When analyte standards are not available, determine unknown peaks based on relative retention times (see appendix A) to standards.

2. Determine the area counts of the analyte peaks as well as any measurable peaks (except for solvent peaks) from the standards and samples.

3. Using the equation given below from the linear regression of the standard curve, calculate the concentration in mg/mL of the analytes. Calculate rebaudioside A using the rebaudioside A curve and all other analytes using the stevioside curve. Alternatively, use the data acquisition software to calculate the concentrations of the analytes based on the calibration curves prepared using the software.

   Conc. (mg/mL) = Area Response x slope + y-intercept

4. Correct the concentrations of each analyte in the samples by multiplying the concentration of each known glycoside by its correction factor (see appendix D). This corrects for the difference in molecular weight between analyte of interest and stevioside. No correction is needed for rebaudioside B because it and stevioside have the same molecular weight.

5. Calculate the w/w % of rebaudioside A and each known glycoside in the samples as follows:

   w/w% = Conc. of the Analyte (mg/mL) x 100 / Sample Conc (mg/mL)

6. Correct rebaudioside A and all known glycosides, above the LOQ, for moisture and solvents (if applicable) by multiplying the wt/wt% by the following factor (F). All analytes should be reported on a dry basis.

   F = 100 / (100- % Moisture and Solvents in Samples)

B-2 HPLC-MS Analysis Method for Enzyme Modified Stevia

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GLG HPLC-MS Analysis Method of Enzyme Modified Stevia (EMS)

File No.: GLG-QA-STD-HPLC-06

Prepared by: Zhang Lei, QA/QC Manager

Issued by: Kevin Li, VP of Technology

Date of Issued: 08/08/2015

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GLG HPLC-MS Analysis Method of Enzyme Modified Stevia (EMS)

1. Analysis method

1.1 Mechanism

Glucoamylase (GA, 1, 4-α-D-glucan glucohydrolase, EC 3.1.2.3) has been widely used in hydrolysis of α-1, 4 and α-1, 6 glucosidic linkages to release β-D-glucose from the non-reducing ends of starch and related oligosaccharides. Transgalactosylated glucosidic linkages in Glycosylated Enzyme Treated Stevia (GETS) (or Enzyme Modified Stevia (EMS)) can be cleaved with GA selectively. This method does not affect glucosidic and mannopyranosyl linkages of steviol glycosides in *Stevia* (stevioside, Rebaudioside A and Rebaudioside C etc.). Therefore, GETS purity can be calculated based on the concentration of glucose and steviol glycosides after complete hydrolysis with GA (1).

$$GETS\ purity = \frac{C_1 + 0.9 \times C_2}{C_0} \times 100\% \qquad (1)$$

Where
C_0 is the amount (g/L) of GETS.
C_1 and C_2 are the amounts (g/L) of steviol glycosides and glucose after complete hydrolysis with GA, respectively.

1.2 Enzymatic hydrolysis of GETS with GA

GETS water solution (5 g/L) and GA (100 U/mL) were mixed in an Erlenmeyer flask, and then shaken at 60 °C for 6 h.

1.3 Assay of glucose concentration

Concentration of glucose in the reaction mixture was determined with SBA-50 Glucose Biosensor (Biology Institute of Shandong Academy of Sciences, Shandong,

[Type here]

PR China).

Concentration of glucose in the reaction mixture also can be determined based on HPLC (Agilent 1200 with a RID). The HPLC conditions were as follows: Shodex SUGAR SH1011 column (8×300 mm, Showa Denko, Japan); column temperature 60 °C; mobile phase 5 mM/L H_2SO_4; flow rate 0.6 mL/min; injection volume 15 μL; detector temperature 30 °C.

1.4 Assay of steviol glycosides with LC-MS

Glycosylated steviol glycosides were identified by LC-MS according to their molecular weights. LC-MS profile was taken from Waters Acquity UPLC system (BEH HILIC column; mobile phase: acetonitrile and water (30:70, 0 min; 100:0, 11 min-13 min); 0.3 mL/min; column temperature: 30 °C; injection volume: 2 μL; collision energy: 6 eV; 200-2000; polarity: ES$^-$).

1.5 Assay of steviol glycosides with HPLC

The reaction mixture was analyzed with HPLC (JECFA method with some modification). Steviol glycosides concentration was obtained by HPLC using standard samples.

Gradient elution in HPLC using the NH_2 column

Time	80% Acetonitrile	20% Acetonitrile	Flow	Curve
0.00	91.7	8.3	1.000	1
0.10	91.7	8.3	1.000	6
2.00	91.7	8.3	1.000	6
15.00	61.7	38.3	1.000	6
20.00	91.7	8.3	1.000	6
25.00	91.7	8.3	1.000	6

[Type here]

2. Result

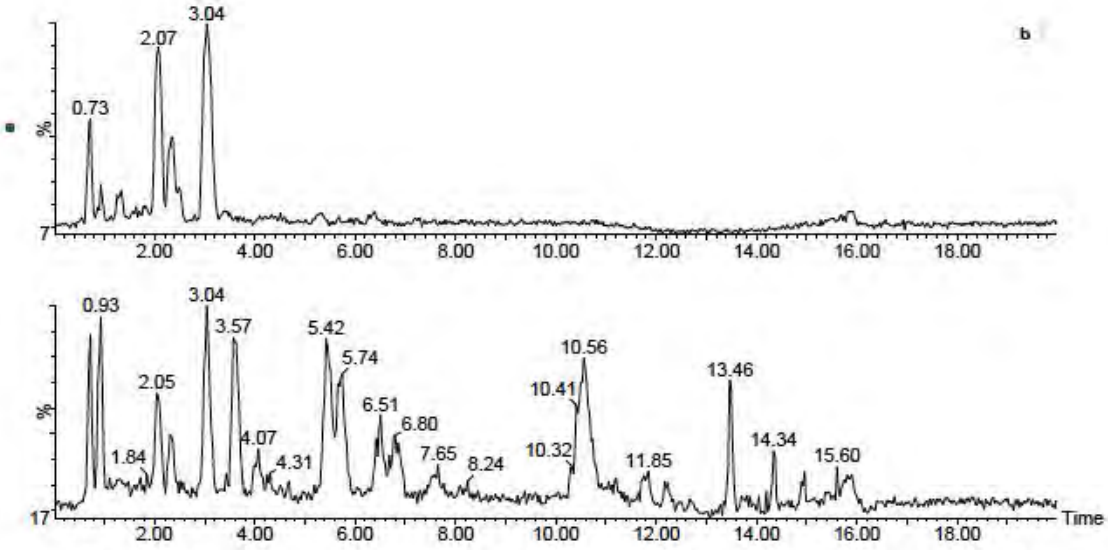


Fig. 1 Total ion chromatography of GETS (a) and its complete hydrolysis sample at 6 h (b)

Table 1 HPLC–MS features of the steviol glycosides and transglycosylation products

Retention Time/min	[M-H]-	compound
0.72	803.2	Reb B
0.93	965.3	Reb B+Glc 1[a]
1.34	641.2	Sbio
2.05	803.2	Stv
2.31	949.2	Reb C
3.04	965.2	Reb A
3.57	965.3	Stv+Glc 1
4.07	1111.3	Reb C+Glc 1
5.45, 5.47, 6.51, 6.77	1127.3	Stv+Glc 2, Reb A+Glc 1
7.65	1273.4	Reb C+Glc 2
10.50, 11.76, 12.17	1289.5	Stv+Glc 3, Reb A+Glc 2
12.70	1435.4	Reb C+Glc 3
13.46, 13.74	1451.5	Stv+Glc 4, Reb A+Glc 3
14.34	1613.5	Stv+Glc 5, Reb A+Glc 4
14.69	1776.4	Stv+Glc 6, Reb A+Glc 5

a: Glc represents glucose

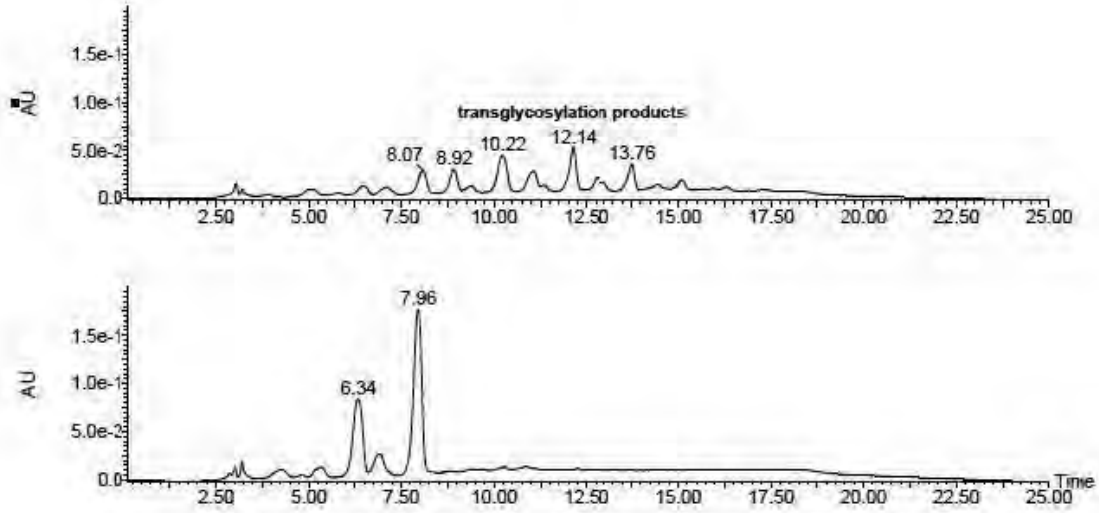


Fig. 2 HPLC of GETS (a) and its complete hydrolysis sample at 6 h (b)

Appendix C HPLC-MS Chromatograms for EMS95

C-1a HPLC-MS Report for EMS95 Lot 20151201 (Pre-hydrolysis)

C-1b HPLC-MS Report for EMS95 Lot 20151201 (Post-hydrolysis)

C-2a HPLC-MS Report for EMS95 Lot 20151203 (Pre-hydrolysis)

C-2b HPLC-MS Report for EMS95 Lot 20151203 (Post-hydrolysis)

C-3a HPLC-MS Report for EMS95 Lot 20151206 (Pre-hydrolysis)

C-3b HPLC-MS Report for EMS95 Lot 20131206 (Post-hydrolysis)

C-4a HPLC-MS Report for EMS95 Lot 20151210 (Pre-hydrolysis)

C-4b HPLC-MS Report for EMS95 Lot 20151210 (Post-hydrolysis)

C-5a HPLC-MS Report for EMS95 Lot 20151212 (Pre-hydrolysis)

C-5b HPLC-MS Report for EMS95 Lot 20151212 (Post-hydrolysis)

C-1a HPLC-MS Report for EMS95 Lot 20151201 (Pre-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151201-0h.raw

Header

Acquired File Name:	C:\MassLynx\Default.PRO\Data\312151216-15.Raw
Acquired Date:	16-Dec-2015
Acquired Time:	21:38:28
Job code:	Default
Task code:	
User Name:	operator 1
Laboratory Name:	
Instrument:	
Conditions:	
Submitter:	
SampleID:	
Bottle Number:	73
Description:	GLG-EMS95-20151201-0h

Instrument Calibration Parameters

MS1 Static:	None
MS1 Scanning:	None
MS1 Scan Speed:	None
MS2 Static:	None
MS2 Scanning:	None
MS2 Scan Speed:	None
Calibration Time:	
Calibration Date:	

Coefficients

MS1 Static:	None
MS2 Static:	None
Function 1:	None

No experimental record file.

ACE Experimental Record

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents

Degasser ContinuousStroke Volume	100.0 Final Flow Settings not being held at End of Run	
A%	91.7	80% acetonitrile
B%	8.3	20% acetonitrile
C%	0.0	
D%	0.0	
Flow (ml/min)	1.000	
Flow Ramp	2.00	
Stop Time (mins)	25.0	
Column Temperature (oC)	35.0	
Column Temperature Limit (oC)	20.0	
Min Pressure (Bar)	0.0	
Max Pressure (Bar)	300.0	
Pre-column Volume (bar)	0.00	
Column Position	Column 1Pre-column Volume (bar)	0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1
0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm)	200.00
End Wavelength (nm)	400.00
Resolution (nm)	1.2
Sampling Rate (spectra/s)	1.000
Filter Response	1
Exposure Time(ms)	Automatic

Interpolate 656 Yes
Acquisition stop time (mins) 25.00
Save to disk: Yes
Waters996 PDA Analog Channel 1

Output Mode Off
Waters996 PDA Analog Channel 2

Output Mode RatioFilter Type HammingWavelength (nm) 254.00
Bandwidth (nm) 2.40
Offset (AU) 0.00
Filter Response Time (s) 1.00
Threshold (AU) 0.1
Ratio Denominator (nm) 254.00

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm) 0.00
Sample Temperature (oC) 35.0
Sample Temperature Limit (oC) 20.0

Purge Loop Volumes 0.00

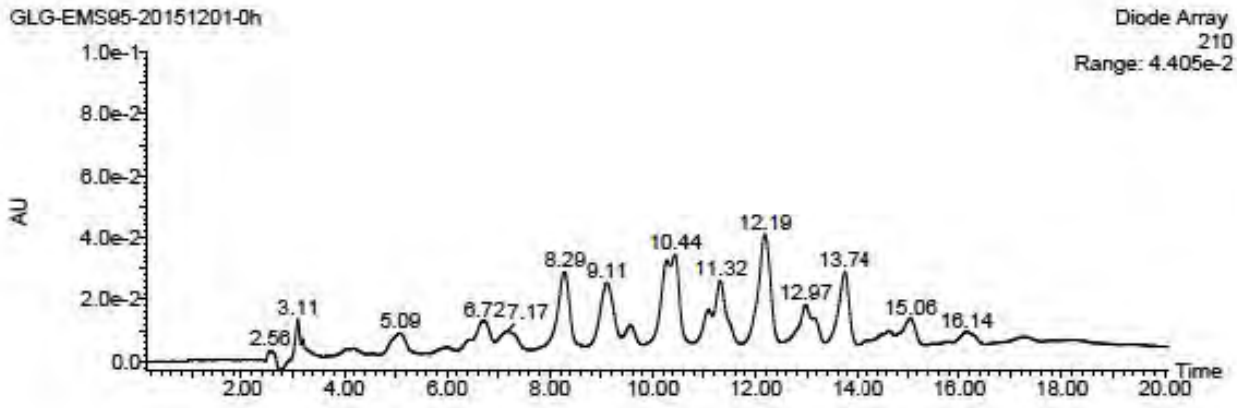
Sample Run Injection Parameter

Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

---------- oOo ----------

End of experimental record.

Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400


GLG-EMS95-20151201-0h
Diode Array
210
Range: 4.405e-2
AU
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8.0e-2
6.0e-2
4.0e-2
2.0e-2
0.0
2.56
3.11
5.09
6.72
7.17
8.29
9.11
10.44
11.32
12.19
12.97
13.74
15.06
16.14
Time
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00

C-1b HPLC-MS Report for EMS95 Lot 20151201 (Post-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151201-6h.raw

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Acquired Time: 23:49:28
Job code: Default
Task code:
User Name: operator 1
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID:
Bottle Number: 78
Description: GLG-EMS95-20151201-6h

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents
Degasser ContinuousStroke Volume 100.0 Final Flow Settings not being held at End of Run
A% 91.7 80% acetonitrile
B% 8.3 20% acetonitrile
C% 0.0
D% 0.0
Flow (ml/min) 1.000
Flow Ramp 2.00
Stop Time (mins) 25.0
Column Temperature (oC) 35.0
Column Temperature Limit (oC) 20.0
Min Pressure (Bar) 0.0
Max Pressure (Bar) 300.0
Pre-column Volume (bar) 0.00
Column Position Column 1Pre-column Volume (bar) 0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1

0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm)	200.00
End Wavelength (nm)	400.00
Resolution (nm)	1.2
Sampling Rate (spectra/s)	1.000
Filter Response	1
Exposure Time(ms)	Automatic
Interpolate 656	Yes
Acquisition stop time (mins)	25.00

Save to disk: Yes
Waters996 PDA Analog Channel 1

Output Mode Off
Waters996 PDA Analog Channel 2

Output Mode	RatioFilter Type	HammingWavelength (nm)	254.00
Bandwidth (nm)	2.40		
Offset (AU)	0.00		
Filter Response Time (s)	1.00		
Threshold (AU)	0.1		
Ratio Denominator (nm)	254.00		

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm)	0.00
Sample Temperature (oC)	35.0
Sample Temperature Limit (oC)	20.0

Purge Loop Volumes 0.00

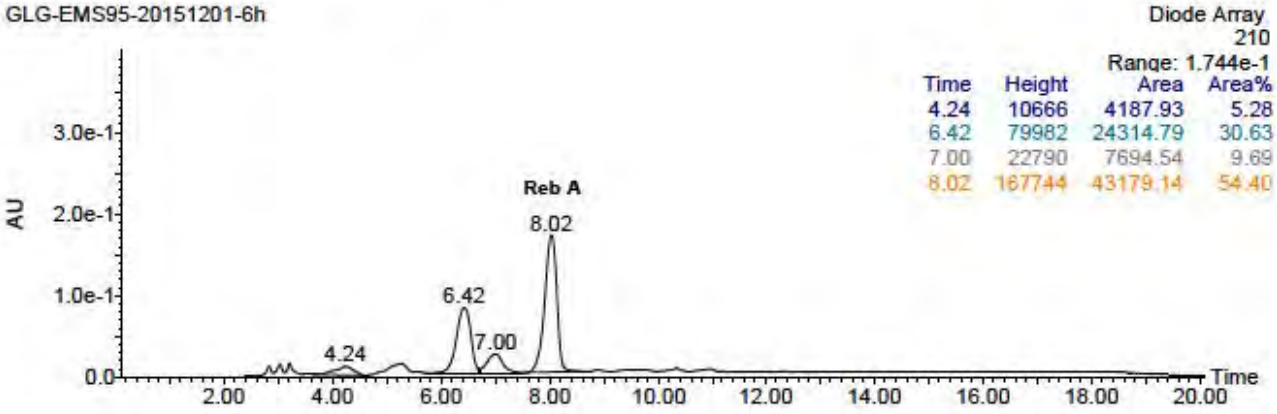
Sample Run Injection Parameter

Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

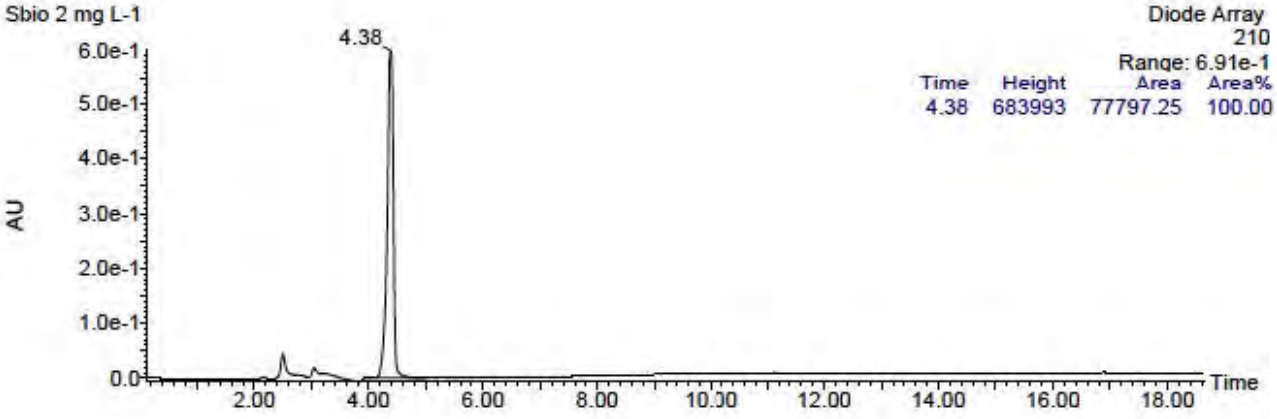
---------- oOo ----------

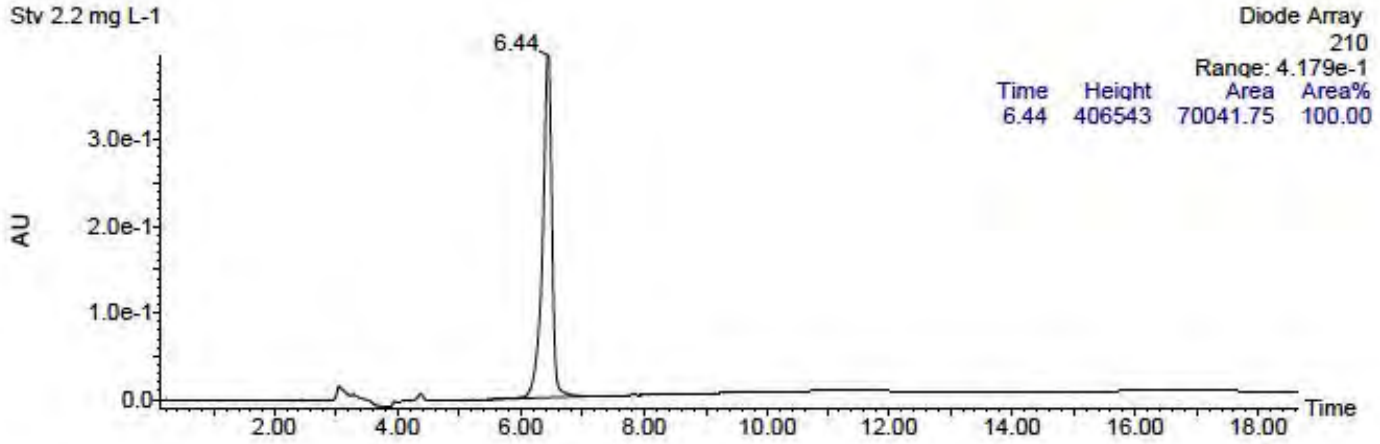
End of experimental record.

Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400



Standard




Stv 2.2 mg L-1
Diode Array
210
Range: 4.179e-1
Time Height Area Area%
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6.44
AU
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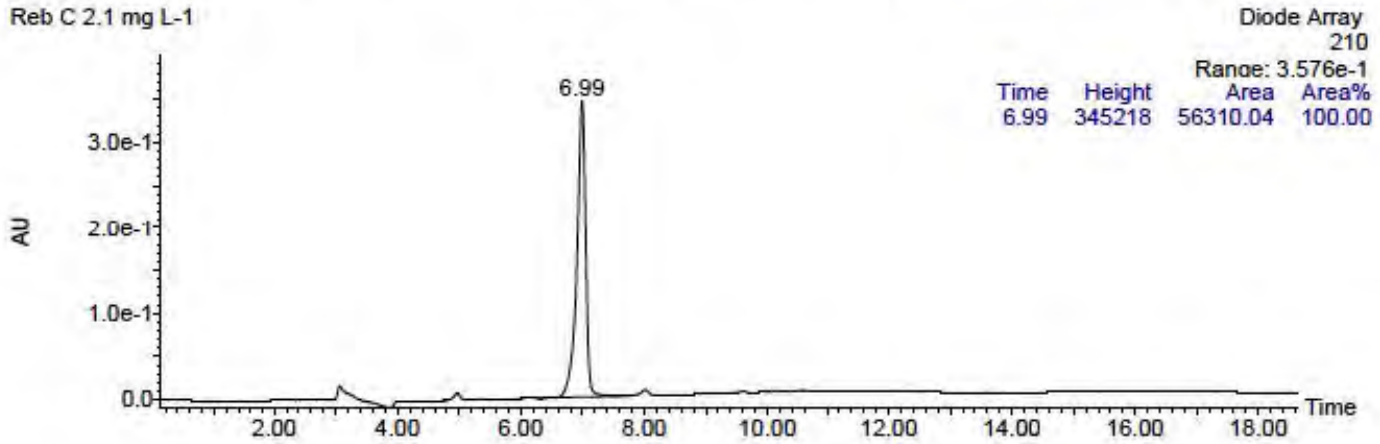
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb C 2.1 mg L-1
Diode Array
210
Range: 3.576e-1
Time Height Area Area%
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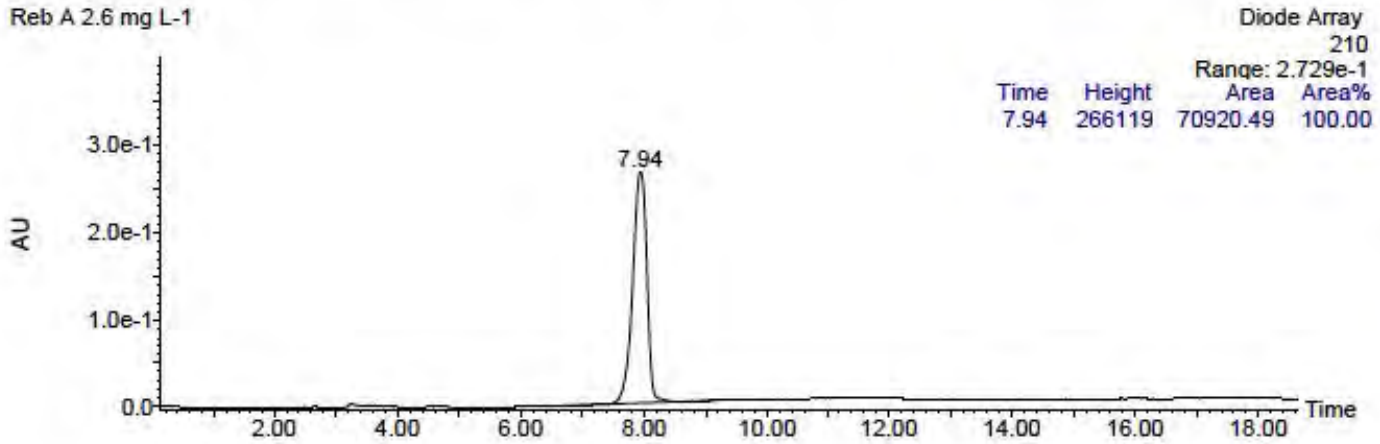
6.99
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb A 2.6 mg L-1
Diode Array
210
Range: 2.729e-1
Time Height Area Area%
7.94 266119 70920.49 100.00
7.94
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time

C-2a HPLC-MS Report for EMS95 Lot 20151203 (Pre-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151203-0h.raw

Header
Acquired File Name: C:\MassLynx\Default.PRO\Data\312151216-16.Raw
Acquired Date: 16-Dec-2015
Acquired Time: 22:04:36
Job code: Default
Task code:
User Name: operator 1
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID:
Bottle Number: 74
Description: GLG-EMS95-20151203-0h

Instrument Calibration
Parameters
MS1 Static: None
MS1 Scanning: None
MS1 Scan Speed: None
MS2 Static: None
MS2 Scanning: None
MS2 Scan Speed: None
Calibration Time:
Calibration Date:
Coefficients
MS1 Static: None
MS2 Static: None
Function 1: None

No experimental record file.

ACE Experimental Record

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents

Degasser	Continuous	Stroke Volume	100.0 Final Flow Settings not being held at End of Run
A%	91.7	80% acetonitrile	
B%	8.3	20% acetonitrile	
C%	0.0		
D%	0.0		
Flow (ml/min)	1.000		
Flow Ramp	2.00		
Stop Time (mins)	25.0		
Column Temperature (oC)	35.0		
Column Temperature Limit (oC)	20.0		
Min Pressure (Bar)	0.0		
Max Pressure (Bar)	300.0		
Pre-column Volume (bar)	0.00		
Column Position	Column 1	Pre-column Volume (bar)	0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1
0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm)	200.00
End Wavelength (nm)	400.00
Resolution (nm)	1.2
Sampling Rate (spectra/s)	1.000
Filter Response	1
Exposure Time(ms)	Automatic

Interpolate 656 Yes
Acquisition stop time (mins) 25.00
Save to disk: Yes
Waters996 PDA Analog Channel 1

Output Mode Off
Waters996 PDA Analog Channel 2

Output Mode RatioFilter Type Hamming Wavelength (nm) 254.00
Bandwidth (nm) 2.40
Offset (AU) 0.00
Filter Response Time (s) 1.00
Threshold (AU) 0.1
Ratio Denominator (nm) 254.00

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm) 0.00
Sample Temperature (oC) 35.0
Sample Temperature Limit (oC) 20.0

Purge Loop Volumes 0.00

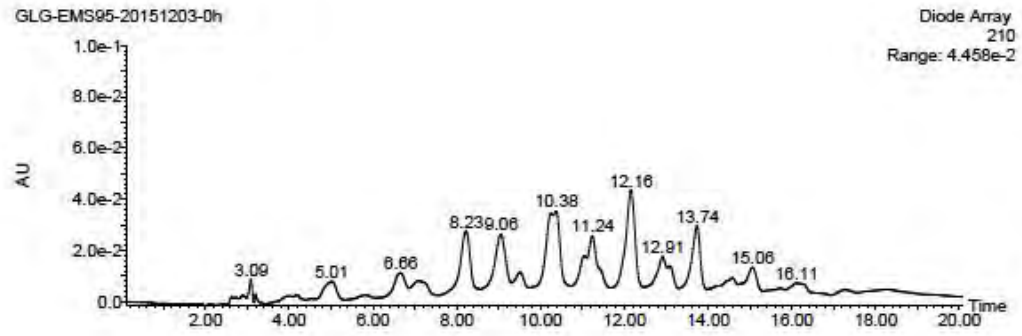
Sample Run Injection Parameter

Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

---------- oOo ----------

End of experimental record.

Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400


GLG-EMS95-20151203-0h
Diode Array
210
Range: 4.458e-2
AU
1.0e-1
8.0e-2
6.0e-2
4.0e-2
2.0e-2
0.0
3.09
5.01
6.66
8.23
9.06
10.38
11.24
12.16
12.91
13.74
15.06
16.11
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Time

C-2b HPLC-MS Report for EMS95 Lot 20151203 (Post-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151203-6h.raw

Header
Acquired File Name: C:\MassLynx\Default.PRO\Data\312151216-21.Raw
Acquired Date: 17-Dec-2015
Acquired Time: 00:15:33
Job code: Default
Task code:
User Name: operator 1
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID:
Bottle Number: 79
Description: GLG-EMS95-20151203-6h

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents
Degasser ContinuousStroke Volume 100.0 Final Flow Settings not being held at End of Run
A% 91.7 80% acetonitrile
B% 8.3 20% acetonitrile
C% 0.0
D% 0.0
Flow (ml/min) 1.000
Flow Ramp 2.00
Stop Time (mins) 25.0
Column Temperature (oC) 35.0
Column Temperature Limit (oC) 20.0
Min Pressure (Bar) 0.0
Max Pressure (Bar) 300.0
Pre-column Volume (bar) 0.00
Column Position Column 1Pre-column Volume (bar) 0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1

0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm) 200.00
End Wavelength (nm) 400.00
Resolution (nm) 1.2
Sampling Rate (spectra/s) 1.000
Filter Response 1
Exposure Time(ms) Automatic
Interpolate 656 Yes
Acquisition stop time (mins) 25.00
Save to disk: Yes
Waters996 PDA Analog Channel 1

Output Mode Off
Waters996 PDA Analog Channel 2

Output Mode Ratio Filter Type Hamming Wavelength (nm) 254.00
Bandwidth (nm) 2.40
Offset (AU) 0.00
Filter Response Time (s) 1.00
Threshold (AU) 0.1
Ratio Denominator (nm) 254.00

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm) 0.00
Sample Temperature (oC) 35.0
Sample Temperature Limit (oC) 20.0

Purge Loop Volumes 0.00

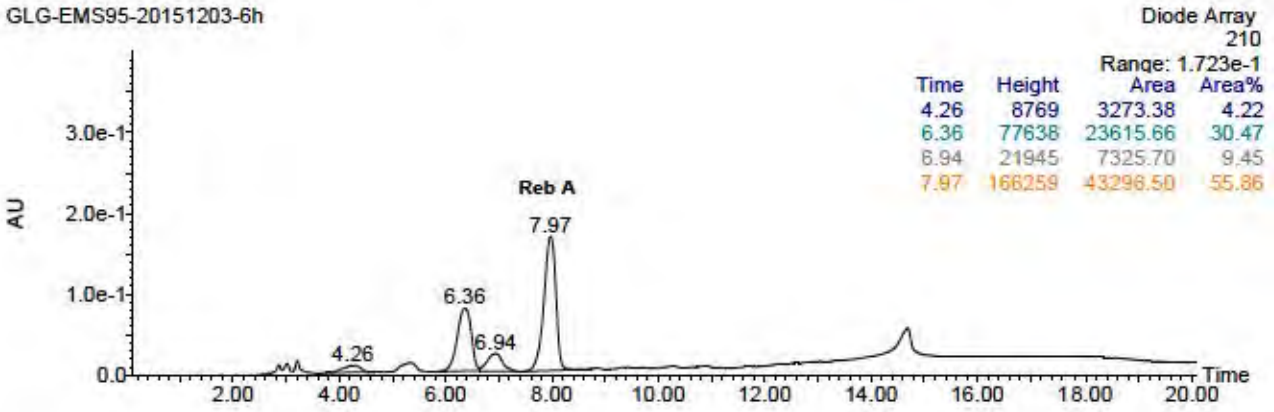
Sample Run Injection Parameter

Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

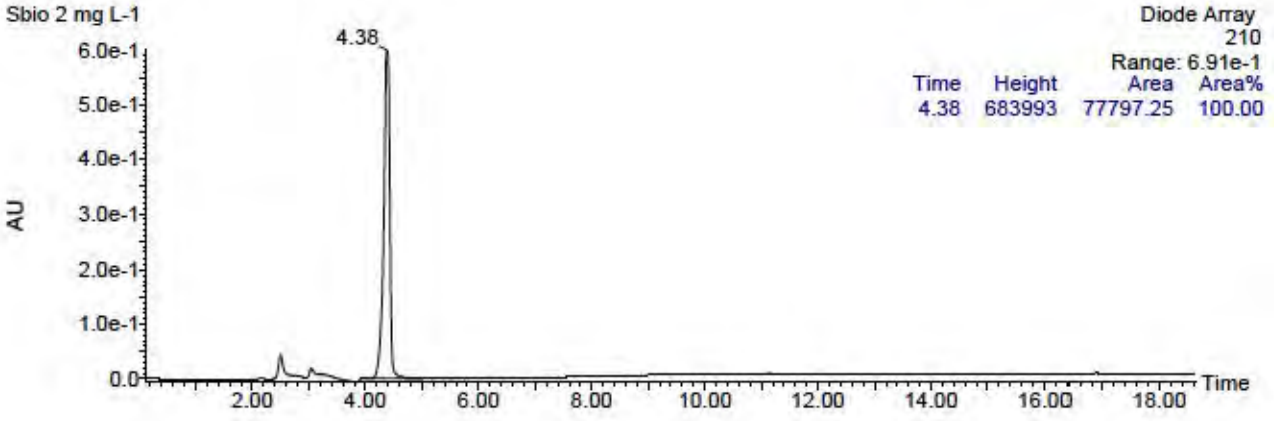
---------- oOo ----------

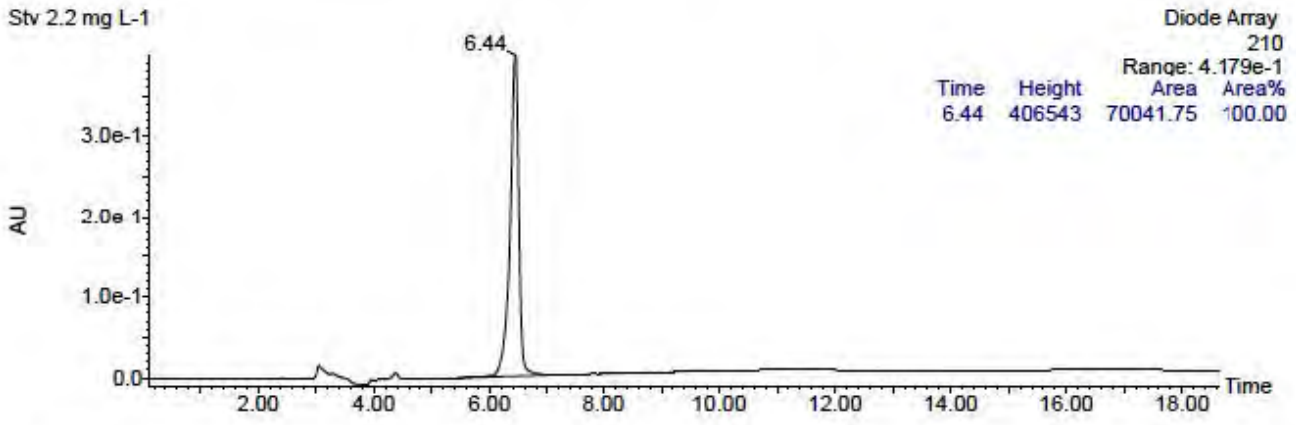
End of experimental record.

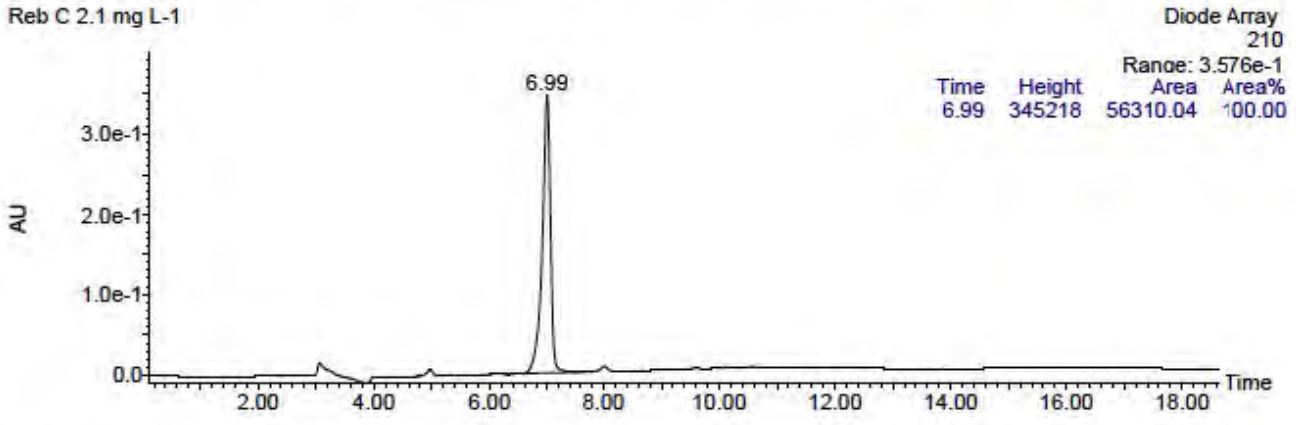
Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400

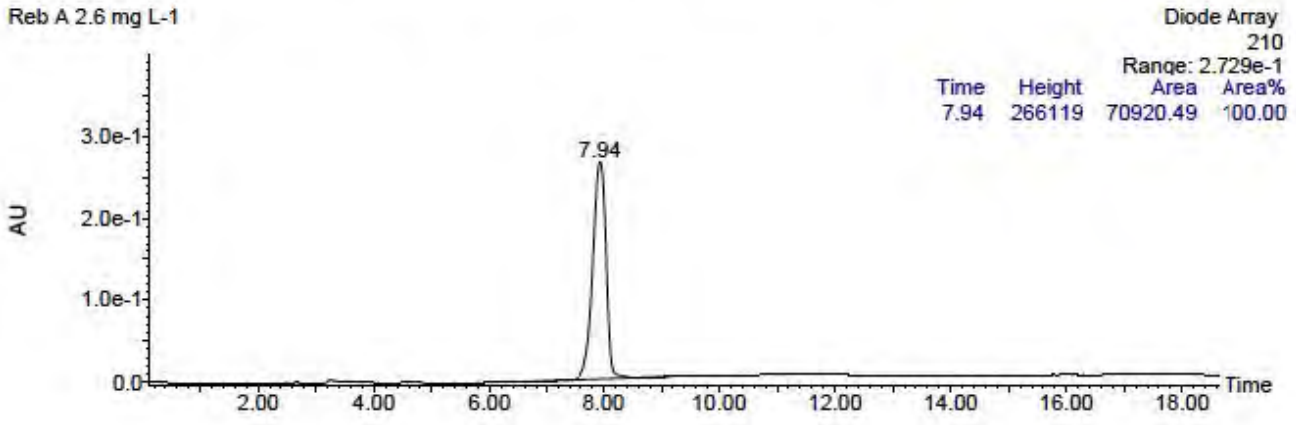


Standard




Stv 2.2 mg L-1
Diode Array
210
Range: 4.179e-1
Time Height Area Area%
6.44 406543 70041.75 100.00
6.44
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb C 2.1 mg L-1
Diode Array
210
Range: 3.576e-1
Time Height Area Area%
6.99 345218 56310.04 100.00
6.99
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb A 2.6 mg L-1
Diode Array
210
Range: 2.729e-1
Time Height Area Area%
7.94 266119 70920.49 100.00
7.94
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time

C-3a HPLC-MS Report for EMS95 Lot 20151206 (Pre-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151206-0h.raw

Header
Acquired File Name: C:\MassLynx\Default.PRO\Data\312151216-17.Raw
Acquired Date: 16-Dec-2015
Acquired Time: 22:30:57
Job code: Default
Task code:
User Name: operator 1
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID:
Bottle Number: 75
Description: GLG-EMS95-20151206-0h

Instrument Calibration
Parameters
MS1 Static: None
MS1 Scanning: None
MS1 Scan Speed: None
MS2 Static: None
MS2 Scanning: None
MS2 Scan Speed: None
Calibration Time:
Calibration Date:
Coefficients
MS1 Static: None
MS2 Static: None
Function 1: None

No experimental record file.

ACE Experimental Record

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents

Degasser	Continuous	Stroke Volume 100.0 Final Flow Settings not being held at End of Run
A%	91.7	80% acetonitrile
B%	8.3	20% acetonitrile
C%	0.0	
D%	0.0	
Flow (ml/min)	1.000	
Flow Ramp	2.00	
Stop Time (mins)	25.0	
Column Temperature (oC)	35.0	
Column Temperature Limit (oC)	20.0	
Min Pressure (Bar)	0.0	
Max Pressure (Bar)	300.0	
Pre-column Volume (bar)	0.00	
Column Position	Column 1	Pre-column Volume (bar) 0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1
0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm)	200.00
End Wavelength (nm)	400.00
Resolution (nm)	1.2
Sampling Rate (spectra/s)	1.000
Filter Response	1
Exposure Time(ms)	Automatic

Interpolate 656 Yes
Acquisition stop time (mins) 25.00
Save to disk: Yes
Waters996 PDA Analog Channel 1

Output Mode Off
Waters996 PDA Analog Channel 2

Output Mode RatioFilter Type HammingWavelength (nm) 254.00
Bandwidth (nm) 2.40
Offset (AU) 0.00
Filter Response Time (s) 1.00
Threshold (AU) 0.1
Ratio Denominator (nm) 254.00

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm) 0.00
Sample Temperature (oC) 35.0
Sample Temperature Limit (oC) 20.0

Purge Loop Volumes 0.00

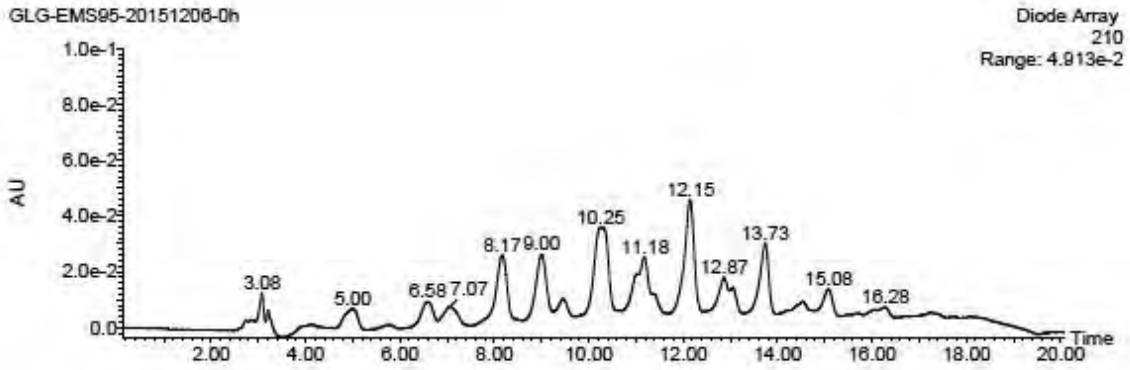
Sample Run Injection Parameter

Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

---------- oOo ----------

End of experimental record.

Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400


GLG-EMS95-20151206-0h
Diode Array
210
Range: 4.913e-2
AU
1.0e-1
8.0e-2
6.0e-2
4.0e-2
2.0e-2
0.0
3.08
5.00
6.58
7.07
8.17
9.00
10.25
11.18
12.15
12.87
13.73
15.08
16.28
Time
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00

C-3b HPLC-MS Report for EMS95 Lot 20131206 (Post-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151206-6h.raw

Header
Acquired File Name: C:\MassLynx\Default.PRO\Data\312151216-22.Raw
Acquired Date: 17-Dec-2015
Acquired Time: 00:41:38
Job code: Default
Task code:
User Name: operator 1
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID:
Bottle Number: 80
Description: GLG-EMS95-20151206-6h

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents
Degasser ContinuousStroke Volume 100.0 袀 Final Flow Settings not being held at End of Run
A% 91.7 80% acetonitrile
B% 8.3 20% acetonitrile
C% 0.0
D% 0.0
Flow (ml/min) 1.000
Flow Ramp 2.00
Stop Time (mins) 25.0
Column Temperature (oC) 35.0
Column Temperature Limit (oC) 20.0
Min Pressure (Bar) 0.0
Max Pressure (Bar) 300.0
Pre-column Volume (bar) 0.00
Column Position Column 1Pre-column Volume (bar) 0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1

0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm) 200.00
End Wavelength (nm) 400.00
Resolution (nm) 1.2
Sampling Rate (spectra/s) 1.000
Filter Response 1
Exposure Time(ms) Automatic
Interpolate 656 Yes
Acquisition stop time (mins) 25.00
Save to disk: Yes
Waters996 PDA Analog Channel 1

Output Mode Off
Waters996 PDA Analog Channel 2

Output Mode RatioFilter Type HammingWavelength (nm) 254.00
Bandwidth (nm) 2.40
Offset (AU) 0.00
Filter Response Time (s) 1.00
Threshold (AU) 0.1
Ratio Denominator (nm) 254.00

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm) 0.00
Sample Temperature (oC) 35.0
Sample Temperature Limit (oC) 20.0

Purge Loop Volumes 0.00

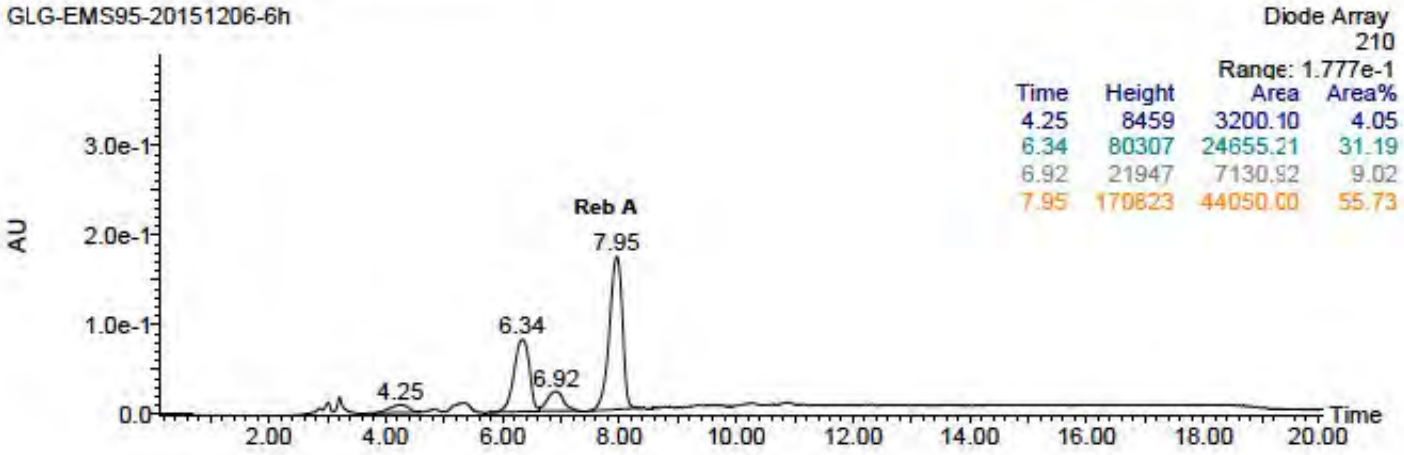
Sample Run Injection Parameter

Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

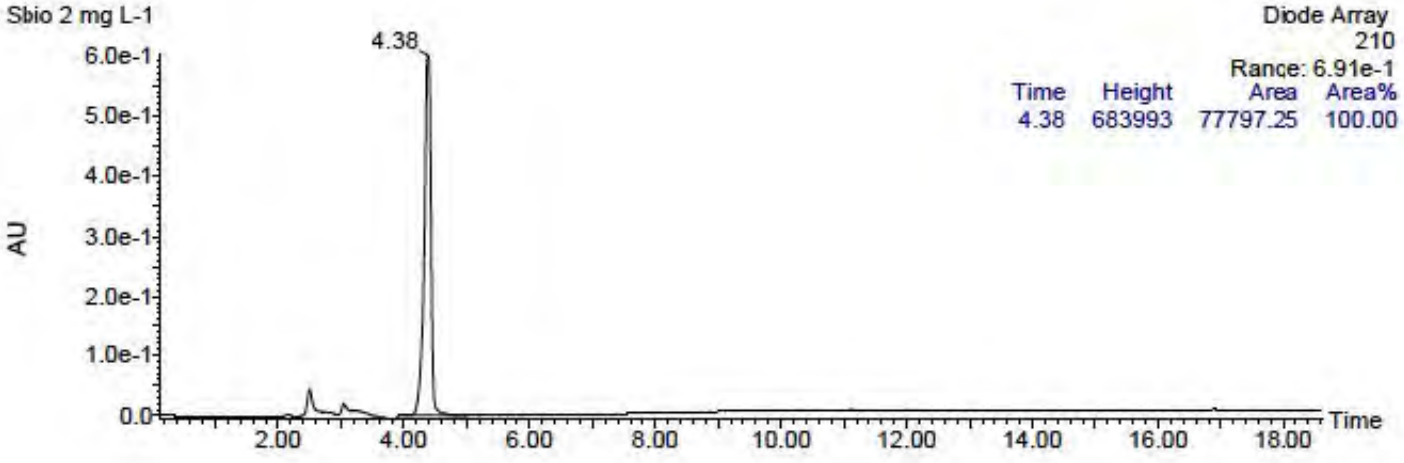
---------- oOo ----------

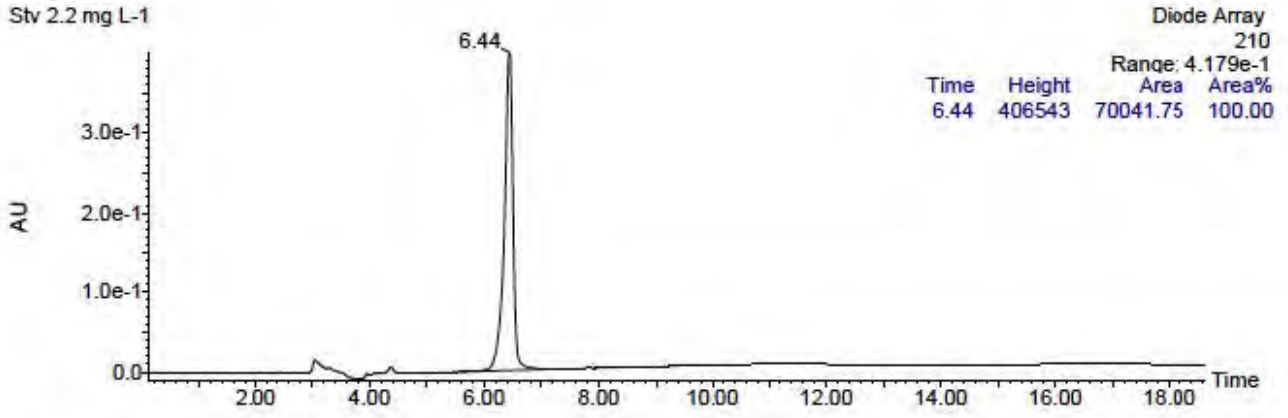
End of experimental record.

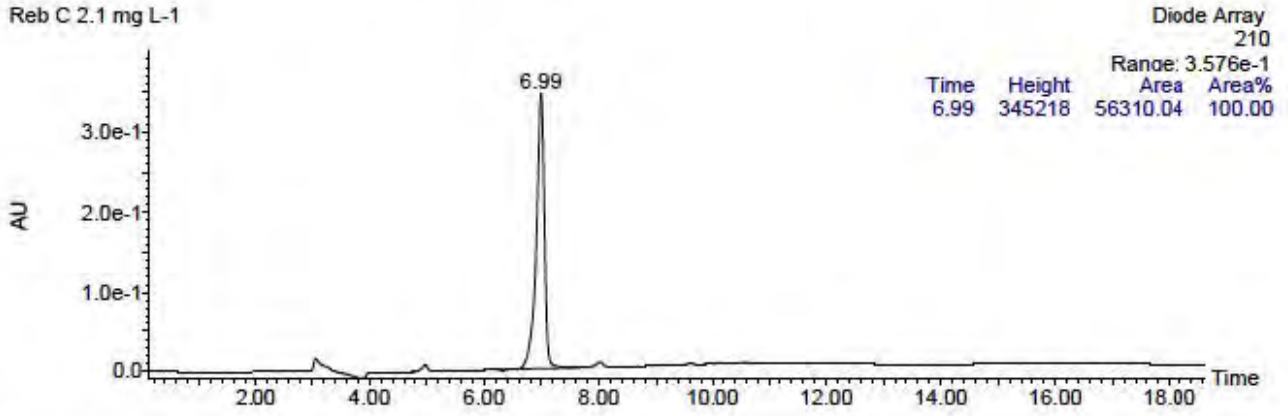
Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400

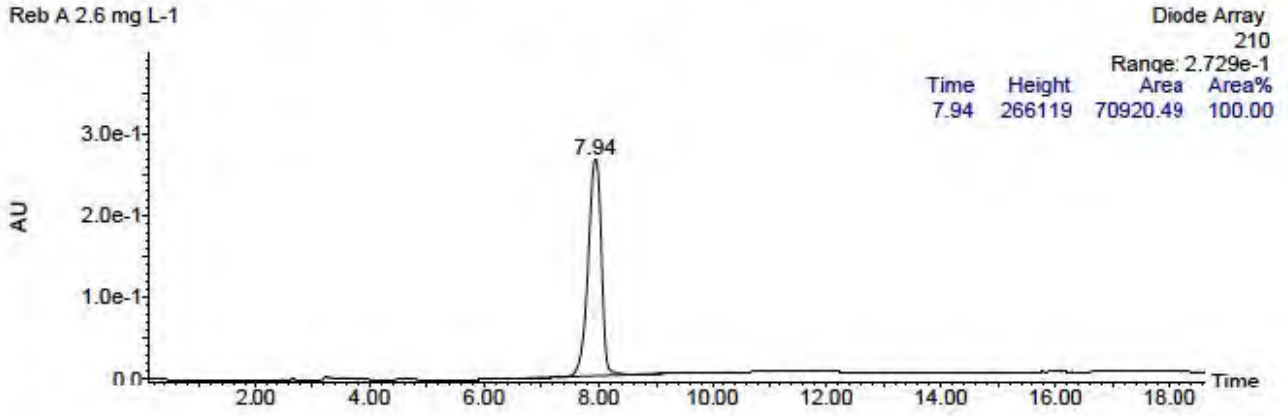


Standard




Stv 2.2 mg L-1
Diode Array
210
Range: 4.179e-1
Time Height Area Area%
6.44 406543 70041.75 100.00
6.44
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb C 2.1 mg L-1
Diode Array
210
Range: 3.576e-1
Time Height Area Area%
6.99 345218 56310.04 100.00
6.99
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb A 2.6 mg L-1
Diode Array
210
Range: 2.729e-1
Time Height Area Area%
7.94 266119 70920.49 100.00
7.94
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time

C-4a HPLC-MS Report for EMS95 Lot 20151210 (Pre-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151210-0h.raw

Header
Acquired File Name: C:\MassLynx\Default.PRO\Data\312151216-18.Raw
Acquired Date: 16-Dec-2015
Acquired Time: 22:57:18
Job code: Default
Task code:
User Name: operator 1
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID:
Bottle Number: 76
Description: GLG-EMS95-20151210-0h

Instrument Calibration
Parameters
MS1 Static: None
MS1 Scanning: None
MS1 Scan Speed: None
MS2 Static: None
MS2 Scanning: None
MS2 Scan Speed: None
Calibration Time:
Calibration Date:
Coefficients
MS1 Static: None
MS2 Static: None
Function 1: None

No experimental record file.

ACE Experimental Record

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents

Degasser	Continuous	Stroke Volume	100.0 Final Flow Settings not being held at End of Run
A%	91.7	80% acetonitrile	
B%	8.3	20% acetonitrile	
C%	0.0		
D%	0.0		
Flow (ml/min)	1.000		
Flow Ramp	2.00		
Stop Time (mins)	25.0		
Column Temperature (oC)	35.0		
Column Temperature Limit (oC)	20.0		
Min Pressure (Bar)	0.0		
Max Pressure (Bar)	300.0		
Pre-column Volume (bar)	0.00		
Column Position	Column 1	Pre-column Volume (bar)	0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1
0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm)	200.00
End Wavelength (nm)	400.00
Resolution (nm)	1.2
Sampling Rate (spectra/s)	1.000
Filter Response	1
Exposure Time(ms)	Automatic

Interpolate 656 Yes
Acquisition stop time (mins) 25.00
Save to disk: Yes
Waters996 PDA Analog Channel 1

Output Mode Off
Waters996 PDA Analog Channel 2

Output Mode RatioFilter Type HammingWavelength (nm) 254.00
Bandwidth (nm) 2.40
Offset (AU) 0.00
Filter Response Time (s) 1.00
Threshold (AU) 0.1
Ratio Denominator (nm) 254.00

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm) 0.00
Sample Temperature (oC) 35.0
Sample Temperature Limit (oC) 20.0

Purge Loop Volumes 0.00

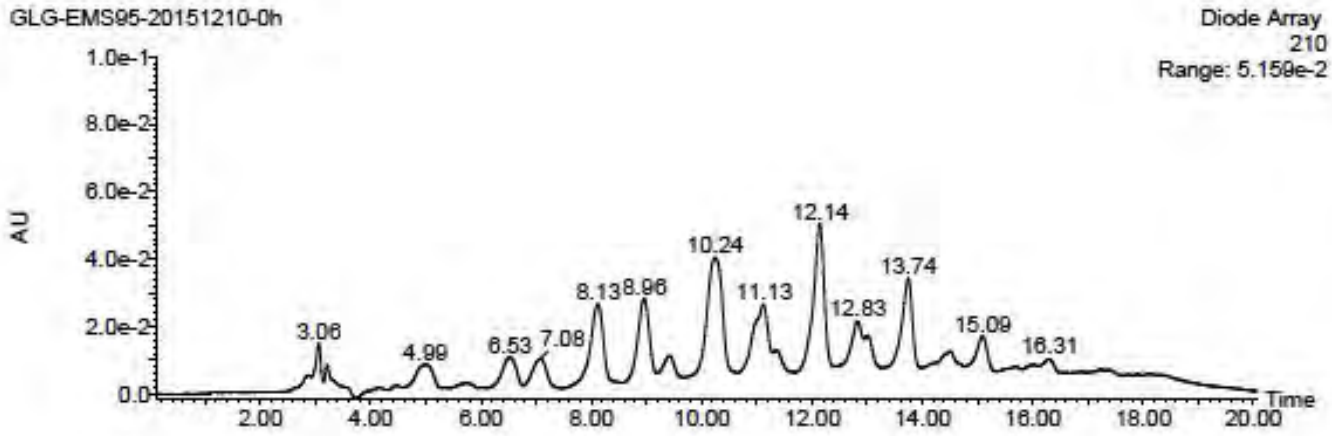
Sample Run Injection Parameter

Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

---------- oOo ----------

End of experimental record.

Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400


GLG-EMS95-20151210-0h
Diode Array
210
Range: 5.159e-2
AU
1.0e-1
8.0e-2
6.0e-2
4.0e-2
2.0e-2
0.0
3.06
4.99
6.53
7.08
8.13
8.96
10.24
11.13
12.14
12.83
13.74
15.09
16.31
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Time

C-4b HPLC-MS Report for EMS95 Lot 20151210 (Post-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151210-6h.raw

Header
Acquired File Name: C:\MassLynx\Default.PRO\Data\312151216-23.Raw
Acquired Date: 17-Dec-2015
Acquired Time: 01:07:43
Job code: Default
Task code:
User Name: operator 1
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID:
Bottle Number: 81
Description: GLG-EMS95-20151210-6h

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents
Degasser ContinuousStroke Volume 100.0 袀 Final Flow Settings not being held at End of Run
A% 91.7 80% acetonitrile
B% 8.3 20% acetonitrile
C% 0.0
D% 0.0
Flow (ml/min) 1.000
Flow Ramp 2.00
Stop Time (mins) 25.0
Column Temperature (oC) 35.0
Column Temperature Limit (oC) 20.0
Min Pressure (Bar) 0.0
Max Pressure (Bar) 300.0
Pre-column Volume (bar) 0.00
Column Position Column 1Pre-column Volume (bar) 0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1

0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm)	200.00
End Wavelength (nm)	400.00
Resolution (nm)	1.2
Sampling Rate (spectra/s)	1.000
Filter Response	1
Exposure Time(ms)	Automatic
Interpolate 656	Yes
Acquisition stop time (mins)	25.00

Save to disk: Yes

Waters996 PDA Analog Channel 1

Output Mode Off

Waters996 PDA Analog Channel 2

Output Mode Ratio	Filter Type	Hamming	Wavelength (nm) 254.00
Bandwidth (nm)	2.40		
Offset (AU)	0.00		
Filter Response Time (s)	1.00		
Threshold (AU)	0.1		
Ratio Denominator (nm)	254.00		

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm)	0.00
Sample Temperature (oC)	35.0
Sample Temperature Limit (oC)	20.0

Purge Loop Volumes 0.00

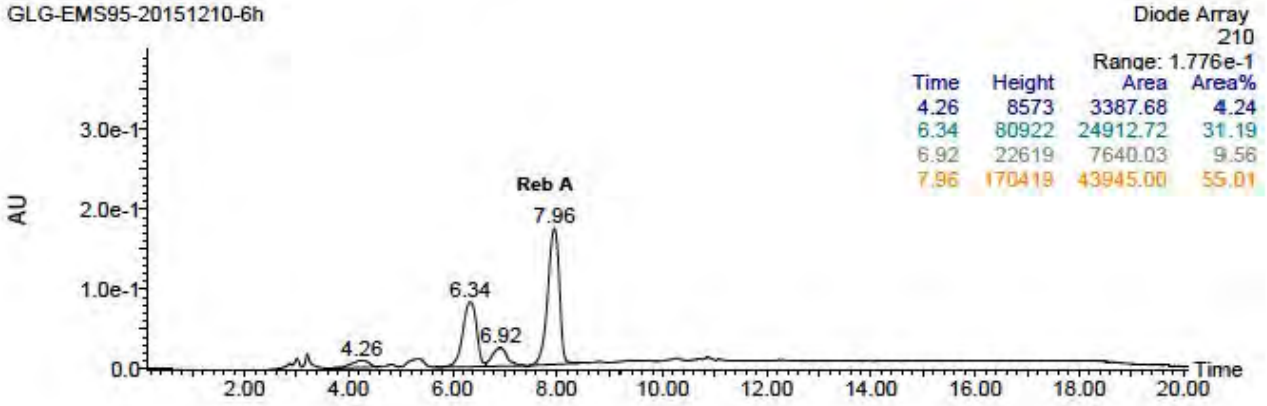
Sample Run Injection Parameter

Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

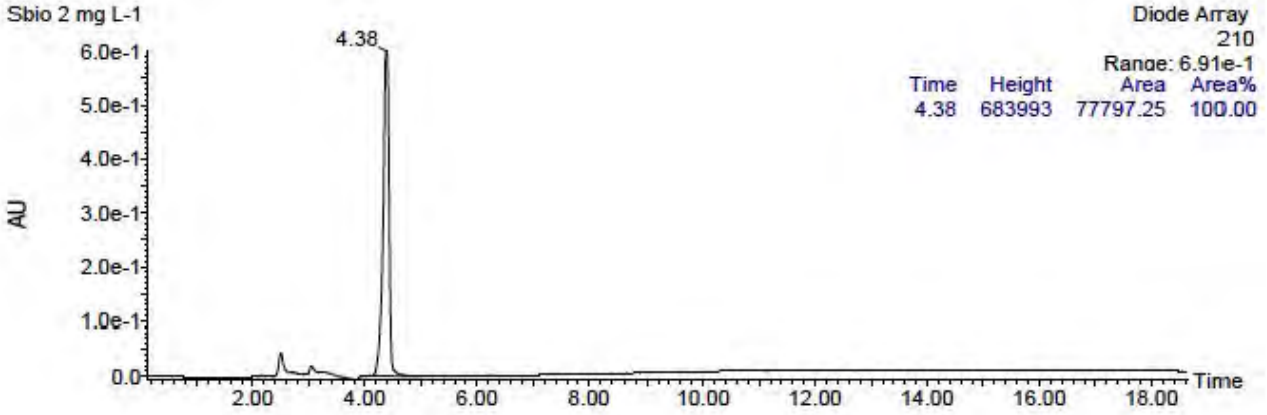
---------- oOo ----------

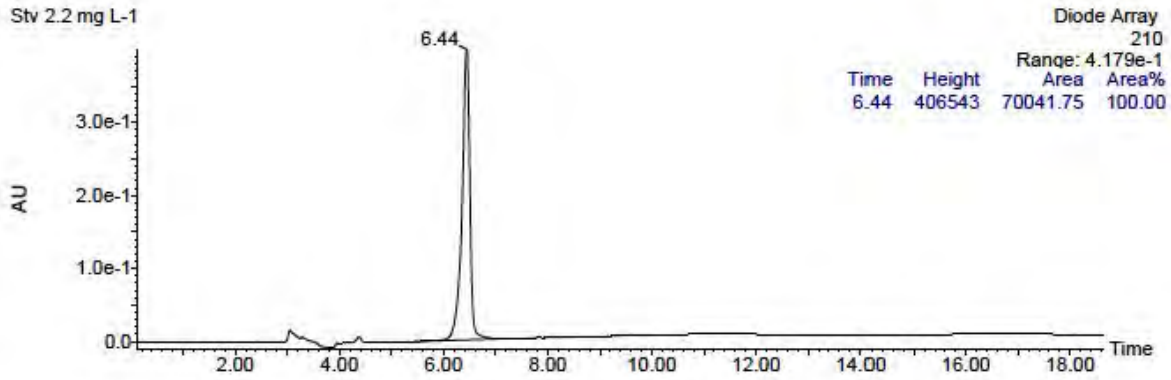
End of experimental record.

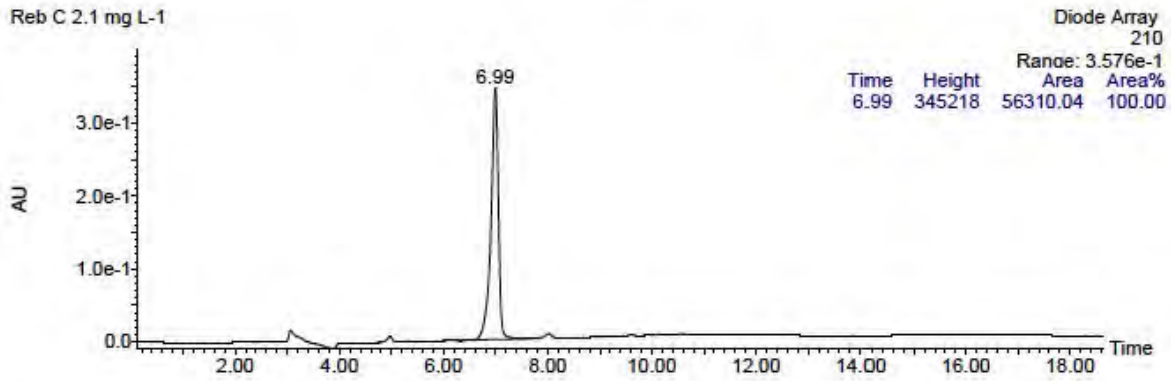
Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400

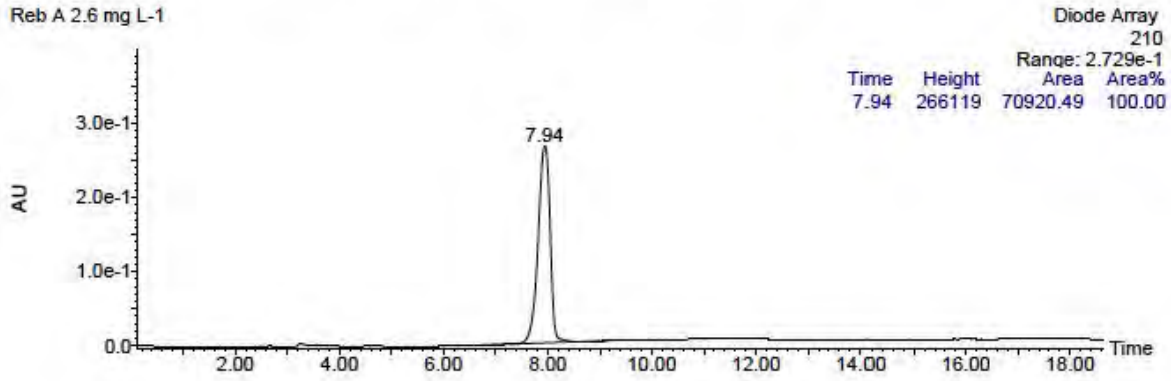


Standard




Stv 2.2 mg L-1
Diode Array
210
Range: 4.179e-1
Time Height Area Area%
6.44 406543 70041.75 100.00
6.44
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb C 2.1 mg L-1
Diode Array
210
Range: 3.576e-1
Time Height Area Area%
6.99 345218 56310.04 100.00
6.99
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb A 2.6 mg L-1
Diode Array
210
Range: 2.729e-1
Time Height Area Area%
7.94 266119 70920.49 100.00
7.94
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time

C-5a HPLC-MS Report for EMS95 Lot 20151212 (Pre-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151212-0h.raw

Header
Acquired File Name: C:\MassLynx\Default.PRO\Data\312151216-19.Raw
Acquired Date: 16-Dec-2015
Acquired Time: 23:23:23
Job code: Default
Task code:
User Name: operator 1
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID:
Bottle Number: 77
Description: GLG-EMS95-20151212-0h

Instrument Calibration
Parameters
MS1 Static: None
MS1 Scanning: None
MS1 Scan Speed: None
MS2 Static: None
MS2 Scanning: None
MS2 Scan Speed: None
Calibration Time:
Calibration Date:
Coefficients
MS1 Static: None
MS2 Static: None
Function 1: None

No experimental record file.

ACE Experimental Record

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents

Degasser	Continuous	Stroke Volume	100.0 Final Flow Settings not being held at End of Run
A%	91.7	80% acetonitrile	
B%	8.3	20% acetonitrile	
C%	0.0		
D%	0.0		
Flow (ml/min)	1.000		
Flow Ramp	2.00		
Stop Time (mins)	25.0		
Column Temperature (oC)	35.0		
Column Temperature Limit (oC)	20.0		
Min Pressure (Bar)	0.0		
Max Pressure (Bar)	300.0		
Pre-column Volume (bar)	0.00		
Column Position	Column 1	Pre-column Volume (bar)	0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1
0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm)	200.00
End Wavelength (nm)	400.00
Resolution (nm)	1.2
Sampling Rate (spectra/s)	1.000
Filter Response	1
Exposure Time(ms)	Automatic

Interpolate 656 Yes
Acquisition stop time (mins) 25.00
Save to disk: Yes
Waters996 PDA Analog Channel 1

Output Mode Off
Waters996 PDA Analog Channel 2

Output Mode RatioFilter Type HammingWavelength (nm) 254.00
Bandwidth (nm) 2.40
Offset (AU) 0.00
Filter Response Time (s) 1.00
Threshold (AU) 0.1
Ratio Denominator (nm) 254.00

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm) 0.00
Sample Temperature (oC) 35.0
Sample Temperature Limit (oC) 20.0

Purge Loop Volumes 0.00

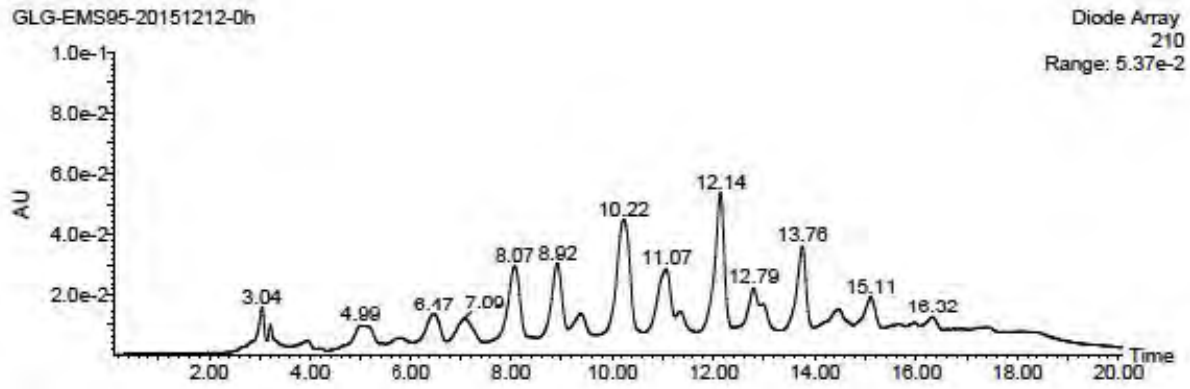
Sample Run Injection Parameter

Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

---------- oOo ----------

End of experimental record.

Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400


GLG-EMS95-20151212-0h
Diode Array
210
Range: 5.37e-2
AU
1.0e-1
8.0e-2
6.0e-2
4.0e-2
2.0e-2
3.04
4.99
6.17
7.09
8.07
8.92
10.22
11.07
12.14
12.79
13.76
15.11
16.32
Time
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00

C-5b HPLC-MS Report for EMS95 Lot 20151212 (Post-hydrolysis)

Acquisition Experiment Report
File:f:\hplc\GLG-EMS95-20151212-6h.raw

Header
Acquired File Name: C:\MassLynx\Default.PRO\Data\312151216-24.Raw
Acquired Date: 17-Dec-2015
Acquired Time: 01:33:48
Job code: Default
Task code:
User Name: operator 1
Laboratory Name:
Instrument:
Conditions:
Submitter:
SampleID:
Bottle Number: 82
Description: GLG-EMS95-20151212-6h

---------- Run method parameters ----------

-- PUMP --

Waters Alliance 2695 HPLC Pump Initial Conditions

Solvents
Degasser ContinuousStroke Volume 100.0 Final Flow Settings not being held at End of Run
A% 91.7 80% acetonitrile
B% 8.3 20% acetonitrile
C% 0.0
D% 0.0
Flow (ml/min) 1.000
Flow Ramp 2.00
Stop Time (mins) 25.0
Column Temperature (oC) 35.0
Column Temperature Limit (oC) 20.0
Min Pressure (Bar) 0.0
Max Pressure (Bar) 300.0
Pre-column Volume (bar) 0.00
Column Position Column 1Pre-column Volume (bar) 0.00

Waters Alliance 2695 HPLC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time	A%	B%	C%	D%	Flow	Curve
0.00	91.7	8.3	0.0	0.0	1.000	1

0.10	91.7	8.3	0.0	0.0	1.000	6
2.00	91.7	8.3	0.0	0.0	1.000	6
15.00	61.7	38.3	0.0	0.0	1.000	6
20.00	91.7	8.3	0.0	0.0	1.000	6
25.00	91.7	8.3	0.0	0.0	1.000	6

Waters Alliance 2695 HPLC Pump External Event Timetable

No Entries in the Pump External Event Timetable.-- END PUMP --

-- DETECTOR --

Waters996 PDA

Start Wavelength (nm)	200.00
End Wavelength (nm)	400.00
Resolution (nm)	1.2
Sampling Rate (spectra/s)	1.000
Filter Response	1
Exposure Time(ms)	Automatic
Interpolate 656	Yes
Acquisition stop time (mins)	25.00

Save to disk: Yes
Waters996 PDA Analog Channel 1

Output Mode Off
Waters996 PDA Analog Channel 2

Output Mode	Ratio	Filter Type	Hamming
Wavelength (nm)	254.00		
Bandwidth (nm)	2.40		
Offset (AU)	0.00		
Filter Response Time (s)	1.00		
Threshold (AU)	0.1		
Ratio Denominator (nm)	254.00		

-- END DETECTOR --

-- AUTOSAMPLER --

Waters Alliance 2695 Autosampler Initial Conditions

Needle Depth (mm)	0.00
Sample Temperature (oC)	35.0
Sample Temperature Limit (oC)	20.0

Purge Loop Volumes 0.00

Sample Run Injection Parameter

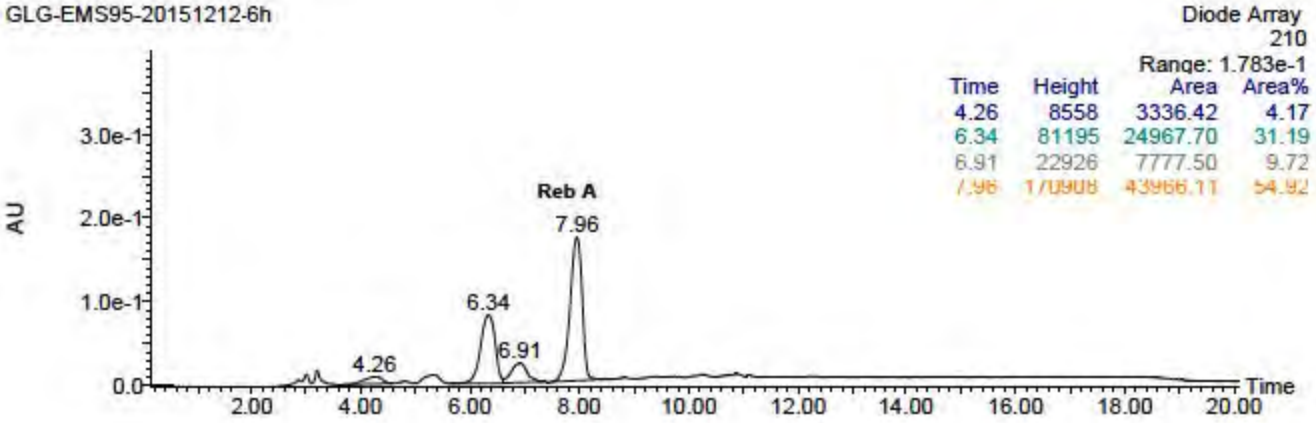
Injection Volume (ul) - 10.00
-- END AUTOSAMPLER --

---------- oOo ----------

End of experimental record.

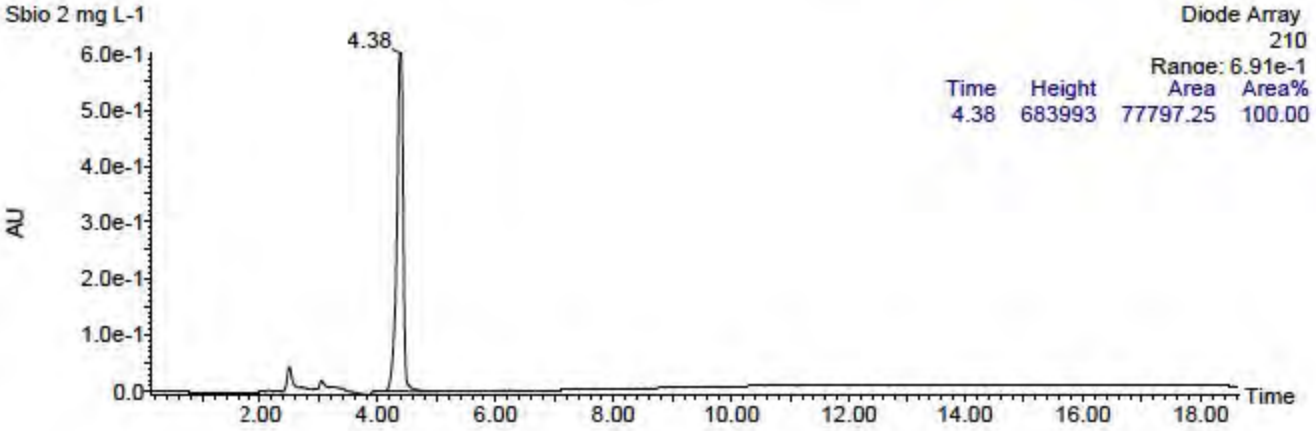
Function 1
Scans in function: 1494
Function type: Diode Array
Wavelength range (nm) : 200 to 400

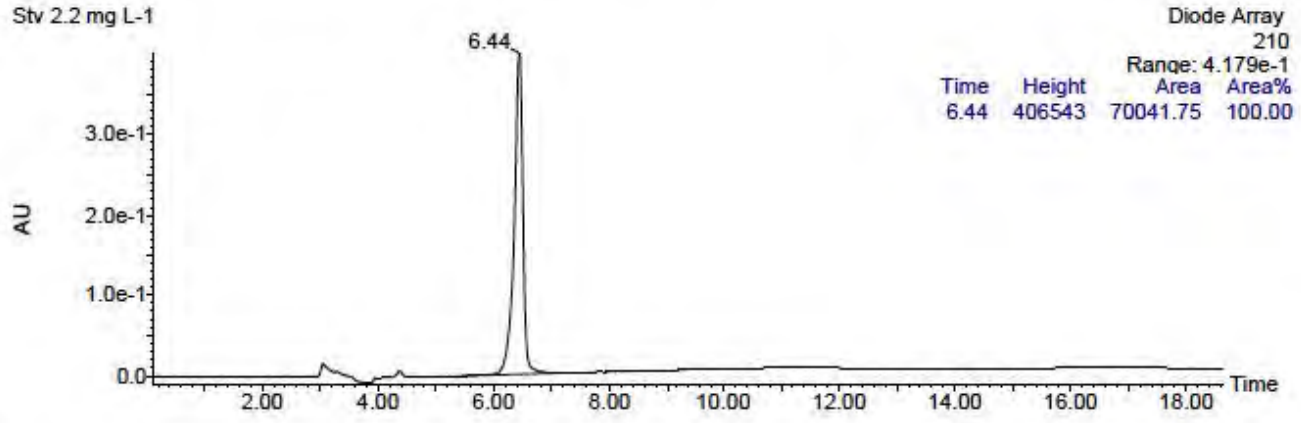
GLG-EMS95-20151212-6h

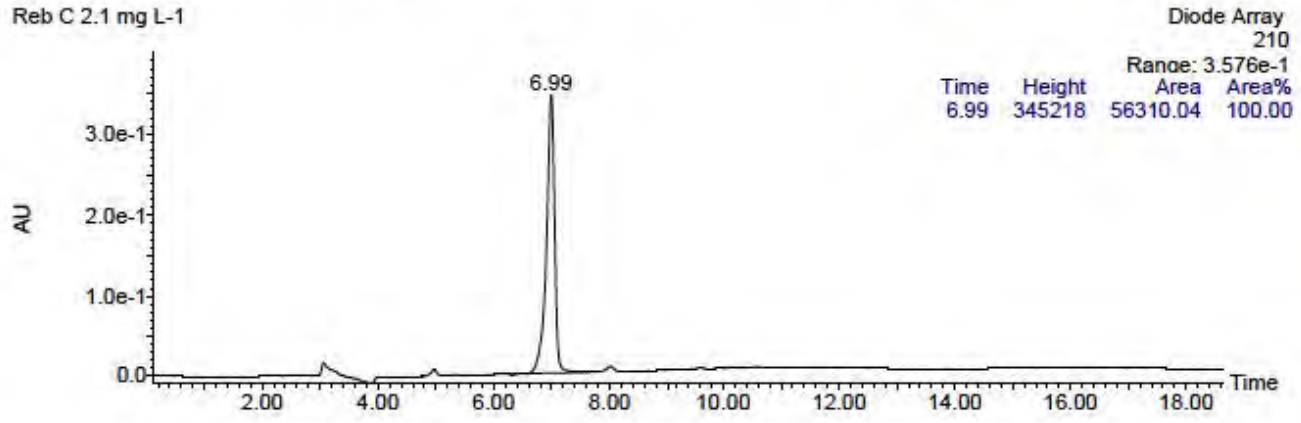


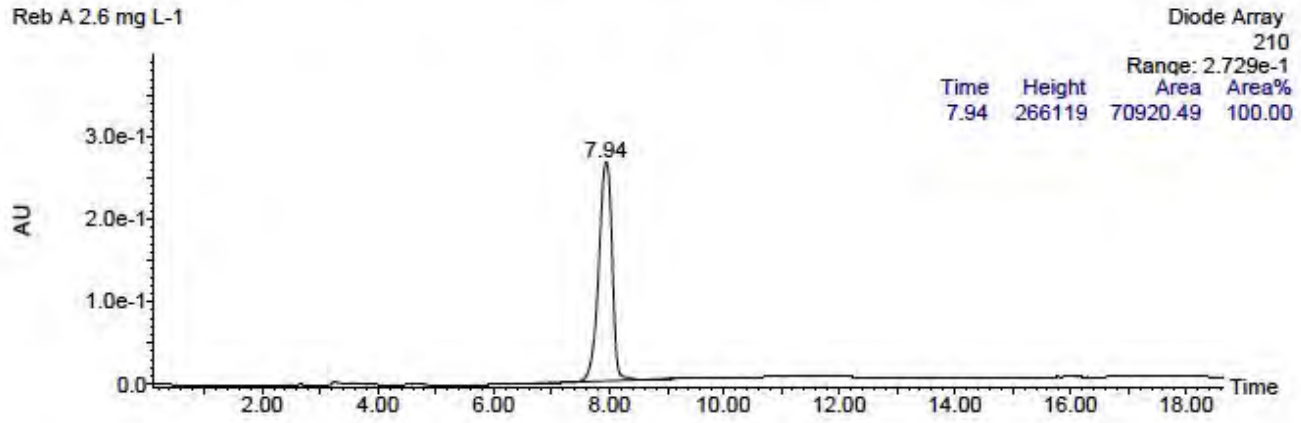
Standard

Sbio 2 mg L-1




Stv 2.2 mg L-1
Diode Array
210
Range: 4.179e-1
Time Height Area Area%
6.44 406543 70041.75 100.00
6.44
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb C 2.1 mg L-1
Diode Array
210
Range: 3.576e-1
Time Height Area Area%
6.99 345218 56310.04 100.00
6.99
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time


Reb A 2.6 mg L-1
Diode Array
210
Range: 2.729e-1
Time Height Area Area%
7.94 266119 70920.49 100.00
7.94
AU
3.0e-1
2.0e-1
1.0e-1
0.0
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00
Time

Appendix D Certificates of Analysis for Multiple Production Batches of EMS95

D-1 Certificate of Analysis for EMS95 Lot 20151201

TASTEBOOST

Certificate of Analysis

GLG
LEADING LIFE TECHNOLOGIES
BETTER STEVIA, MONK FRUIT AND MORE

Research and Development
GLG Life Tech Corporation
www.glglifetech.com
GLG-QA-COA-80

Product: TasteBoost™ EMS95
Lot Number: GLG-EMS95-20151201
Manufacturing Date: Dec. 1st, 2015
Country of Origin: CHINA
Shelf Life: 2 Years

Product Description: TasteBoost™ EMS95 is white to light yellow powder, with a refreshing sweet taste.

Distributed By:
GLG Life Tech Corporation
Suite 2168-1050 West Pender Street
Vancouver, B.C. V6E 3S7
Canada
Phone: 1.604.669.2602
Fax: 1.604.662.8858
Email: sales@glglifetech.com
Web: www.glglifetech.com

Manufacturing By:
Qingdao Runde Biotechnology Co., Ltd.
Lingshanwei Town, Jiaonan County,
Qingdao, Shandong, 266427 China
Phone: +86.532.68018636
Fax: +86.532.68018626

Qingdao Runde Biotechnology Co., Ltd. is a wholly owned foreign subsidiary of GLG Life Tech Corporation.

Date of Analysis: Dec. 16th, 2015

INSPECTION ITEM	SPECIFICATION	RESULT	METHOD
Appearance	White powder	White powder	Organoleptic AS IS
Solubility	Freely soluble in water	pass	JECFA 2010
Unreacted steviol glycosides, (wt/wt)	≤15.0% (on dry basis)	13.1%	JECFA 2010
Content of a-glucosyl steviol glycosides, (wt/wt)	≥80.0% (on dry basis)	82.1%	JECFA 2010
Total Steviol Glycosides, (wt/wt)	≥95.0% (on dry basis)	95.2%	JECFA 2010
Loss on Drying	≤4.0%	3.68%	JECFA Vol.4
PH	4.5-7.0	5.25	JECFA Vol.4
Residue on Ignition	≤1.0%	0.08%	JECFA Vol.4
Total Metals	<10.0 ppm	<10.0 ppm	USP231
Lead (Pb)	≤1.0 ppm	0.06ppm	JECFA Vol.4
Arsenic (As)	≤1.0 ppm	0.03ppm	JECFA Vol.4
Residual Solvents - Ethanol	≤0.5%	pass	JECFA Vol.4
- Methanol	≤0.02%	pass	JECFA Vol.4
Total Plate Count	<1000 CFU/g	<10 CFU/g	FDA-BAM chapter 3
Yeast & Mold	<100 CFU/g	<10 CFU/g	FDA-BAM chapter 18
E.coli	<3 MPN/g	<3 MPN/g	FDA-BAM chapter 4
Staphylococcus Aureus	<10 CFU/g	<10 CFU/g	FDA-BAM chapter 12
Salmonella (/25g)	Negative	Negative	FDA-BAM chapter 5

Conclusion	QUALIFIED

Note: This product should be stored sealed in a cool, dry place.

Analyzed by: [redacted] **Date:** 16/12/2015

Checked by: [redacted] **Date:** 16/12/2015

Approved by: [redacted] (Quality Manager) **Date:** 16/12/2015

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Suite 2168 - 1050 West Pender Street *Vancouver, B.C. *Canada * V6E 3S7
Phone: 604.669.2602 * Fax: 604.662.8858 *Email: sales@glglifetech.com * W: www.glglifetech.com

D-2 Certificate of Analysis for EMS95 Lot 20151203

TASTEBOOST

Certificate of Analysis

GLG

Research and Development
GLG Life Tech Corporation
www.glglifetech.com
GLG-QA-COA-80

Product: TasteBoost™ EMS95
Lot Number: GLG-EMS95-20151203
Manufacturing Date: Dec. 3rd ,2015
Country of Origin: CHINA
Shelf Life: 2 Years

Product Description: TasteBoost™ EMS95 is white to light yellow powder, with a refreshing sweet taste.

Distributed By: GLG Life Tech Corporation
Suite 2168-1050 West Pender Street
Vancouver, B.C. V6E 3S7
Canada
Phone: 1.604.669.2602
Fax: 1.604.662.8858
Email: sales@glglifetech.com
Web: www.glglifetech.com

Manufacturing By: Qingdao Runde Biotechnology Co., Ltd.
Lingshanwei Town, Jiaonan County,
Qingdao, Shandong, 266427 China
Phone: +86.532.68018636
Fax: +86.532.68018626

Qingdao Runde Biotechnology Co., Ltd. is a wholly owned foreign subsidiary of GLG Life Tech Corporation.

Date of Analysis: Dec. 17th ,2015

INSPECTION ITEM	SPECIFICATION	RESULT	METHOD
Appearance	White powder	White powder	Organoleptic AS IS
Solubility	Freely soluble in water	Pass	JECFA 2010
Unreacted steviol glycosides, (wt/wt)	≤15.0% (on dry basis)	12.8%	JECFA 2010
Content of a-glucosyl steviol glycosides, (wt/wt)	≥80.0% (on dry basis)	82.4%	JECFA 2010
Total Steviol Glycosides, (wt/wt)	≥95.0% (on dry basis)	95.2%	JECFA 2010
Loss on Drying	≤4.0%	3.32%	JECFA Vol.4
PH	4.5-7.0	5.38	JECFA Vol.4
Residue on Ignition	≤1.0%	0.09%	JECFA Vol.4
Total Metals	<10.0 ppm	<10.0 ppm	USP231
Lead (Pb)	≤1.0 ppm	0.02ppm	JECFA Vol.4
Arsenic (As)	≤1.0 ppm	0.04ppm	JECFA Vol.4
Residual Solvents - Ethanol	≤0.5%	Pass	JECFA Vol.4
- Methanol	≤0.02%	Pass	JECFA Vol.4
Total Plate Count	<1000 CFU/g	<10 CFU/g	FDA-BAM chapter 3
Yeast & Mold	<100 CFU/g	<10 CFU/g	FDA-BAM chapter 18
E.coli	<3 MPN/g	<3 MPN/g	FDA-BAM chapter 4
Staphylococcus Aureus	<10 CFU/g	<10 CFU/g	FDA-BAM chapter 12
Salmonella (/25g)	Negative	Negative	FDA-BAM chapter 5

Conclusion	QUALIFIED

Note: This product should be stored sealed in a cool, dry place.

Analyzed by: [redacted] **Date:** 17/12/2015

Checked by: [redacted] **Date:** 17/12/2015

Approved by: [redacted] (Quality Manager) **Date:** 17/12/2015

Suite 2168 - 1050 West Pender Street *Vancouver, B.C.*Canada * V6E 3S7
Phone: 604.669.2602 * Fax: 604.662.8858*Email: sales@glglifetech.com * W: www.glglifetech.com

D-3 Certificate of Analysis for EMS95 Lot 20151206

TASTEBOOST

Certificate of Analysis

GLG
LEADING LIFE TECHNOLOGIES
BETTER STEVIL MORE FRUIT AHEAD

Research and Development
GLG Life Tech Corporation
www.glglifetech.com
GLG-QA-COA-80

Product: TasteBoost™ EMS95
Lot Number: GLG-EMS95-20151206
Manufacturing Date: Dec. 6th ,2015
Country of Origin: CHINA
Shelf Life: 2 Years

Product Description: TasteBoost™ EMS95 is white to light yellow powder, with a refreshing sweet taste.

Distributed By:
GLG Life Tech Corporation
Suite 2168-1050 West Pender Street
Vancouver, B.C. V6E 3S7
Canada
Phone: 1.604.669.2602
Fax: 1.604.662.8858
Email: sales@glglifetech.com
Web: www.glglifetech.com

Manufacturing By:
Qingdao Runde Biotechnology Co., Ltd.
Lingshanwei Town, Jiaonan County,
Qingdao, Shandong, 266427 China
Phone: +86.532.68018636
Fax: +86.532.68018626

Qingdao Runde Biotechnology Co., Ltd. is a wholly owned foreign subsidiary of GLG Life Tech Corporation.

Date of Analysis: Dec. 17th ,2015

INSPECTION ITEM	SPECIFICATION	RESULT	METHOD
Appearance	White powder	White powder	Organoleptic AS IS
Solubility	Freely soluble in water	Pass	JECFA 2010
Unreacted steviol glycosides, (wt/wt)	≤15.0% (on dry basis)	13.8%	JECFA 2010
Content of a-glucosyl steviol glycosides, (wt/wt)	≥80.0% (on dry basis)	81.5%	JECFA 2010
Total Steviol Glycosides, (wt/wt)	≥95.0% (on dry basis)	95.3%	JECFA 2010
Loss on Drying	≤4.0%	3.29%	JECFA Vol.4
PH	4.5-7.0	5.30	JECFA Vol.4
Residue on Ignition	≤1.0%	0.09%	JECFA Vol.4
Total Metals	<10.0 ppm	<10.0 ppm	USP231
Lead (Pb)	≤1.0 ppm	0.04ppm	JECFA Vol.4
Arsenic (As)	≤1.0 ppm	0.02ppm	JECFA Vol.4
Residual Solvents - Ethanol	≤0.5%	Pass	JECFA Vol.4
- Methanol	≤0.02%	Pass	JECFA Vol.4
Total Plate Count	<1000 CFU/g	<10 CFU/g	FDA-BAM chapter 3
Yeast & Mold	<100 CFU/g	<10 CFU/g	FDA-BAM chapter 18
E.coli	<3 MPN/g	<3 MPN/g	FDA-BAM chapter 4
Staphylococcus Aureus	<10 CFU/g	<10 CFU/g	FDA-BAM chapter 12
Salmonella (/25g)	Negative	Negative	FDA-BAM chapter 5

Conclusion	QUALIFIED

Note: This product should be stored sealed in a cool, dry place.

Analyzed by: [redacted] **Date:** 17/12/2015
Checked by: [redacted] **Date:** 17/12/2015
Approved by: [redacted] uality Manager) **Date:** 17/12/2015

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Phone: 604.669.2602 * Fax: 604.662.8858*Email: sales@glglifetech.com * W: www.glglifetech.com

D-4 Certificate of Analysis for EMS95 Lot 20151210

TASTEBOOST

Certificate of Analysis

GLG

Research and Development
GLG Life Tech Corporation
www.glglifetech.com
GLG-QA-COA-80

Product: TasteBoost™ EMS95
Lot Number: GLG-EMS-20151210
Manufacturing Date: Dec. 10th, 2015
Country of Origin: CHINA
Shelf Life: 2 Years

Product Description: TasteBoost™ EMS95 is white to light yellow powder, with a refreshing sweet taste.

Distributed By: GLG Life Tech Corporation
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Web: www.glglifetech.com

Manufacturing By: Qingdao Runde Biotechnology Co., Ltd.
Lingshanwei Town, Jiaonan County,
Qingdao, Shandong, 266427 China
Phone: +86.532.68018636
Fax: +86.532.68018626

Qingdao Runde Biotechnology Co., Ltd. is a wholly owned foreign subsidiary of GLG Life Tech Corporation.

Date of Analysis: Dec. 17th, 2015

INSPECTION ITEM	SPECIFICATION	RESULT	METHOD
Appearance	White powder	White powder	Organoleptic AS IS
Solubility	Freely soluble in water	Pass	JECFA 2010
Unreacted steviol glycosides, (wt/wt)	≤15.0% (on dry basis)	13.1%	JECFA 2010
Content of a-glucosyl steviol glycosides, (wt/wt)	≥80.0% (on dry basis)	82.1%	JECFA 2010
Total Steviol Glycosides, (wt/wt)	≥95.0% (on dry basis)	95.2%	JECFA 2010
Loss on Drying	≤4.0%	3.38%	JECFA Vol.4
PH	4.5-7.0	5.19	JECFA Vol.4
Residue on Ignition	≤1.0%	0.06%	JECFA Vol.4
Total Metals	<10.0 ppm	<10.0 ppm	USP231
Lead (Pb)	≤1.0 ppm	0.05ppm	JECFA Vol.4
Arsenic (As)	≤1.0 ppm	0.03ppm	JECFA Vol.4
Residual Solvents - Ethanol	≤0.5%	Pass	JECFA Vol.4
- Methanol	≤0.02%	Pass	JECFA Vol.4
Total Plate Count	<1000 CFU/g	<10 CFU/g	FDA-BAM chapter 3
Yeast & Mold	<100 CFU/g	<10 CFU/g	FDA-BAM chapter 18
E.coli	<3 MPN/g	<3 MPN/g	FDA-BAM chapter 4
Staphylococcus Aureus	<10 CFU/g	<10 CFU/g	FDA-BAM chapter 12
Salmonella (/25g)	Negative	Negative	FDA-BAM chapter 5

Conclusion	QUALIFIED

Note: This product should be stored sealed in a cool, dry place.

Analyzed by: [redacted] **Date:** 17/12/2015

Checked by: [redacted] **Date:** 17/12/2015

Approved by: [redacted] (Quality Manager) **Date:** 17/12/2015

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D-5 Certificate of Analysis for EMS95 Lot 20151212

TASTEBOOST

Certificate of Analysis

GLG

Research and Development
GLG Life Tech Corporation
www.glglifetech.com
GLG-QA-COA-80

Product: TasteBoost™ EMS95
Lot Number: GLG-EMS95-20151212
Manufacturing Date: Dec. 12th, 2015
Country of Origin: CHINA
Shelf Life: 2 Years

Product Description: TasteBoost™ EMS95 is white to light yellow powder, with a refreshing sweet taste.

Distributed By: GLG Life Tech Corporation
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Web: www.glglifetech.com

Manufacturing By: Qingdao Runde Biotechnology Co., Ltd.
Lingshanwei Town, Jiaonan County,
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Phone: +86.532.68018636
Fax: +86.532.68018626

Qingdao Runde Biotechnology Co., Ltd. is a wholly owned foreign subsidiary of GLG Life Tech Corporation.

Date of Analysis: Dec. 17th, 2015

INSPECTION ITEM	SPECIFICATION	RESULT	METHOD
Appearance	White powder	White powder	Organoleptic AS IS
Solubility	Freely soluble in water	Pass	JECFA 2010
Unreacted steviol glycosides, (wt/wt)	≤15.0% (on dry basis)	12.8%	JECFA 2010
Content of a-glucosyl steviol glycosides, (wt/wt)	≥80.0% (on dry basis)	82.5%	JECFA 2010
Total Steviol Glycosides, (wt/wt)	≥95.0% (on dry basis)	95.3%	JECFA 2010
Loss on Drying	≤4.0%	3.41%	JECFA Vol.4
PH	4.5-7.0	5.36	JECFA Vol.4
Residue on Ignition	≤1.0%	0.08%	JECFA Vol.4
Total Metals	<10.0 ppm	<10.0 ppm	USP231
Lead (Pb)	≤1.0 ppm	0.06ppm	JECFA Vol.4
Arsenic (As)	≤1.0 ppm	0.05ppm	JECFA Vol.4
Residual Solvents - Ethanol	≤0.5%	Pass	JECFA Vol.4
- Methanol	≤0.02%	Pass	JECFA Vol.4
Total Plate Count	<1000 CFU/g	<10 CFU/g	FDA-BAM chapter 3
Yeast & Mold	<100 CFU/g	<10 CFU/g	FDA-BAM chapter 18
E.coli	<3 MPN/g	<3 MPN/g	FDA-BAM chapter 4
Staphylococcus Aureus	<10 CFU/g	<10 CFU/g	FDA-BAM chapter 12
Salmonella (/25g)	Negative	Negative	FDA-BAM chapter 5

Conclusion	QUALIFIED

Note: This product should be stored sealed in a cool, dry place.

Analyzed by: [redacted] **Date:** 17/12/2015

Checked by: [redacted] **Date:** 17/12/2015

Approved by: [redacted] (Quality Manager) **Date:** 17/12/2015

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Appendix E Pesticide Analytical Report for EMS95 from Intertek

Test Report for Pesticides for Lot 20151201

Intertek

Test Report Number: SHAH00650456

Applicant: QINGDAO RUNDE BIOTECHNOLOGY CO, LTD
Lingshanwei Town, Jiaonan City Qingdao,
Shandong,China 266427

Date: 01 Mar, 2016

Sample Description:

Sample Name	:	Enzymatically Modified Stevia/EMS95
Test Purpose	:	Entrust Test
Sample Specification	:	RA97
Sample Quantity	:	100g
Batch No./Code	:	GLG-EMS95-20151201

Tests Conducted:
As requested by the applicant, for details refer to attached page(s).

To be continued

Authorized By:
For Intertek Testing Services Ltd., Shanghai
(b) (6)

Cheng Jun
General Manager

上海天祥质量技术服务有限公司 检验检测专用章

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Intertek

Test Report Number: SHAH00650456

Tests Conducted

1 Test Result:

GC/MS/MS(GC/MS) Results Table
Pesticides Found and Concentration

No.	Test Item	Limit (mg/kg)	Result (mg/kg)	LOQ (mg/kg)	Conclusion
1	2,4'-DDD	—	ND	0.01	—
2	2,4'-DDE	—	ND	0.01	—
3	2,4'-DDT	—	ND	0.01	—
4	2-phenylphenol	—	ND	0.01	—
5	4,4'-DDD	—	ND	0.01	—
6	4,4'-DDE	—	ND	0.01	—
7	4,4'-DDT	—	ND	0.01	—
8	Acetochlor	—	ND	0.01	—
9	Aldrin	—	ND	0.01	—
10	Ametryn	—	ND	0.01	—
11	Amitraz	—	ND	0.01	—
12	Atrazine	—	ND	0.01	—
13	Benalaxyl	—	ND	0.01	—
14	Benfluralin	—	ND	0.01	—
15	Benoxacor	—	ND	0.01	—
16	Bifenthrin	—	ND	0.01	—
17	Bromopropylate	—	ND	0.01	—
18	Bupirimate	—	ND	0.01	—
19	Buprofezin	—	ND	0.01	—
20	Butachlor	—	ND	0.01	—
21	Cadusafos	—	ND	0.01	—
22	Captan	—	ND	0.05	—
23	Chlordane	—	ND	0.01	—
24	Chlorfenapyr	—	ND	0.01	—
25	Chlorfenvinphos	—	ND	0.01	—
26	Chlorfluazuron	—	ND	0.01	—
27	Chlorothalonil	—	ND	0.01	—
28	Chlorpropham	—	ND	0.01	—
29	Chlorpyrifos	—	ND	0.01	—
30	Chlorpyrifos-methyl	—	ND	0.01	—
31	Clethodim	—	ND	0.05	—
32	Coumaphos	—	ND	0.01	—
33	Cyanazine	—	ND	0.01	—
34	Cyflufenamid	—	ND	0.05	—
35	Cyfluthrin	—	ND	0.1	—
36	Cypermethrin	—	ND	0.1	—
37	Cyprodinil	—	ND	0.01	—
38	Deltamethrin	—	ND	0.01	—
39	Diazinon	—	ND	0.01	—
40	Dichlofluanid	—	ND	0.01	—
41	Dichlorvos	—	ND	0.01	—
42	Dicloran	—	ND	0.01	—
43	Dicofol	—	ND	0.01	—
44	Dieldrin	—	ND	0.01	—
45	Diethofencarb	—	ND	0.01	—
46	Difenoconazole	—	ND	0.01	—
47	Diphenylamine	—	ND	0.01	—
48	Edifenphos	—	ND	0.01	—
49	Endosulfan-1	—	ND	0.01	—
50	Endosulfan-2	—	ND	0.01	—
51	Endosulfan-sulfate	—	ND	0.01	—
52	Endrin	—	ND	0.01	—

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Intertek

Test Report Number: SHAH00650456

Tests Conducted

No.	Test Item	Limit (mg/kg)	Result (mg/kg)	LOQ (mg/kg)	Conclusion
53	EPN	—	ND	0.01	—
54	Epoxiconazole	—	ND	0.01	—
55	Ethion	—	ND	0.01	—
56	Ethoprophos	—	ND	0.01	—
57	Etofenprox	—	ND	0.1	—
58	Etrimfos	—	ND	0.01	—
59	Fenamiphos	—	ND	0.01	—
60	Fenarimol	—	ND	0.01	—
61	Fenitrothion	—	ND	0.01	—
62	Fenobucarb	—	ND	0.01	—
63	Fenoxycarb	—	ND	0.01	—
64	Fenpropathrin	—	ND	0.1	—
65	Fenpropimorph	—	ND	0.01	—
66	Fenthion	—	ND	0.01	—
67	Fenvalerate	—	ND	0.01	—
68	Fipronil	—	ND	0.01	—
69	Fluazifop-butyl	—	ND	0.01	—
70	Flucythrinate	—	ND	0.1	—
71	Flufenoxuron	—	ND	0.01	—
72	Flumioxazin	—	ND	0.01	—
73	Flusilazole	—	ND	0.01	—
74	Fluvalinate	—	ND	0.01	—
75	Gamma-HCH	—	ND	0.01	—
76	Halfenprox	—	ND	0.01	—
77	HCH(BHC)	—	ND	0.05	—
78	Heptachlor	—	ND	0.01	—
79	Hexachlorobenzene	—	ND	0.01	—
80	Hexythiazox	—	ND	0.05	—
81	Imazalil	—	ND	0.01	—
82	Iprobenfos	—	ND	0.01	—
83	Iprodione	—	ND	0.01	—
84	Isocarbophos	—	ND	0.01	—
85	Isofenphos	—	ND	0.01	—
86	Isoprocarb	—	ND	0.01	—
87	Isoprothiolane	—	ND	0.01	—
88	Kresoxim-methyl	—	ND	0.01	—
89	Lambda-Cyhalothrin	—	ND	0.01	—
90	Malathion	—	ND	0.01	—
91	Mefenoxam	—	ND	0.01	—
92	Metalaxyl	—	ND	0.01	—
93	Metamitron	—	ND	0.01	—
94	Methidathion	—	ND	0.01	—
95	Metolachlor	—	ND	0.01	—
96	Mevinphos	—	ND	0.01	—
97	Myclobutanil	—	ND	0.01	—
98	Napropamide	—	ND	0.01	—
99	Nitrofen	—	ND	0.01	—
100	Nitrothal-isopropyl	—	ND	0.01	—
101	Oxadixyl	—	ND	0.1	—
102	Oxadiazon	—	ND	0.01	—
103	Oxyfluorfen	—	ND	0.01	—
104	Paclobutrazol	—	ND	0.01	—
105	Paraoxon-ethyl	—	ND	0.01	—
106	Parathion	—	ND	0.01	—
107	Parathion-methyl	—	ND	0.01	—

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Intertek

Test Report Number: SHAH00650456

Tests Conducted

No.	Test Item	Limit (mg/kg)	Result (mg/kg)	LOQ (mg/kg)	Conclusion
108	Penconazole	—	ND	0.01	—
109	Pendimethalin	—	ND	0.01	—
110	Permethrin	—	ND	0.01	—
111	Perthan	—	ND	0.01	—
112	Phenthoate	—	ND	0.01	—
113	Phorate	—	ND	0.01	—
114	Phosalone	—	ND	0.01	—
115	Phosmet	—	ND	0.01	—
116	Pirimicarb	—	ND	0.01	—
117	Pirimiphos-ethyl	—	ND	0.01	—
118	Pirimiphos-methyl	—	ND	0.01	—
119	Prochloraz	—	ND	0.01	—
120	Procymidone	—	ND	0.01	—
121	Profenofos	—	ND	0.01	—
122	Prometryn	—	ND	0.01	—
123	Propargite	—	ND	0.01	—
124	Propetamphos	—	ND	0.01	—
125	Propham	—	ND	0.01	—
126	Propiconazole	—	ND	0.01	—
127	Propoxur	—	ND	0.01	—
128	Propyzamide	—	ND	0.01	—
129	Pyrazophos	—	ND	0.01	—
130	Pyridaben	—	ND	0.01	—
131	Pyridaphenthion	—	ND	0.01	—
132	Pyrimethanil	—	ND	0.01	—
133	Quinalphos	—	ND	0.01	—
134	Quintozene	—	ND	0.01	—
135	S-421	—	ND	0.01	—
136	Simazine	—	ND	0.01	—
137	Spiroxamine	—	ND	0.01	—
138	Tebuconazole	—	ND	0.01	—
139	Tecnazene	—	ND	0.01	—
140	Tetrachlorvinphos	—	ND	0.01	—
141	Tetradifon	—	ND	0.01	—
142	Thiamethoxam	—	ND	0.05	—
143	Tolclofos-methyl	—	ND	0.01	—
144	Tolyfluanid	—	ND	0.01	—
145	Triadimefon	—	ND	0.01	—
146	Triadimenol	—	ND	0.01	—
147	Triazophos	—	ND	0.01	—
148	Trifloxystrobin	—	ND	0.01	—
149	Trifluralin	—	ND	0.01	—
150	Vinclozolin	—	ND	0.01	—

To be continued

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Intertek

Test Report Number: SHAH00650456

Tests Conducted

2 Test Result:

LC/MS/MS Results Table
Pesticides Found and Concentration

No.	Test Item	Limit (mg/kg)	Result (mg/kg)	LOQ (mg/kg)	Conclusion
1	3-hydroxycarbofuran	—	ND	0.01	—
2	Acephate	—	ND	0.05	—
3	Acetamiprid	—	ND	0.01	—
4	Acifluorfen	—	ND	0.01	—
5	Aldicarb	—	ND	0.01	—
6	Aldicarb sulfone	—	ND	0.01	—
7	Aldicarb sulfoxide	—	ND	0.01	—
8	Amidosulfuron	—	ND	0.01	—
9	Anilazine	—	ND	0.01	—
10	Azinphos-methyl	—	ND	0.01	—
11	Azoxystrobin	—	ND	0.005	—
12	Bendiocarb	—	ND	0.005	—
13	Benfuracarb	—	ND	0.01	—
14	Bensulfuron-methyl	—	ND	0.005	—
15	Boscalid	—	ND	0.005	—
16	Butocarboxim	—	ND	0.01	—
17	Carbaryl	—	ND	0.005	—
18	Carbendazim	—	ND	0.005	—
19	Carbofuran	—	ND	0.01	—
20	Chlorbenzuron	—	ND	0.01	—
21	Chlorotoluron	—	ND	0.005	—
22	Clofentezine	—	ND	0.005	—
23	Cymoxanil	—	ND	0.01	—
24	Cyromazine	—	ND	0.005	—
25	Daminozide	—	ND	0.01	—
26	Dicrotophos	—	ND	0.01	—
27	Dimethoate	—	ND	0.005	—
28	Dimethomorph	—	ND	0.005	—
29	Emamectin benzoate	—	ND	0.01	—
30	Ethiofencarb	—	ND	0.005	—
31	Ethoxyquin	—	ND	0.1	—
32	Fenhexamid	—	ND	0.01	—
33	Fomesafen	—	ND	0.01	—
34	Furathiocarb	—	ND	0.005	—
35	Heptenophos	—	ND	0.005	—
36	Imidacloprid	—	ND	0.01	—
37	Indoxacarb	—	ND	0.005	—
38	Iprovalicarb	—	ND	0.005	—
39	Isofenphos-methyl	—	ND	0.005	—
40	Isoproturon	—	ND	0.002	—
41	Isouron	—	ND	0.01	—
42	Linuron	—	ND	0.002	—
43	Methamidophos	—	ND	0.05	—
44	Methiocarb	—	ND	0.005	—
45	Methomyl	—	ND	0.01	—
46	Methoxyfenozide	—	ND	0.005	—
47	Monocrotophos	—	ND	0.005	—
48	Naled	—	ND	0.1	—
49	Nicosulfuron	—	ND	0.005	—
50	Omethoate	—	ND	0.002	—
51	Oxydemeton-methyl	—	ND	0.01	—
52	Phosphamidon	—	ND	0.01	—
53	Phoxim	—	ND	0.005	—

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www.intertek.com www.intertek.com.cn

Intertek

Test Report Number: SHAH00650456

Tests Conducted

No.	Test Item	Limit (mg/kg)	Result (mg/kg)	LOQ (mg/kg)	Conclusion
54	Promecarb	—	ND	0.01	—
55	Propamocarb	—	ND	0.005	—
56	Pymetrozin	—	ND	0.05	—
57	Quizalofop-ethyl	—	ND	0.005	—
58	Rimsulfuron	—	ND	0.01	—
59	Spinosad	—	ND	0.01	—
60	Tebufenozide	—	ND	0.01	—
61	Thiabendazole	—	ND	0.01	—
62	Thiacloprid	—	ND	0.005	—
63	Thifensulfuron-methyl	—	ND	0.01	—
64	Thiodicarb	—	ND	0.01	—
65	Thiofanox-sulfone	—	ND	0.005	—
66	Thiofanox-sulfoxid	—	ND	0.01	—
67	Thiophanate-methyl	—	ND	0.01	—
68	Triasulfuron	—	ND	0.01	—
69	Trichlorfon	—	ND	0.005	—
70	Triflumizole	—	ND	0.005	—
71	Triflusulfuron-methyl	—	ND	0.01	—
72	Vamidothion	—	ND	0.01	—

Remark: LOQ = Limit of quantity
ND = Not Detected (Less than limitation of quantity)

Date Sample Received: 24 FEB,2016
Testing Period: 24 FEB,2016 To 1 March, 2016

****

End of report

Intertek Testing Services Ltd., Shanghai
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Appendix F Nutritional Analysis Report for EMS95

SGS

Test Report **Report No: QDAFF160100845** **Date: Jan 29 2016**

Client name:	Qingdao Runde Biotechnology Co., Ltd
Client address:	Lingshanwei Town, Jiaonan County Qingdao, Shandong, 266427 China
Sample name:	Enzymatically Modified Stevia/EMS95
Sample Batch No.:	GLG-EMS95-20151201
Product Date:	2015.12.1
Manufacturer:	/

Above information and sample(s) was/were submitted and certified by the client, SGS quoted the information with no responsibility as to the accuracy, adequacy and/or completeness.

SGS Sample No.:	QDAFF160100845
SGS reference No.:	TAOFD1600386901/TAOFD1600387501/TAOFD1600386301
Date of sample received:	Jan 26 2016
Testing period:	Jan 26 2016 ~ Jan 29 2016

TEST(S) REQUESTED:
Selected test(s) as requested by applicant

TEST RESULT(S):
Please refer to the next page(s)

The results shown in this test report refer only to the sample(s) tested, and for clients internal use only.

(b) (6)

SGS Authorized Signature

SGS-CSTC Standards Technical Services Co., Ltd. Qingdao Branch
Page 1 of 11 RAND:2620922

SGS

Test Report **Report No: QDAFF160100845** **Date: Jan 29 2016**

Test item(s)	Unit(s)	Test method(s)	Test result(s)	Method detection limit(s)
Protein	g/100g	GB 5009.5-2010 I	<0.1	/
Ash	g/100g	GB 5009.4-2010	0.15	/
K	mg/kg	GB/T 5009.91-2003	49.15	1.0
Na	mg/kg	GB/T 5009.91-2003	225.83	1.0
Ca	mg/kg	GB/T 5009.92-2003	391.39	1.0
Fe	mg/kg	GB/T 5009.90-2003	3.01	1.0
P	mg/100g	GB/T 5009.87-2003 I	<50	/
Total fat	g/100g	AOAC 996.06	ND	0.01
Saturated fatty acid	g/100g	AOAC 996.06	ND	0.01
Saturated fat	g/100g	AOAC 996.06	ND	0.01
Mono-unsaturated fatty acid	g/100g	AOAC 996.06	ND	0.01
Mono-unsaturated fat	g/100g	AOAC 996.06	ND	0.01
Multi-unsaturated fatty acid	g/100g	AOAC 996.06	ND	0.01
Multi-unsaturated fat	g/100g	AOAC 996.06	ND	0.01
Trans fatty acid	g/100g	AOAC 996.06	ND	0.01
Cholesterol	mg/100g	GB/T 22220-2008	ND	2.6
Fructose	g/100g	GB/T 22221-2008	ND	0.4
Glucose	g/100g	GB/T 22221-2008	ND	2
Sucrose	g/100g	GB/T 22221-2008	ND	0.4
Maltose	g/100g	GB/T 22221-2008	ND	0.4
Total sugars (Fructose+Glucose+ Sucrose+Maltose)	g/100g	GB/T 22221-2008	ND	/
Vitamin C	mg/100g	GB/T 5009.86-2003 I	ND	0.3
Moisture	g/100g	GB 5009.3-2010 I	4.83	/

SGS-CSTC Standards Technical Services Co., Ltd. Qingdao Branch
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SGS

Test Report **Report No: QDAFF160100845** **Date: Jan 29 2016**

CALCULATION RESULT(S):

Calculate item(s)	Unit(s)	Calculate method(s)	Calculate result(s)
Carbohydrate	g/100g	Refer to GB/Z 21922-2008	95.0
Energy	kJ/100g	Refer to GB/Z 21922-2008	1615

Remark:
1. The test was carried out by a SGS laboratory.
2. Conversion factor of nitrogen to protein is 6.25.
3. ND: Not detected
4. NR: No recoveried
5. Total fat = Saturated fat + Mono-unsaturated fat + Multi-unsaturated fat + Trans fat

SAMPLE DESCRIPTION: Sample in bag

*** End of Report***

Appendix G Stability Testing Report for EMS95

GLG LIFE TECH CORPORATION	Issue Date:10/01/2016
GLG Storage Stability Data of Enzymatically Modified Stevia / EMS95	File No: GLG-QA-SSD-EMS

GLG Storage Stability Data for Enzymatically Modified Stevia/EMS 95

Prepared by: Zhang Lei (QA/QC Manager, GLG Life Tech Corporation)

Date: 28/03/2016

Approved by: Kevin Li (VP of Technology, GLG Life Tech Corporation)

Date: 29/03/2016

GLG LIFE TECH CORPORATION	Issue Date: 10/01/2016
GLG Storage Stability Data of Enzymatically Modified Stevia / EMS95	File No: GLG-QA-SSD-EMS

Objective

To determine storage stability of Enzymatically Modified Stevia / EMS95 produced by GLG.

Samples

One sample representing commercial lot of Enzymatically Modified Stevia / EMS95 labeled as "GLG-EMS95-20131201"

Standards

Rebaudioside A Standard; (Chromadex Inc. Irvine, CA USA);
Stevioside Standard; (Chromadex Inc. Irvine, CA USA);
Rebaudioside C Standard; (Chromadex Inc. Irvine, CA USA);

Solvents and Reagents

Acetonitrile, HPLC grade (Merck, Germany);
Water, HPLC grade (Millipore, Germany);
Ammonium acetate, reagent grade (Merck, Germany);
Acetic acid, reagent grade (Merck, Germany).

Apparatus

1.Waters 2695 HPLC system equipped with binary pump, auto sampler, UV detector, (Waters, USA);
2. Analytical column, Luna NH2 250×4.6×5um (Phenomenex, USA)
3. SBA-50 Glucose Biosensor (Biology Institute of Shandong Academy of Sciences, Shandong, PR China).
4. Analytical balance, XS205, (Mettler Toledo, USA);
5. Volumetric (class ***A)*** and Laboratory glassware.

Sample Storage

Sample was stored in original packaging at 25°C±5°C and 60%±5% relative humidity.

Solution Preparation

Sample solutions were prepared at approx. 5g/l concentration in Diluent

The assay results are summarized in Table 1

Table 1

Lot# GLG-EMS95-20131201, Enzymatically Modified Stevia , % dry basis					
Duration	**TSG**	**Total plate count**	**Salmonella**	**E.Coli**	**Staphylococcus**
t=0	95.9	＜10cfu/g	Negative	Negative	Negative
3 months	95.9	＜10cfu/g	Negative	Negative	Negative
6 months	95.8	＜10cfu/g	Negative	Negative	Negative
12months	95.6	＜10cfu/g	Negative	Negative	Negative
18months	95.4	＜10cfu/g	Negative	Negative	Negative
24months	95.2	10cfu/g	Negative	Negative	Negative

Appendix H Sweetness Intensity Test Report for EMS95

GLG
LEADING LIFE TECHNOLOGIES.
BETTER STEVIA, MONK FRUIT AND MORE.

Sweetness Intensity Data for Enzymatically Modified Stevia/EMS 95

File No.: GLG-QA-SD-125

Prepared by: Zhang Lei, QA/QC Manager

Issued by: Kevin Li, VP of Technology

Date of Issue: 08/01/2016

Suite 2168 – 1050 West Pender Street *Vancouver, BC. *Canada *V6E 3S7
Phone: 604.669.2602 * Fax:604.662.8858 *Email: info@glglifetech.com *W: www.glglifetech.com

Sweetness Intensity Data for Enzymatically Modified Stevia/EMS 95

OBJECTIVE

To determine sweetness intensity of Enzymatically Modified Stevia / EMS95 manufactured by *GLG Life Tech Corporation*.

SAMPLES

Samples representing commercial lot of Enzymatically Modified Stevia / EMS95 labeled as "GLG-EMS95-20131201"

SOLVENTS and REAGENTS

- Sucrose
- Purified water,
- Unsalted crackers

APPARATUS

1. Analytical balance, XS205, (Mettler Toledo, USA);
2. Volumetric (class A) and Laboratory glassware.

ASSAY and PROCEDURES

The sweetness intensity tests are following with "ISO 8587:2006 Sensory Analysis Methodology Ranking" testing method.
28 panelists have been previously qualified for taste acuity and trained for the sweetness intensity test. The panelists were presented with 5 samples (5.0% of sucrose water solution, Enzymatically Modified Stevia / EMS95 water solutions with different concentrations).

TEST RESULTS

Test results, see Table 1.

Table 1. Sweetness Potency of Enzymatically Modified Stevia / EMS95

Sample	Steviol glycosides concentration % (Sweetness equivalent to 5.0% of sucrose at 20 °C)	Sweetness Intensity
EMS95	0.039	130 times sweeter than sucrose

Appendix I Estimated Daily Intake Levels of Steviol Glycosides

There have been continuing studies to estimate the intake of steviol glycosides. Most recently, Dewinter et al. (2016) investigated the dietary intake of non-nutritive sweeteners, including steviol glycosides, in children with type 1 diabetes. Using a phased tier approach, the tier 2 (maximum concentration) and tier 3 (maximum used concentrations) exposures were assessed based on survey data obtained from patients at the Paediatrics Department of the University Hospitals Leuven (Belgium). In both tier 2 and tier 3 exposure assessments, high consumers (P95) aged 4-6 years old were estimated to have a steviol glycosides intake higher than the ADI, calculated at 119% of ADI. The authors noted that the exposure assessment is a worst-case scenario since "it is assumed that all processed foods in which the food additive is authorized contain the food additive at the [maximum permitted levels]." Furthermore, Dewinter et al. conclude that there is little chance that children with type 1 diabetes will exceed ADIs for steviol glycosides.

A. Food Uses as Addressed by JECFA, Merisant & Cargill

As part of its safety deliberations, JECFA reviewed various estimates of possible daily intake of steviol glycosides (WHO, 2006). These estimates are presented in Table I-1. Merisant also listed intended use levels of rebaudioside A for various food applications in their GRAS Notification (Table I-2). Merisant utilized food consumption survey data from 2003-2004 NHANES to determine the estimated daily intake from the proposed uses of rebaudioside A. On a per user basis, the mean and 90th precentile daily consumption levels of rebaudioside A were estimated as 2.0 and 4.7 mg per kg bw per day, respectively. In its notification, Cargill (2008) utilized a different approach in estimating dietary intake figures for rebaudioside A when incorporated as a general sweetener in a broad cross-section of processed foods. Cargill considered that, with a few minor exceptions, rebaudioside A uses and use levels would be comparable to those of aspartame uses in the US. Using post-market surveillance consumption data and published data for consumption of aspartame and other high intensity sweeteners (A. G. Renwick, 2008), Cargill performed a side-by-side consumption analysis for rebaudioside A versus aspartame. Findings from the above-described different sources along with FSANZ estimates and the intake estimates are presented in Table I-3.

B. Estimated Daily Intake

The very conservative consumer intake estimates provided by JECFA as shown in Table I-1 were utilized to gauge the potential human exposures of rebaudioside A and steviol glycosides and in foods as reported in the US and in other countries. As rebaudioside A is about twice as sweet as the mixed glycosides, these levels can be adjusted accordingly.

Table I-1. Food Uses of Steviol Glycosides Reported to JECFA with Calculated Steviol Equivalents

Food Type	Maximum Use Level Reported[a] (mg steviol glycosides /kg of food)	Maximum Use Level Calculated for rebaudioside A[b] mg rebaudioside A /kg of food	Maximum Use Level Calculated for rebaudioside A[b] mg Steviol Equivalents /kg of food
Desserts	500	250	83
Cold confectionery	500	250	83
Pickles	1000	500	167
Sweet corn	200	100	33
Biscuits	300	150	50
Beverages	500	250	83
Yogurt	500	250	83
Sauces	1000	500	167
Delicacies	1000	500	167
Bread	160	80	27

[a] Reproduced from WHO (2006).

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

Table I-2. Proposed Uses & Levels of Rebaudioside A by Merisant[a]

Food Uses	Reb A (ppm)
Tabletop sweeteners	30,000[b]
Sweetened ready-to-drink teas	90-450
Fruit juice drinks	150-500
Diet soft drinks	150-500
Energy drinks	150
Flavored water	150
Cereals (oatmeal, cold cereal, cereal bars)	150

[a] Merisant (2008)

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

Further consideration was given to anticipated human exposures as projected independently and with different approaches by JECFA (WHO, 2006), Merisant (2008), and Cargill (2008). As described below, the multiple approaches tended to converge to yield estimated daily intakes

(EDIs) in the range of 1.3 – 4.7 mg per kg bw per day that, when compared to the acceptable daily intake (ADI), constitutes supporting information in the subject GRAS evaluation.

JECFA evaluated information on exposure to steviol glycosides as submitted by Japan and China. Additional information was available from a report on *Stevia rebaudiana* Bertoni plants and leaves that were prepared for the European Commission by the Scientific Committee on Food. JECFA used the GEMS/Food database to prepare international estimates of exposure to steviol glycosides (as steviol). JECFA assumed that steviol glycosides would replace all dietary sugars at the lowest reported relative sweetness ratio for steviol glycosides and sucrose, which is 200:1. The intakes ranged from 1.3 mg per kg bw per day with the African diet to 3.5 mg per kg bw per day with the European diet. Additionally, JECFA also estimated the per capita exposure derived from disappearance (poundage) data supplied by Japan and China. The Committee evaluated exposures to steviol glycosides by assuming full replacement of all dietary sugars in the diets for Japan and the US. The exposures to steviol glycosides (as steviol) as evaluated or derived by the Committee are summarized in Table I-4.

JECFA concluded that the replacement estimates were highly conservative---that is, the calculated dietary exposure overestimates likely consumption---and that true dietary intakes of steviol glycosides (as steviol) would probably be 20 – 30% of these values or 1.0 - 1.5 mg per kg bw per day on a steviol basis or 3.0 – 4.5 mg per kg bw per day for rebaudioside A based on the molecular weight adjustment. Similarly, FSANZ (2008) estimated steviol glycoside dietary intake for adult consumers in New Zealand, assuming a full sugar replacement scenario, which resulted in estimated exposures of 0.3 - 1.0 mg per kg bw per day for the mean and 90th percentile consumer, or 0.5 – 1.5 mg per kg bw per day for rebaudioside A when making both the molecular weight and sweetness equivalency calculations. FSANZ examined consumption in other age groups and concluded that there were no safety concerns for children of any age. Merisant also calculated a dietary estimate for Reb A of 2.0 mg per kg bw per day for the average consumer and 4.7 mg per kg bw per day for a 90th percentile consumer. On a steviol equivalent basis, the Merisant estimates would be 0.7 and 1.6 mg per kg bw per day, respectively. In another review conducted on behalf of Cargill and included in their GRAS notification, the intake of rebaudioside A when used as a complete sugar replacement was estimated at 1.3 – 3.4 mg per kg bw per day when calculated as Reb A (A. G. Renwick, 2008).

Table I-3. Summary of Estimated Daily Intake Assessments for Rebaudioside A & Calculation of Rebaudioside A Values from JECFA & FSANZ Estimates of EDI

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

[a] Published values for mixed steviol glycosides consumption listed in this column were used for the calculation of Reb A consumption values appearing in next two columns.

[b] Estimates for Reb A consumption were calculated from JECFA and FSANZ estimates as steviol by multiplying by 3 to correct for the molecular weight of Reb A compared to steviol and by subsequently dividing by 2 because of the increased inherent sweetness of Reb A compared to the mixed steviol glycosides.

[c] Total daily intake figures were calculated for a 60 kg adult.

[d] Published values are shown for comparison purposes.

Table I-4. Summary of Estimates of Exposure to Steviol Glycosides (as Steviol)

Estimate	Exposure (mg/kg bw/day)
GEMS/Food (International)[a]	1.3 -3.5 (for a 60 kg person)
Japan, Per Capita	0.04
Japan, Replacement Estimate[b]	3
US, Replacement Estimate[b]	5

[a] WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme.

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

In October 2009, Cargill applied to FSANZ to increase the maximum usage levels of high purity steviol glycosides in the high volume food categories of ice cream and various beverages. Cargill supported its application with increased usage levels by presenting market share analyses that overestimate actual intake while remaining well below the generally accepted ADI. In December 2010, FSANZ recommended accepting the increased usage levels as requested since no public health and safety issues were identified (FSANZ, 2010). Subsequently, FSANZ approved the Cargill application to increase the allowed maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages and flavored soy beverages up to 200 mg per kg and in plain soy beverages up to 100 mg per kg (FSANZ, 2011).

On January 13, 2011, EFSA revised its dietary exposure assessment of steviol glycosides. For high consumers, revised exposure estimates to steviol glycosides remain above the established acceptable daily intake (ADI) of 4 mg per kg bw (steviol equivalent). For European children aged 1-14, revised intake estimates ranged from 1.7 to 16.3 mg per kg bw per day, and for adults, the range was reported to be from 5.6 to 6.8 mg per kg bw per day (EFSA, 2011b).

There have been many scholarly estimates of potential dietary intake of replacement sweeteners---including steviol glycosides---that have been published (FSANZ, 2008; A. G. Renwick, 2008; WHO, 2003) or submitted to FDA (Merisant, 2008). In GRN 301, a simplified estimate was proposed to and accepted by FDA based on the estimates of exposure in “sucrose equivalents” (A. G. Renwick, 2008) and the sweetness intensity of any particular sweetener (BioVittoria, 2009). As summarized in GRN 301, the 90th percentile consumer of a sweetener which is 100 times as sweet as sucrose when used as a total sugar replacement would be a maximum of 9.9 mg per kg bw per day for any population subgroup.

Appendix J Summary of Published Safety Reviews

1. Summary of JECFA Reviews

At an early review during its 51st meeting, JECFA (WHO, 2000) expressed the following reservations about the safety data available at that time for steviol glycosides:

> The Committee noted several shortcomings in the information available on stevioside. In some studies, the material tested (stevioside or steviol) was poorly specified or of variable quality, and no information was available on other constituents or contaminants. Furthermore, no studies of human metabolism of stevioside and steviol were available. In addition, data on long-term toxicity and carcinogenicity were available for stevioside in only one species. The mutagenic potential of steviol has been tested sufficiently only *in vitro.*
>
> In view of the absence of information for the elaboration of specifications for stevioside and since the evaluation of the available toxicological data revealed several limitations, the Committee was unable to relate the results of the toxicological investigations to the commercial product and could not allocate an ADI to stevioside.
>
> Before reviewing stevioside again, the Committee considered that it would be necessary to develop specifications to ensure that the material tested was representative of the commercial product. Further information on the nature of the substance that was tested, data on the metabolism of stevioside in humans and the results of suitable *in vivo* genotoxicity studies with steviol would also be necessary.

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

> The Committee noted that most of the data requested at its fifty-first meeting, e.g., data on the metabolism of stevioside in humans, and on the activity of steviol in suitable studies of genotoxicity *in vivo*, had been made available. The Committee concluded that stevioside and rebaudioside A are

not genotoxic *in vitro* or *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives *in vitro* is not expressed *in vivo*.

The NOEL for stevioside was 970 mg per kg bw per day in a long-term study (Toyoda et al., 1997) evaluated by the Committee at its fifty-first meeting. The Committee noted that stevioside has shown some evidence of pharmacological effects in patients with hypertension or with type-2 diabetes at doses corresponding to about 12.5–25 mg per kg bw per day (equivalent to 5–10 mg per kg bw per day expressed as steviol). The evidence available at present was inadequate to assess whether these pharmacological effects would also occur at lower levels of dietary exposure, which could lead to adverse effects in some individuals (e.g., those with hypotension or diabetes).

The Committee therefore decided to allocate a temporary ADI, pending submission of further data on the pharmacological effects of steviol glycosides in humans. A temporary ADI of 0–2 mg per kg bw was established for steviol glycosides, expressed as steviol, on the basis of the NOEL for stevioside of 970 mg per kg bw per day (or 383 mg per kg bw per day, expressed as steviol) in the 2-year study in rats and a safety factor of 200. This safety factor incorporates a factor of 100 for inter- and intra-species differences and an additional factor of 2 because of the need for further information. The Committee noted that this temporary ADI only applies to products complying with the specifications.

The Committee required additional information, to be provided by 2007, on the pharmacological effects of steviol glycosides in humans. These studies should involve repeated exposure to dietary and therapeutic doses, in normotensive and hypotensive individuals and in insulin-dependent and insulin-independent diabetics.

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

In response to the call for data on "stevioside" for the 63rd meeting of the Committee, submissions from several countries showed that the main components of the commercially available extracts of stevia are stevioside and rebaudioside A, in various amounts ranging from about 10-70% stevioside and 20-70% rebaudioside A. The information indicated that most commercial products contained more than 90% steviol glycosides with the two main steviol glycosides comprising about 80% of the material. The 63rd JECFA required that the summed content of stevioside and rebaudioside A was not less than 70% and established a minimum purity of 95% total steviol glycosides. Analytical data showed that most of the remaining 5% could be accounted for by saccharides other than those associated with the individual steviol glycosides.

Noting that the additive could be produced with high purity (at least 95%) and that all the steviol glycosides hydrolyze upon ingestion to steviol, on which the temporary ADI is based, the 68th JECFA decided it was unnecessary to maintain a limit for the sum of stevioside and rebaudioside content.

> The Committee recognized that the newly revised specifications would cover a range of compositions that could include, on the dried basis, product that was at least 95% stevioside or at least 95% rebaudioside A.

In 2008, based on additional clinical studies, at its 69th meeting, JECFA finalized the evaluation of steviol glycosides (WHO, 2008), raised the ADI to 0 – 4 mg per kg bw per day, and removed the "temporary" designation. The summary of the Committee's key conclusions in the final toxicology monograph addendum (WHO, 2009) were stated as follows:

> From a long-term study with stevioside, which had already been discussed by the Committee at its fifty-first meeting, a NOEL of 970 mg per kg bw per day was identified. At its sixty-third meeting, the Committee set a temporary ADI of 0–2 mg per kg bw for steviol glycosides, expressed as steviol, on the basis of this NOEL for stevioside of 970 mg per kg bw per day (383 mg per kg bw per day expressed as steviol) and a safety factor of 200, pending further information. The further information was required because the Committee had noted that stevioside had shown some evidence of pharmacological effects in patients with hypertension or with type 2 diabetes at doses corresponding to about 12.5–25.0 mg per kg bw per day (5–10 mg per kg bw per day expressed as steviol).
>
> The results of the new studies presented to the Committee at its present meeting have shown no adverse effects of steviol glycosides when taken at doses of about 4 mg per kg bw per day, expressed as steviol, for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal or low-normal blood pressure for 4 weeks. The Committee concluded that the new data were sufficient to allow the additional safety factor of 2 and the temporary designation to be removed and established an ADI for steviol glycosides of 0–4 mg per kg bw expressed as steviol.
>
> The Committee noted that some estimates of high-percentile dietary exposure to steviol glycosides exceeded the ADI, particularly when assuming complete replacement of caloric sweeteners with steviol glycosides, but recognized that these estimates were highly conservative and that actual intakes were likely to be within the ADI range.

2. Summary of FSANZ Review of Steviol Glycosides

In 2008, FSANZ completed a review of the safety of steviol glycosides for use as a sweetener in foods. FSANZ concluded that steviol glycosides are well tolerated and unlikely to have adverse effects on blood pressure, blood glucose, or other parameters in normal, hypotensive, or diabetic subjects at doses up to 11 mg per kg bw per day. FSANZ agreed with JECFA in setting an ADI of 4 mg steviol equivalents per kg bw per day, which was derived by applying a 100-fold safety factor to the NOEL of 970 mg per kg bw per day established by a 2-year rat study (Toyoda et al., 1997). The FSANZ review discussed the adequacy of the existing database and several new studies, including the clinical studies reviewed by JECFA in the summer of 2007, most notably the work of L. A. Barriocanal et al. (2008), which was later published in 2008.

In their draft document, FSANZ also indicated that the new data in humans provides a basis for revising the uncertainty factors that were used by JECFA to derive the temporary ADI for steviol

glycosides in 2005. In particular, the evidence surrounding the pharmacological effects of steviol glycosides on blood pressure and blood glucose has been strengthened so that the additional 2-fold safety factor for uncertainty related to effects in normotensive or diabetic individuals is no longer required. Therefore, FSANZ established an ADI of 4 mg per kg bw per day for steviol glycosides as steviol equivalents, derived by applying a 100-fold safety factor to the NOEL of 970 mg per kg bw per day (equivalent to 383 mg per kg bw per day steviol) in a 2-year rat study (FSANZ, 2008). In December 2010, FSANZ recommended accepting the increased usage levels since no public health and safety issues were identified (FSANZ, 2010). Subsequently, FSANZ approved an increase in the maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages and flavored soy beverages up to 200 mg per kg and in plain soy beverages up to 100 mg per kg (FSANZ, 2011).

3. Summary of EFSA Review of Steviol Glycosides

On March 10, 2010, EFSA adopted a scientific opinion on the safety of steviol glycosides (mixtures that comprise not less than 95% of stevioside and/or rebaudioside A) as a food additive. Earlier---in 1984, 1989 and 1999---the Scientific Committee for Food (SCF) evaluated stevioside as a sweetener. At the time, the SCF concluded that the use of stevioside was “toxicologically not acceptable” due to insufficient available data to assess its safety. However, in light of JECFA’s 2008 findings, and in response to a June 2008 request by the European Commission, EFSA reevaluated the safety of steviol glycosides as a sweetener.

As both rebaudioside A and stevioside are metabolized and excreted by similar pathways, with steviol being the common metabolite for both glycosides, the EFSA Panel agreed that the results of toxicology studies on either stevioside or rebaudioside A are applicable for the safety assessment of steviol glycosides. Considering the available safety data (*in vitro* and *in vivo* animal studies and some human tolerance studies), the EFSA Panel concluded that steviol glycosides, complying with JECFA specifications, are not carcinogenic, genotoxic, or associated with any reproductive/developmental toxicity. The EFSA Panel established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg per kg bw per day based on the application of a 100-fold uncertainty factor to the NOAEL in the 2-year carcinogenicity study in the rat when administering 2.5% stevioside in the diet. This is equal to 967 mg stevioside per kg bw per day (corresponding to approximately 388 mg steviol equivalents per kg bw per day). Conservative estimates of steviol glycosides exposures both in adults and in children suggest that the ADI could possibly be exceeded by European consumers of certain ages and geographies at the maximum proposed use levels.

Recently, EFSA (2011b) revised its exposure assessment of steviol glycosides from its uses as a food additive for children and adults, and published the reduced usage levels in 16 foods by a factor of 1.5 to 3, with no changes for 12 food groups. Additionally, 15 other foods were removed, mainly within the category of desserts and other products, while 3 new food uses were added. The

mean estimated exposure to steviol glycosides (equivalents) in European children (aged 1-14 years) ranged from 0.4 to 6.4 mg per kg bw per day and from 1.7 to 16.3 mg per kg bw per day at the 95th percentile. A correction was considered to be necessary for the consumption of non-alcoholic flavored drinks (soft drinks) by children, and the corrected exposure estimate at the 95th percentile for children ranged from 1.0 to 12.7 mg per kg bw per day. For adults, the mean and 97.5th percentile intakes were estimated to range from 1.9 to 2.3 and 5.6 to 6.8 mg per kg bw per day, respectively. Non-alcoholic flavored drinks (soft drinks) are the main contributors to the total anticipated exposure to steviol glycosides for both consumer categories. For high consumers, EFSA noted that revised exposure estimates to steviol glycosides remain above the established ADI of 4 mg per kg bw (steviol equivalent).

In addition, EFSA (2011a) recently accepted rebaudioside A as a flavoring agent in a variety of foods. EFSA reviewed the available safety data on rebaudioside A and agreed that the ADI of 4 mg per kg bw per day established for steviol glycosides applied also to rebaudioside A in a purified form. The dietary intake for use as a flavoring agent was calculated by two different methods, and EFSA determined that the worst-case exposure would be 10,888 microgram per person per day, which is equivalent to 181 microgram rebaudioside A per kg bw per day, for a person weighing 60 kg. This corresponds to a daily intake of 60 microgram steviol per kg bw per day, using a conversion factor of 0.33 for converting the amount of rebaudioside A into steviol equivalents.

4. Other Published Reviews

Stevia and steviol glycosides have been extensively investigated for their biological, toxicological, and clinical effects (M. C. Carakostas et al., 2008; J. M. C. Geuns, 2003; Huxtable, 2002). Four additional reviews have appeared on the toxicology and biological activity of stevia extracts and steviol glycosides (Brahmachari et al., 2011; Brown & Rother, 2012; Chatsudthipong & Muanprasat, 2009; Yadav & Guleria, 2012). In reviewing these studies, caution is warranted since these reviews do not differentiate well between studies on crude stevia extract and purified steivol glycosides. In addition, many of the reviewed studies on biological activity used routes of administration other than oral, and they may have used doses that are much higher than expected dietary exposures of steviol glycosides as a sweetener. In a letter to the editor of the Journal of Pharmacology and Therapeutics, Ashley Roberts and Munro (2009) criticized the Chatsudthipong and Muanprasat (2009) review with some important points that are applicable in general to these four reviews. Important excerpts from this letter are as follows:

> “It is well established that some stevia extracts are crude mixtures that contain multiple components of the stevia leaf, including those components that do not provide a sweet taste. These mixtures also vary considerably in quality, purity, and composition. Therefore, it is not surprising that sometimes these crude and uncharacterized materials may contain substances that possess some degree of pharmacologic activity but any such effects cannot be attributed specifically to the steviol glycosides. In contrast to studies conducted with less pure steviol glycoside preparations, studies conducted with purified preparations do not indicate any evidence of pharmacological effects.”

> "The authors consistently cite pharmacological, toxicological, and biochemical effects from in vitro studies or from studies in which animals were dosed intravenously (e.g., Melis, 1992 a,b,c). Steviol glycosides are hydrolyzed completely by the gut microflora to steviolprior to absorption, with no systemic absorption of the glycone form following oral exposure. Therefore, the results of in vitro and intravenous, intraperitoneal, or subcutaneous dosing studies of the glycone form are not relevant to the safety of steviol glycosides consumed orally."
>
> "Collectively, the report of Chatsudthipong and Muanprasat (2009) is incomplete and lacking discussion of key studies of the safety of stevioside and rebaudioside A. It focuses on alleged effects of stevia and steviol glycosides of low or unknown purity, fails to consider the route of exposure in relation to metabolism and safety assessment and does not include recent opinions expressed by world wide regulatory authorities affirming the safety of purified forms of stevioside and rebaudioside A as a food ingredient."

Most recently, J. D. Urban, Carakostas, and Taylor (2015) reviewed the potential allergenicity of steviol glycosides. The authors noted that: "hypersensitivity reactions to stevia in any form are rare" and concluded that current data do not support claims that steviol glycosides are allergenic. In addition, the authors stated that there is "little substantiated scientific evidence" to warrant consumer warning labels for highly purified stevia extracts (J. D. Urban et al., 2015).

Appendix K Studies on Steviol Glycosides Preparations That Are Primarily Mixtures of Stevioside & Rebaudioside A

This appendix summarizes studies on stevioside or stevia extracts that were identified compositionally as predominantly stevioside. In some of the published literature, the terms stevia, stevioside, and stevia glycoside are used interchangeably. However, wherever possible, an attempt has been made to identify the specific substance studied.

1. Absorption, Distribution, Metabolism & Excretion (ADME) Studies

Several studies in rats (E. Koyama et al., 2003; Nakayama, Kasahara, & Yamamoto, 1986; Wingard Jr et al., 1980) and other animal models, including chickens (J. M. Geuns, Malheiros, et al., 2003), hamsters (A. Hutapea, Toskulkao, Wilairat, & Buddhasukh, 1999), and pigs (J. M. Geuns, Augustijns, Mols, Buyse, & Driessen, 2003), indicate that stevioside is not readily absorbed from the GI tract. Available evidence from *in vitro* metabolism studies suggests that bacteria in the colon of rats and humans can transform various stevia glycosides into steviol (Gardana et al., 2003). Steviol was shown to be more readily transported with *in vitro* intestinal preparations than various steviosides (J. M. C. Geuns, 2003; E. Koyama et al., 2003). Slow absorption of steviol was indicated by detection in the plasma of rats given oral stevioside (Wang, Goh, Fan, & Lee, 2004). However, Sung (2002) did not detect plasma steviol following oral administration of steviosides to rats. In studies with human and rat liver extracts, E. Koyama et al. (2003) demonstrated that steviol can be converted to various glucuronides. Excretion of metabolites of stevioside after oral doses has been shown in urine and feces in rats (Sung, 2002) and hamsters (A. Hutapea et al., 1999). Oral doses in pigs led to the detection of metabolites in feces but not in urine (J. M. Geuns, Augustijns, et al., 2003).

E Koyama et al. (2003) published an *in vitro* study in which α-glucosylated steviol glycosides were degraded by fecal microflora to steviol glycosides. These are subsequently hydrolyzed to the aglycone, steviol, demonstrating that the metabolic fate of α-glucosylated steviol glycosides follows that of non-modified steviol glycosides. Due to the similarities in metabolic fate, the safety of α-glucosylated steviol glycosides can be established based on studies conducted with non-modified steviol glycosides. Furthermore, as individual steviol glycosides show similar pharmacokinetics in the rat and humans, the results of toxicology studies on individual steviol glycosides are applicable to the safety of steviol glycosides in general.

In a human study with 10 healthy subjects, J. M. Geuns et al. (2006) measured blood, urine, and fecal metabolites in subjects that received 3 doses of 250 mg of purified stevioside (>97%) three times a day for 3 days. Urine was collected for 24 hours on day 3, and blood and fecal samples were also taken on day 3. Free steviol was detected in feces but not in blood or urine. Steviol glucuronide was detected in blood, urine, and feces. Approximately 76% of the total steviol equivalents dosed were recovered in urine and feces. Based on these measurements, the authors

concluded that there was complete conversion of stevioside in the colon to steviol, which was absorbed and rapidly converted to the glucuronide.

In a recent publication, A. Renwick and Tarka (2008) reviewed studies on microbial hydrolysis of steviol glycosides. The reviewers concluded that stevioside and Reb A are not absorbed directly, and both are converted to steviol by gut microbiota in rats and in humans. This hydrolysis occurs more slowly for Reb A than for stevioside. Studies have shown that steviol-16,17-epoxide is not a microbial metabolite. Given the similarity in the microbial metabolism of stevioside and rebaudioside A, with the formation of steviol as the single hydrolysis product that is absorbed from the intestinal tract, these investigators concluded that the toxicological data on stevioside are relevant to the risk assessment of rebaudioside C.

Table K-1. Mutagenicity & Genotoxicity Studies on Rebaudioside A

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
Bacterial Mutagenicity	5 *Salmonella* strains with and without exogenous metabolic activation system	Reb A	99.5	1.5, 5.0, 15, 50, 150, 500, 1,500 and 5,000 µg per plate	No mutagenic response	Wagner and Van Dyke (2006)
Bacterial Mutagenicity	5 *Salmonella* strains and 1 *E. coli* strain with and without exogenous metabolic activation system	Reb A		Up to 5,000 µg per plate	No mutagenic response	L. D. Williams and Burdock (2009)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence and presence of exogenous metabolic activation system	Reb A	99.5	Cloning conc. of 500, 1,000, 2,000, 3,000, 4,000 and 5,000 µg/mL	No mutagenic or clastogenic response	Clarke (2006)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence and presence of exogenous metabolic activation system	Reb A		Up to 5,000 µg/mL	No mutagenic or clastogenic response	L. D. Williams and Burdock (2009)
Chromosome Aberration	Chinese Hamster V79 cells	Reb A		Up to 5,000 µg/mL		L. D. Williams and

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
						Burdock (2009)
Mouse Micronucleus	Micronucleus study consisted of 7 groups, each containing 5 male and 5 female ICR mice.	Reb A	99.5	500, 1,000 and 2,000 mg/kg bw	No increase in micronuclei formation	Krsmanovic and Huston (2006)
Mouse Micronucleus		Reb A		Up to 750 mg/kg bw	No increase in micronuclei formation	L. D. Williams and Burdock (2009)
Unscheduled DNA Synthesis	*In vivo* rat	Reb A		Up to 2,000 mg/kg bw	No increase in unscheduled DNA synthesis	L. D. Williams and Burdock (2009)
DNA damage (comet assay)	*Male BDF1 mouse stomach, colon, liver*	Stevia extract	Stevio-side, 52%; Reb A, 22%	250 – 2,000 mg/kg bw	Negative[a]	Sekihashi, Saitoh, and Sasaki (2002)
Chromosomal aberration	*CHL/IU Chinese hamster lung fibroblasts*	Reb A	NS	1.2 - 55 mg/mL	Negative[b]	Nakajima (2000a)
Micronucleus formation	*BDF1 mouse bone marrow*	Reb A	NS	500-2,000 mg/kg bw per day for 2 days	Negative[c]	Nakajima (2000b)
Forward mutation	*S. typhimurium* TM677	Reb A	NS	10 mg/plate	Negative[b]	Pezzuto, Compadre, Swanson, Nanayakkara, and Kinghorn (1985)

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

1. Acute Toxicity Studies

The oral LD_{50} studies of stevioside (purity, 96%) following administration of a single dose to rodents are summarized in Table K-2. No lethality was noted within 14 days after the administration, and no clinical signs of toxicity, or morphological or histopathological changes were found, indicating that stevioside is relatively harmless.

Table K-2. Acute Toxicity of Stevioside (Purity 96%) Given Orally to Rodents

Species	Sex	LD_{50} (g/kg bw)	Reference
Mouse	Male and Female	>15	Toskulkac, Chaturat, Temcharoen, and Glinsukon (1997)
Mouse	Male	> 2	Medon et al. (1982)
Rat	Male and Female	>15	Toskulkac et al. (1997)
Hamster	Male and Female	>15	Toskulkac et al. (1997)

2. Subchronic Toxicity Studies

In five published studies, subchronic toxicity of stevioside was investigated in rats following oral administration. In addition, a reproduction study in hamsters included subchronic phases on the F_0, F_1, and F_2 generations. These studies are summarized in Table K-3. One of these studies was particularly important because it served as a range-finding study for two subsequent chronic studies. In this 13-week toxicity study, Fischer 344 rats (10 per sex per group) were given doses of 0, 0.31, 0.62, 1.25, 2.5, or 5% in the diet (equivalent to 160, 310, 630, 1,300, and 2,500 mg per kg bw per day) to determine the appropriate doses for a two-year carcinogenicity study. None of the animals died during the administration period, and there was no difference in body-weight gain between the control and treated groups during administration or in food consumption in the latter part of the study. The activity of lactic dehydrogenase and the incidence of single-cell necrosis in the liver were increased in all groups of treated males. The authors considered these effects to be nonspecific, because of the lack of a clear dose-response relationship, the relatively low severity, and their limitation to males. Other statistically significant differences in hematological and biochemical parameters were also considered to be of minor toxicological significance. The authors concluded that a concentration of 5% in the diet was a suitable maximum tolerable dose of stevioside for a two-year study in rats (Aze et al., 1990).

In earlier 3-month rat studies reviewed by J. M. C. Geuns (2003)---the sample purity, doses, strain of rat were not reported---a no effect level was determined to be in excess of 2,500 mg per kg bw per day and 7% of the diet, apparently due to lack of effects at the highest dose tested in both studies (Akashi & Yokoyama, 1975).

In a recently published exploratory subchronic toxicity study, Awney et al. (2011) investigated the effects of 97% pure stevioside on body weight, organ relative weight, hematological and biochemical parameters, and enzyme activities in Sprague Dawley rats. In this 12-week toxicity study, groups of male rats (8 per group) were given drinking water containing stevioside. The

groups were assigned to drink distilled water (control), low-dose stevioside solution (15 mg per kg per day), high-dose stevioside solution (1,500 mg per kg per day), or low-dose stevioside (15 mg per kg per day) plus inulin solution for 12 weeks as the sole source of liquid. Fluid intake was recorded daily, and levels of test articles were adjusted weekly to receive the appropriate target concentration. Low-dose stevioside (15 mg per kg bw per day) administration, with or without inulin, for 12 weeks did not reveal any adverse effects on body weight, organs relative weight, hematological and biochemical parameters, or enzyme activities. However, treatment with high-dose stevioside was reported to cause significant changes in several investigated toxicological parameters. Among the hematological parameters, significant changes were noted in all except WBCs, RBCs, and PCV%, and in all clinical chemistry parameters except proteins, total lipids, serum ATL and AST. These data support the NOEL of 15 mg per kg per day. However, critical review of the publication reveals that the study was poorly designed and implemented. Design deficiencies include: insufficient numbers of animals; group-housing with the potential for stress-related changes; unreliable access to steviol *via* drinking water, resulting in suspect dosing calculations in group-housed cages; no indication of fasting prior to blood collection, which affects many chemistry and hematological values; no urine collection; and no histopathological evaluations for confirmation of findings beyond the controls. In addition to these study design deficiencies, the report fails to adequately present mean or individual organ weight data and, in general, there appears to be inadequate comparison of study findings against laboratory historical control data. Any one of these oversights could have adversely affected the results and/or interpretation of the hematological and chemistry data.

In addition to the above-described parameters, tartrate-resistant alkaline phosphatase (TRAP) levels were measured and found to be significantly decreased (Awney et al., 2011). TRAP is an enzyme that is expressed by bone-resorbing osteoclasts, inflammatory macrophages, and dendritic cells. This enzyme was not measured in any previous steviol glycosides studies nor has it been adequately vetted for application in toxicological studies. These investigators did not identify the specific TRAP isomer measured, the methodology employed, the handling of the samples, or any historical data on TRAP levels. The significance and relevance of this poorly documented toxicological endpoint, which lacks histopathological confirmation, does not appear to have a distinct role in determining the toxicological profile of a material in a test animal. The data presented by Awney et al. (2011) are probably not representative of changes due to the subchronic dietary administration of steviol glycosides because of overall inadequate study design and reliance on the findings of the untested enzyme TRAP. The preponderance of the data from several well designed studies on steviol glycosides suggest that differences noted in hematological and chemistry data are probably random, nonspecific, and not toxicologically significant.

Critical reviews of the publication by M. Carakostas (2012) and Waddell (2011) revealed a poor study design that included: insufficient numbers of animals; group-housing with the potential for stress-related changes; unreliable access to steviol *via* drinking water resulting in suspect dosing calculations in group-housed cages; no indication of fasting prior to blood collection, which affects

many chemistry and hematological values; no urine collection; and no histopathological evaluations for confirmation of findings beyond the controls. Additionally, the report did not adequately describe mean or individual organ weight data and lacked comparison of study findings against laboratory historical control data.

Table K-3. Summary of Subchronic Studies on Stevioside

Study	Animal Model/ Group size	Test Material/ Sample Purity	Doses / Duration	Author Assigned NOAEL (mg/kg bw/day)	Results and Remarks
Aze et al. (1990)[a]	F344 rat/ 10 females & 10 males in each of 6 groups	Stevioside/ Not reported	0, 0.31, 0.62, 1.25, 2.5, 5% in diet/13 weeks	Not reported	No effects observed on mortality, body weight or food consumption. Clinical chemistry investigation revealed increased LDH levels & histopathological investigation indicated increased incidence of single-cell liver necrosis in all male treated groups, but not in clear dose-response relationship. Investigators did not consider these changes to be treatment related due to small magnitude & low severity of changes, the lack of clear dose relationship & limitation to males only. Organ weights, urine chemistry & gross necropsy not discussed. Authors concluded that 5% stevioside in diet is tolerable dose for 2 year study.
Yodyingyuad and Bunyawong (1991)[a]	Hamster/ four groups of 20 (10 male, 10 female)	Stevioside/ 90%	0, 0.5, 1.0, 2.5 g/kg bw/day/ duration unclear/ 3 months	2,500	F_0, F_1 & F_2 generations in reproductive study dosed for 90 days. Histological examination showed no effect at any dose. Weights of organs, blood analysis, urine chemistry & gross necropsy not discussed. The F_1 & F_2 hamsters continued to receive stevioside (via drinking water for one month, then at same dose as parents).
Mitsuhashi (1976)[b]	Rat (strain not reported)	Stevioside/ Not reported	Dietary concentrations up to 7%/ 3 months	Not reported	No effects noted at all doses tested. Experimental details such as body weight, organ weight, blood analysis, urine chemistry, gross necropsy & histopathology not discussed.
Akashi and Yokoyama (1975)[b]	Rat (strain not reported)	Stevioside/ Not reported	Oral doses up to 2,500 mg/kg bw/3 months	2,500	No effects noted at all doses tested. Experimental details such as body weight, organ weight, blood analysis, urine chemistry, gross necropsy & histopathology not discussed.
Awney et al. (2011)	Sprague Dawley rats	Stevioside 97%	Drinking water (15, 1,500 mg/kg bw /day)	15	Treatment with high dose stevioside caused significant changes in several investigated toxicological parameters. Among hematological parameters, significant changes noted in all except WBCs, RBCs& PCV% & in all clinical chemistry parameters except proteins, total lipids, ATL and AST.

[a] Abstract only. [b] As reported by J. M. C. Geuns (2003).

3. Chronic Toxicity Studies

Chronic effects of stevioside have been studied in three separate studies (Table K-4). No treatment-related increase in tumor incidence was seen in any of these studies. In the most recent and well-documented study {additional study details were presented to JECFA in 2006 (WHO, 2006), the apparent no observed adverse effect level (NOAEL) in F344 rats was the dietary level of 2.5% [test sample purity 96%, Toyoda et al. (1997)]}. At 5% of the diet, statistically significant decreases in body weight, percent survival, and kidney weight were noted. The authors attributed these effects to various factors. The decrease in body weight was attributed to an inhibition of glucose utilization. The decrease in survival seemed to have been caused by an unusual late onset of large granular lymphocyte leukemia in high dose males. The authors reported that this tumor is rather common in F344 rats and that the overall incidence in male rats was actually within the historical control range experienced in the laboratory where studies were conducted. The authors attributed the decrease in kidney weight as probably due to a decrease in chronic inflammation found in the histopathological examination relative to control animals.

Table K-4. Summary of Chronic Toxicity Studies on Stevioside

Study	Animal Model/ Group size	Test Material/ Sample Purity	Doses / Duration	Author Assigned NOAEL (mg/kg bw/day)	Results and Remarks
Toyoda et al. (1997)	F344 rat/ 50 per sex per group	95.6% Stevioside	*Ad libitum* 0,2.5, 5% of diet/~24 months (104 weeks)	Author did not assign a NOAEL. (Mid-dose calculates to 970 in males; JECFA, 2006)	Significant decrease in survival rates in males receiving 5%. General condition, body weight, food intake, mortality, hematological, histopathological & organ weights observed. Body weight gains dose-dependently decreased in both sexes. Kidney weights significantly lower in 5% males& ovary, kidney, & brain weights significantly increased in 5% females. Tumors& non-neoplastic lesions found in all groups& not correlated to treatment. Conclusion--stevioside is not carcinogenic under these experimental conditions.
Xili et al. (1992)[a]	Wistar rat/ 45 per sex per group	85% Stevioside	0, 0.2, 0.6, 1.2 % of diet/24 months	794 (high dose)	After 6, 12 & 24 months 5 rats from each group sacrificed for analysis. No effects observed on growth, food utilization, general appearance, mortality, or lifespan. No changes in hematological, urinary, or clinical biochemical values. Histopathological analysis showed that the neoplastic and non-neoplastic lesions unrelated to level of stevioside in diet.
Yamada et al. (1985)	F344 rat/ 70 per sex per group, 30 per sex per group in low-dose	95.2% Steviol glycosides (75% stevioside; 16% Reb A)	0.1, 0.3, 1% of diet/22 months for males, 24 months for females	550 (high dose)	At 6 &12 months, 10 males & 10 females sacrificed for analysis. General behavior, growth & mortality were same among groups throughout experiment. At 6 months, protein urea significantly increased in females, & blood glucose increased in both sexes, although urinary glucose not detected. Weights of liver, kidney, heart, prostate & testes increased in males at 6 months, &weight of ovaries decreased in females in dose-dependent manner. Histopathological examination showed differences in various organs at 6 months that were unrelated to stevioside dose. These differences not found at 12 months. Authors concluded that there were no significant changes after 2 years.

[a] Only abstract available.

4. Reproductive & Developmental Toxicity Studies

The use of *S. rebaudiana* as an oral contraceptive has been reported by Indians in Paraguay (Planas & Kuć, 1968; Schvartaman, Krimer, & Azorero, 1977). In experimental studies in rats, crude stevia leaf extract has been shown to inhibit fertility (Planas & Kuć, 1968). Reproductive toxicity studies have been conducted with orally administered purified stevioside. No effect on fertility or reproductive parameters was seen in a three-generation study in hamsters at doses up to 2,500 mg per kg per day (Yodyingyuad & Bunyawong, 1991). There was an absence of statistically significant effects at doses up to 3% [equivalent to 3,000 mg per kg bw per day; sample purity 96%; Mori, Sakanoue, Takeuchi, Shimpo, and Tanabe (1981)]. Similar results were observed in an additional rat study that was reviewed by J. M. C. Geuns (2003) where limited information is available in English (Usami, Sakemi, Kawashima, Tsuda, & Ohno, 1994).

Groups of 20 pregnant golden hamsters were given steviol (purity, 90%) at doses of 0, 250, 500, 750, or 1,000 mg per kg bw per day (only 12 animals at the highest dose) by gavage in corn oil on days 6 - 10 of gestation. A significant decrease in body weight gain and increased mortality (1/20, 7/20, and 5/12) were observed at the three highest doses, and the number of live fetuses per litter and mean fetal weight decreased in parallel. Histopathological examination of the maternal kidneys showed a dose-dependent increase in the severity of effects on the convoluted tubules (dilatation, hyaline droplets). However, no dose-dependent teratogenic effects were seen. The NOEL was 250 mg per kg bw per day for both maternal and developmental toxicity (Wasuntarawat et al., 1998).

No effect on pregnancy or developmental parameters were observed in Swiss albino mice with stevioside or aqueous stevia extract at doses up to 800 mg per kg bw per day in female mice (Kumar & Oommen, 2008). Further details on these studies to the extent available are presented in Table K-5.

Table K-5. Summary of Reproductive Toxicity Studies on Steviol Glycosides

Study	Animal Model/ Group size	Test Sample Purity Stevioside (UNLESS Otherwise Noted)	Doses / Duration	Author Assigned NOAEL (mg/kg bw/day)	Results & Remarks
Kumar and Oommen (2008)	Swiss albino mice/ 4 groups of 5 females	Not reported	500 & 800 mg/kg bw/15 days	800	Stevioside & stevia extract (purity & composition not reported) did not have any effect on reproductive parameters in mice when administered to female mice before or during pregnancy. No changes seen in number of implantations or uterine resorptions. No gross anatomical or histopathologic effects seen in 16-day embryos.
Usami et al. (1994)[a]	Wistar Rat/4 groups of 25 or 26 pregnant rats	95.6%[b]	0, 250, 500, 1,000 mg/kg bw/10 days	1,000	Pregnant rats given doses of stevioside by gavage once/day on days 6-15 of gestation & were sacrificed on day 20 of gestation. Fetuses examined for malformations in addition to maternal & fetal body weight, number of live fetuses, sex distribution& numbers of resorptions or dead fetuses. No treatment-related effects observed. Authors concluded that orally administered stevioside not teratogenic in rats.
Yodyingyuad and Bunyawong (1991)	Hamster/ 10 male, 10 female per group (40 total)	90%	0, 500, 1,000, 2,500 mg/kg bw/day/ duration unclear/ 3 months	2,500	Males from each group mated to females from respective dose group. Each female allowed to bear 3 litters during course of experiment. Stevioside had no effect on pregnancies of females at any dose. The F_1 & F_2 hamsters continued to receive stevioside (via drinking water for one month, then at same dose as parents); showed normal growth & fertility. Histological examination showed no effect on reproductive organs at any dose.
Oliveira-Filho, Uehara, Minetti, and Valle (1989)[a]	Rat/ number not reported	Not reported (Dried Stevia Leaves)	0 or 0.67 g dried leaves/mL, 2 mL twice per day/ 60 days	Not reported	Prepubertal rats (25-30 days old) tested for glycemia; serum concentrations of thyroxine; tri-iodothyroxine; available binding sites in thyroid hormone-binding proteins; binding of ^{3}H-methyltrienolone (a specific ligand of androgen receptors) to prostate cytosol; zinc content of prostate, testis, submandibular salivary gland, & pancreas; water content of testes & prostate; body-weight gain; & final weights of testes, prostate, seminal vesicle, submandibular salivary gland& adrenal. Only difference due to treatment was seminal vesicle weight, which fell to 60% compared to control.
Mori et al. (1981)	Rat/11 male, 11 female per group (44 total)	96%	0, 0.15, 0.75 or 3 % of feed/60 days	2,000	Males given stevioside dose in diet for 60 days before & during mating with females who received same diet (as mated male) 14 days before mating & 7 days during gestation. No effect due to treatment on fertility or mating performance& no effect of fetal development. Rats of each sex had slightly decreased body weight gain at highest dose with non-significant increase in number of dead & resorbed fetuses at highest dose.
Planas and Kuć (1968)	Rat/14 per group (28 total)	Not reported (Crude stevia extract)	0 or 5% Crude stevia extract /18 days	Not reported	Extract given orally to adult female rats for 12 days, who were mated with untreated males during last 6 days. Fertility reduced to 21% of fertility in control rats & remained reduced in a 50-60 day recovery. Histological examination, weights of organs, blood analysis, urine chemistry and & necropsy not discussed.

[a] Only abstract available. [b] As reported by EuropeanCommission (1999b).

5. Mutagenicity & Genotoxicity Studies

In a series of studies, mutagenic and genotoxic effects of various stevia extracts and various preparations of stevioside were investigated. These studies are summarized in Table K-6. All studies were negative with the exception of a comet assay done in rats (A. Nunes et al., 2007). The methodology used in this study, and the resulting conclusions, have been questioned by J. M. Geuns (2007b), G. M. Williams (2007), and Brusick (2008), and responded to by the authors (A. P. M. Nunes et al., 2007a, 2007b).

In a recent review, J. Urban et al. (2013) examined the extensive genotoxicity database on steviol glycosides because some concern has been expressed in two recent publications (Brahmachari et al., 2011; Tandel, 2011) in which the authors concluded that additional testing is necessary to adequately address the genotoxicity profile (J. Urban et al., 2013). The review aimed to address this matter by evaluating the specific genotoxicity studies of concern, while evaluating the adequacy of the database that includes more recent genotoxicity data not noted in these publications. The results of this literature review showed that the current database of *in vitro* and *in vivo* studies for steviol glycosides is robust, and does not indicate that either stevioside or rebaudioside A are genotoxic. This finding, combined with lack of carcinogenic activity in several rat bioassays, establishes the safety of all steviol glycosides with respect to their genotoxic/carcinogenic potential.

Table K-6. Mutagenicity & Genotoxicity Studies on Stevia Extracts & Stevioside

End-Point	Test System	Material	Purity (%)	Concentration / Dose	Result	Reference
In Vitro						
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, TA1537	Stevioside	83	5 mg/plate[a] 1 mg/plate[b]	Negative	Matsui et al. (1996)
Reverse mutation	*S. typhimurium* TA98, TA100	Stevioside	99	50 mg/plate	Negative[c]	Suttajit et al. (1993)
Reverse mutation	*S. typhimurium* TA98, TA100	Stevioside	NS	50 mg/plate	Negative	Klongpanichpak, Temcharoen, Toskulkao, Apibal, and Glinsukon (1997)
Forward mutation	*S. typhimurium* TM677	Stevioside	83	10 mg/plate	Negative[c]	Matsui et al. (1996)
Forward mutation	*S .typhimurium* TM677	Stevioside	NS	10 mg/plate	Negative[c]	Pezzuto et al. (1985)
Forward mutation	*S. typhimurium* TM677	Stevioside	NS	Not specified	Negative[c]	Medon et al. (1982)
Gene mutation	Mouse lymphoma L5178Y cells, TK^- locus	Stevioside	NS	5 mg/mL	Negative[c,d]	Oh et al. (1999)
Gene mutation (umu)	*S. typhimurium* TA1535/pSK1002	Stevioside	83	5 mg/plate	Negative[c]	Matsui et al. (1996)
Gene mutation	*B. subtilis* H17 rec+, M45 rec-	Stevioside	83	10 mg/disk	Negative[c]	Matsui et al. (1996)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	83	8 mg/mL 12 mg/mL	Negative	Matsui et al. (1996)

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
Chromosomal aberration	Human lymphocytes	Stevioside	NS	10 mg/mL	Negative	Suttajit et al. (1993)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	85	12 mg/mL	Negative[a]	Ishidate et al. (1984)
In Vivo						
DNA damage (comet assay)	Wistar rats; liver, brain and spleen	Stevioside	88.62	4 mg/L (estimated to be 80 - 500 mg/kg bw/day) in drinking water for 45 days	Positive in all tissues examined, most notably in liver	A. Nunes et al. (2007)
DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver	Stevia extract	Stevioside, 52; Reb A, 22	250 – 2,000 mg/kg bw	Negative[e]	Sekihashi et al. (2002)
DNA damage (comet assay)	Male ddY mouse stomach, colon, liver, kidney, bladder, lung, brain, bone marrow	Stevia	NS	2,000 mg/kg bw	Negative[e]	Sasaki et al. (2002)
Micronucleus formation	ddY mouse bone marrow and regenerating liver	Stevioside	NS	62.5 - 250 mg/kg bw	Negative	Oh et al. (1999)
Mutation	*D. melanogaster* Muller 5 strain	Stevioside	NS	2% in feed	Negative	Kerr, Mello, and Bonadio (1983)

NS = Not specified. [a] Without metabolic activation. [b] As calculated by G. M. Williams (2007). [c] With and without metabolic activation (source not specified in original monograph). [d] Inadequate detail available. [e] Sacrificed at 3 hours and 24 hours.

6. Clinical Studies & Other Reports in Humans

In several studies, pharmacological and biochemical effects of crude extracts of stevia leaves and purified steviol glycosides have been investigated. The effects noted included glucose uptake, insulin secretion, and blood pressure (J. M. Geuns, Augustijns, et al., 2003). In South America, stevioside is used as a treatment for type 2 diabetes. These effects were key concerns for JECFA. In 2006, JECFA summarized the available clinical studies of stevioside and further studies were recommended (WHO, 2006). Subsequently, several studies were conducted, and in 2009, JECFA reviewed these new studies (WHO, 2009). JECFA's summaries of the key studies are included below.

a. Studies Summarized in 2006

In a study by Curi et al. (1986), aqueous extracts of 5 grams of *S. rebaudiana* leaves were administered to 16 volunteers at 6 hour intervals for three days, and glucose tolerance tests were performed before and after the administration. Another six volunteers were given an aqueous solution of arabinose in order to eliminate possible effects of stress. The extract increased glucose

tolerance and significantly decreased plasma glucose concentrations during the test and after overnight fasting in all volunteers.

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

In a follow-up multi-center randomized, double-blind, placebo-controlled trial was conducted in hypertensive Chinese men and women (aged 20–75 years), 85 patients were given capsules containing 500 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 1,500 mg of stevioside per day [equivalent to 21 mg per kg bw per day, as calculated by FSANZ (2008)]. Eighty-nine patients were given a placebo. During the course of study, three patients in each group withdrew. There were no significant changes in body mass index or blood biochemistry parameters throughout the study. In the group receiving stevioside, mean systolic and diastolic blood pressures were significantly decreased compared with the baseline, commencing from about 1 week after the start of treatment. After 2 years, 6 out of 52 patients (11.5%) in the group receiving stevioside had left ventricular hypertrophy compared with 17 of 50 patients (34%) in the group receiving the placebo ($p < 0.001$). Eight patients in each group reported minor side effects (nausea, dizziness and asthenia), which led two patients in each group to withdraw from the study. Four patients in the group receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved and the patients remained in the study (Hsieh et al., 2003).

In a randomized, double-blind trial designed, 48 hyperlipidemic volunteers were recruited to investigate the hypolipidemic and hepatotoxic potential of steviol glycoside extract. The extract used in this study was a product containing stevioside (73 ± 2%), rebaudioside A (24 ± 2%), and other plant polysaccharides (3%). The subjects were given two capsules, each containing 50 mg of steviol glycoside extract or placebo, twice daily (i.e., 200 mg per day, equivalent to 3.3 mg per kg bw per day assuming an average body weight of 60 kg), for 3 months. One subject from placebo group and three from treatment group failed to complete the study for personal reasons, not related to adverse reactions. At the end of the study, both groups showed decreased serum concentrations of total cholesterol and of low-density lipoproteins. Analyses of serum concentrations of triglycerides, liver-derived enzymes, and glucose indicated no adverse effects.

The authors questioned the subjects' compliance with the dosing regimen, in view of the similarity of effect between treatment and placebo (Anonymous, 2004a). In a follow-up study, 12 patients were given steviol glycosides extract in incremental doses of 3.25, 7.5, and 15 mg per kg bw per day for 30 days per dose. Preliminary results indicated no adverse responses in blood and urine biochemical parameters (Anonymous, 2004b).

In a paired cross-over study, 12 patients with type 2 diabetes were given either 1 gram of stevioside (stevioside, 91%; other stevia glycosides, 9%) or 1 gram of maize starch (control group), which was taken with a standard carbohydrate-rich test meal. Blood samples were drawn at 30 minutes before, and for 240 minutes after, ingestion of the test meal. Stevioside reduced postprandial blood glucose concentrations by an average of 18% and increased the insulinogenic index by an average of 40%, indicating beneficial effects on glucose metabolism. Insulin secretion was not significantly increased. No hypoglycemic or adverse effects were reported by the patients or observed by the investigators. Systolic and diastolic blood pressure was not altered by stevioside administration (Gregersen et al., 2004).

b. Studies Summarized in 2009

In a short-term study of stevioside in healthy subjects, 4 male and 5 female healthy volunteers (aged 21–29 years) were provided with capsules containing 250 mg stevioside (97% purity) to be consumed 3 times per day for 3 days (Temme, Vankeirsblick, & Buyse, 2004). Doses, expressed as steviol, were 288 mg per day, or 4.4 mg per kg bw per day for females and 3.9 mg per kg bw per day for males. Twenty-four hour urine samples were taken before dosing on day 1 and after dosing on day 3. Fasting blood samples were taken before dosing on day 1, and six samples were taken at different time points on day 3 after dosing. Fasting blood pressure measurements were taken before the first capsule and at six different time intervals after the first dose. Urine was analyzed for creatinine, sodium, potassium, calcium, and urea. Blood was analyzed for plasma glucose, plasma insulin, alkaline phosphatase, alanine transaminase (ALT), glutamic-pyruvate transaminase (GPT), creatine kinase, and lactate dehydrogenase. The clinical analyses of blood, blood pressure, and urine showed no differences between samples taken before or after dosing.

In an unpublished double-blind, placebo-controlled trial study reviewed at the 68th JECFA meeting, 250 mg of a product containing 91.7% total steviol glycosides, including 64.5% stevioside and 18.9% rebaudioside A, was administered to groups of type 1 (*n* = 8) and type 2 diabetics (*n* = 15), and non-diabetics (*n* = 15), 3 times daily for 3 months. Control groups with the same number of subjects received a placebo. After 3 months, there were no significant changes in systolic or diastolic blood pressure, glycated haemoglobin (HbA1c), blood lipids, or renal or hepatic function. No adverse effects were reported. This study was approved by the local ethics committee and met the requirements of the Declaration of Helsinki (L. Barriocanal et al., 2006; L. A. Barriocanal et al., 2008). The Committee previously noted that this product did not meet the proposed specification of "not less than 95% steviol glycosides" and that the study was conducted in a small number of subjects.

In a follow-up study, L. A. Barriocanal et al. (2008) evaluated the effects of steviol glycosides on blood glucose and blood pressure (BP) for three months in subjects with type 1 diabetes, subjects with type 2 diabetes, and subjects without diabetes and with normal/low-normal BP levels. Patients in each group received either 250 mg t.d.s. (total dissolved solids) steviol glycoside, stevioside, or placebo treatment. The purity of the steviol glycosides was ≥ 92%. Three months of follow up revealed no changes in systolic BP, diastolic BP, glucose, or glycated hemoglobin from baseline. In placebo type 1 diabetics, there was a significant difference in systolic BP and glucose. There were no adverse effects observed in either treatment group, and the authors concluded that oral steviol glycosides are well-tolerated and have no pharmacological effect.

A study of antihypertensive effects was conducted in previously untreated mild hypertensive patients with crude stevioside obtained from the leaves of *S. rebaudiana*. Patients with essential hypertension were subjected to a placebo phase for 4 weeks and then received either capsules containing placebo for 24 weeks or crude stevioside at consecutive doses of 3.75 mg per kg bw per day (7 weeks), 7.5 mg per kg bw per day (11 weeks) and 15 mg per kg bw per day (6 weeks). Comparison of patients receiving stevioside with those on placebo showed neither antihypertensive nor adverse effects of stevioside. This study was approved by the local ethics committee and met the requirements of the Declaration of Helsinki (Ferri et al., 2006). The product in this study also did not meet the proposed specification.

A placebo-controlled double-blind trial was carried out in 49 hyperlipidemic patients (aged 20–70 years, number of males and females not supplied) not undergoing treatment. The study was approved by the local ethics committee and complied with the principles of the Declaration of Helsinki. Individuals were divided into two groups, with 24 subjects receiving placebo capsules and 25 receiving capsules containing a dose of 50 mg steviol glycosides (70% stevioside, 20% Rebaudioside A), equivalent to 1.04 mg steviol per kg bw per day, using the mean body weight of the treatment group, 72.7 kg. Two capsules were taken before lunch, and two before dinner, each day for 90 days. Six subjects withdrew from the study, four in the placebo group and two in the test group. Self-reported adverse reactions were recorded, and fasting blood samples were taken at the end of the study and analyzed for alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low density lipoprotein (VLDL), and triglycerides. No effects of treatment on ALT, AST, or GGT were found. Decreases in the total cholesterol and LDL were observed in both the stevioside group and the placebo group, which were not treatment related. No adverse effects were observed (Silva et al., 2006). The Committee noted at its 68th meeting that the product used in this study did not meet the proposed specification.

In a long-term, randomized, double blinded, placebo-controlled study, Jeppesen et al. (2006) investigated the efficacy and tolerability of oral stevioside in patients with type 2 diabetes. In this study, 55 subjects received 500 mg stevioside (purity unspecified), or placebo (maize starch), 3 times daily for 3 months. Compared with the placebo, stevioside did not reduce the incremental area under the glucose response curve and maintained the insulin response, HbA1c, and fasting

blood glucose levels. HbA1c is an indicator of mean glucose levels and is used in identifying effects on the control of diabetes. No differences in lipids or blood pressure were observed. It is not clear whether this study was approved by the local ethics committee or met the requirements of the Declaration of Helsinki (Jeppesen et al., 2006).

Appendix L Summary of Studies on Steviol Glycosides Preparations That Are Primarily Rebaudioside A

Safety Data on Rebaudioside A[13]

Since 2008, several well-designed toxicology studies that followed the current regulatory and scientific guidelines for such studies have been reported on purified rebaudioside A, although it is uncertain whether or not these studies were considered by JECFA during its 2008 deliberations. These recent investigations included additional subchronic studies in rats and one in dogs, mutagenicity studies, reproduction and developmental studies in rats, and comparative pharmacokinetic studies with stevioside in rats and humans, as well as additional clinical studies. These studies confirm that rebaudioside A is metabolized similarly to other steviol glycosides, and they exhibited an absence of toxicological effects in the key studies reviewed by JECFA. It should be noted that rebaudioside A, as the steviol glycoside with high sweetness intensity and relatively high prevalence in the stevia leaves, remains an active topic of scientific research. For example, a study found in a recent literature search examined the anti-hyperglycemic activity of rebaudioside A in diabetic rats (Saravanan & Ramachandran, 2012). These investigators found that the effects of streptozotocin-induced diabetes on glucose and insulin levels were at least partially reversed in a dose-dependent manner with oral administration of rebaudioside A at doses in the range of 50-200 mg per kg bw. The doses used are 10-40 times higher than expected from the use of rebaudioside A as a sweetener. The known anti-hyperglycemic activity of steviol glycosides led JECFA to require clinical studies at reasonably high doses to show that—at levels used in food—there would be no effect on glucose homeostasis or blood pressure in human consumers. The clinical studies described below on rebaudioside A (Maki, Curry, Carakostas, et al., 2008; Maki, Curry, Reeves, et al., 2008) the lack of these pharmacological effects of rebaudioside A at expected levels of consumption.

1. Absorption, Distribution, Metabolism & Excretion (ADME) Studies

Studies investigating the ADME of extracts from stevia are available on stevioside, Reb A, and other steviol glycosides. Data evaluating the absorption and fate of these extracts from various animal species and humans indicate that one can extrapolate these results from rats to humans. Stevioside is metabolized to steviol *via* intestinal microflora, and the absorption of stevioside after oral administration has been shown to be very low (J. M. Geuns, Augustijns, et al., 2003; J. M. Geuns, Malheiros, et al., 2003; E. Koyama et al., 2003).

[13] Questions about the safety of rebaudioside A were previously raised by Huxtable (2002), and Kobylewski and Eckhert (2008). Their respective concerns, as well as opposing views supporting the safety of designated food uses of rebaudioside A expressed by Expert Panels, have been outlined in other GRAS notifications that were submitted to FDA. A more detailed account can be found in GRAS notifications 278, 287, 303, and 304. This matter is discussed by the Expert Panel in Section VI.C.

Studies investigating the hydrolysis of steviol glycosides by intestinal microflora have demonstrated that both stevioside and Reb A are hydrolyzed to steviol following *in vitro* incubation with various cecal microflora (Gardana et al., 2003; J. M. Geuns, Augustijns, et al., 2003; A. M. Hutapea et al., 1997; Wingard Jr et al., 1980). In addition, the *in vitro* hydrolysis of Reb A to steviol was found to be slower than that of stevioside (E Koyama et al., 2003), which is thought to be partly due to the presence of one additional glucose moiety and to differences in structural complexities. E Koyama et al. (2003) suggest that the major pathway for Reb A is conversion to stevioside with a minor pathway of conversion to Reb B prior to being ultimately converted to steviol. Stevioside is further converted to steviolbioside, steviolmonosides, and finally steviol, with glucose being released with each subsequent hydrolysis.

In three recently completed studies, absorption and fate of rebaudioside A were systematically investigated in rats and humans.

For comparative purposes to determine whether toxicological studies conducted previously with stevioside would be applicable to the structurally-related glycoside, rebaudioside A, toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol were examined in rats (A Roberts & Renwick, 2008). Orally administered single doses of the radiolabeled compounds were extensively and rapidly absorbed with plasma concentration-time profiles following similar patterns for stevioside and rebaudioside A.

A Roberts and Renwick (2008) identified free steviol (82 to 86%), steviol, glucuronide (10 to 12%), and two unidentified metabolites (5-6%) in rat plasma following treatment with either stevioside or Reb A eight hours post-oral administration. A comparable pharmacokinetic profile was noted following oral treatment of rats with radiolabeled Reb A or stevioside, with the time of maximum plasma concentration (T_{max}) for radioactivity ranging between 2 and 8 hours. In comparison, steviol T_{max} for plasma was noted within 30 minutes of oral administration. All plasma samples had similar metabolite profiles; the predominant radioactive component in all samples was steviol, with lower amounts of steviol glucuronide(s) and low levels of one or two unidentified metabolites. It is believed that this delay between the occurrence of radioactivity in the plasma and time of administration of steviol glycosides is due to the fact that the Reb A and stevioside are first cleaved to steviol before absorption.

Within 72 hours of administration, elimination of radioactivity from plasma was essentially complete. Following elimination in the bile, steviol is available to be released again from its conjugated form by microflora activity and may enter enterohepatic circulation. Consequently, free and conjugated steviol are secreted in the feces along with any unhydrolyzed fraction of the administered glycosides. Following Reb A treatment, significant amounts of unchanged rebaudioside A (29% in males and 19% in females) and stevioside (3% in males and 4% in females) were excreted in the feces. Following oral stevioside administration, unchanged stevioside was excreted in rat feces. Other unidentified metabolites are also present in fecal samples of rats treated with either glycoside. Rebaudioside A, stevioside, and steviol were

metabolized and excreted rapidly, with ~60% of the radioactivity eliminated in the feces within 48 hours. Urinary excretion accounted for less than 2% of the administered dose for all compounds in both intact and bile duct-cannulated rats, and the majority of the absorbed dose was excreted *via* the bile. After administration of the compounds to intact and bile duct-cannulated rats, radioactivity in the feces was present primarily as steviol. The predominant radioactive compound detected in the bile of all cannulated rats was steviol glucuronide (A Roberts & Renwick, 2008).

In summary, A Roberts and Renwick (2008) found that steviol was the predominant component found in plasma samples after oral administration of Reb A, stevioside, and steviol in rats. Lower amounts of steviol glucuronide(s) and one or two unidentified metabolites were also found. The majority of all samples were found to be excreted rapidly---primarily in the feces---within 48 hours. This is in agreement with the previous *in vitro* hydrolysis data that indicated that both Reb A and stevioside are metabolized to steviol by intestinal microflora. The predominant compound detected in the bile was steviol glucuronide, while the prominent material in the intestine was steviol, which the authors suggest indicates that deconjugation occurs in the lower intestine. The authors concluded that the overall data on toxicokinetics and metabolism indicate that rebaudioside A and stevioside are handled in an almost identical manner in the rat after oral dosing.

In a randomized, double blind, cross-over study in healthy male subjects, Wheeler et al. (2008) assessed the comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A and stevioside. Following administration of rebaudioside A or stevioside, steviol glucuronide appeared in the plasma of all subjects, with median T_{max} values of 12.00 and 8.00 hours post-dose, respectively. Steviol glucuronide was eliminated from the plasma, with similar $t_{1/2}$ values of approximately 14 hours for each compound. Administration of rebaudioside A resulted in a significantly (~22%) lower steviol glucuronide geometric mean C_{max} value (1,472 ng per mL) than administration of stevioside (1,886 ng per mL). The geometric mean AUC_{0-t} value for steviol glucuronide after administration of rebaudioside A (30,788 ng*hr per mL) was approximately 10% lower than after administration of stevioside (34,090 ng*hr per mL). Steviol glucuronide was excreted primarily in the urine of the subjects during the 72-hour collection period, accounting for 59% and 62% of the rebaudioside A and stevioside doses, respectively. No steviol glucuronide was detected in feces. Pharmacokinetic analysis indicated that both rebaudioside A and stevioside were hydrolyzed to steviol in the gastrointestinal tract prior to absorption. The majority of circulatory steviol was in the form of steviol glucuronide, indicating rapid first-pass conjugation prior to urinary excretion. Only a small amount of steviol was detected in urine (rebaudioside A: 0.04%; stevioside: 0.02%). The investigators concluded that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans, with steviol glucuronide excreted primarily in the urine and steviol in the feces. No safety concerns were noted as determined by reporting of adverse events, laboratory assessments of safety, or vital signs (Wheeler et al., 2008).

Another pharmacokinetic investigation was done as a toxicokinetic (TK) phase of a dietary study to determine the potential of rebaudioside A toxicity in rats at levels up to 2,000 mg per kg bw per day (Sloter, 2008a). Extremely low levels of rebaudioside A and total steviol were detected in

peripheral blood of rats during daily administration of 2,000 mg per kg bw per day of rebaudioside A, with mean plasma concentrations of approximately 0.6 and 12 μg per mL, respectively. Estimates of absorbed dose for rebaudioside A and total steviol were approximately 0.02% and 0.06%, respectively, based on the amounts measured in urine collected over 24 hours in comparison to daily administered dietary dose to rats. Mean fecal rebaudioside A and measured hydrolysis products, expressed as Total Rebaudioside A Equivalents, compared to daily administered dose results in an estimated dose recovery of approximately 84%.

2. Subchronic Toxicity Studies

Curry and Roberts (2008) reported the results of two repeat dose studies of rebaudioside A in Wistar rats. The results of these investigations suggest that administration of rebaudioside A to Han Wistar rats at dietary concentrations of up to 100,000 ppm (9,938 and 11,728 mg per kg bw per day for males and females, respectively) for 4 weeks, or 50,000 ppm (4,161 and 4,645 mg per kg bw per day for males and females, respectively) for 13 weeks, did not present any evidence of systemic toxicity. In the 4-week study, rebaudioside A (97% purity) was administered at dietary concentrations of 0, 25,000, 50,000, 75,000, and 100,000 ppm to male and female rats. The NOAEL, including an evaluation of testes histopathology, was determined to be 100,000 ppm. In the 13-week study, Wistar rats were fed diets containing rebaudioside A at dietary concentrations of 0, 12,500, 25,000, and 50,000 ppm. In high-dose male and females groups, reductions in body weight gain attributable to initial taste aversion and lower caloric density of the feed were observed. Inconsistent reductions in serum bile acids and cholesterol were attributed to physiological changes in bile acid metabolism due to excretion of high levels of rebaudioside A *via* the liver. All other hepatic function test results and liver histopathology were within normal limits. No significant changes in other clinical pathology results, organ weights, and functional observational battery test results were noted. Macroscopic and microscopic examinations of all organs were unremarkable with respect to treatment-related findings. The NOAEL in the 13-week toxicity study was considered to be 50,000 ppm, or approximately 4,161 and 4,645 mg per kg bw per day in male and female rats, respectively (Curry & Roberts, 2008).

In another 90-day dietary admix toxicity study, effects of rebaudioside A (99.5% purity) at target exposure levels of 500, 1,000, and 2,000 mg per kg bw per day were tested in Crl:CD(SD) rats (Eapen, 2007; Nikiforov & Eapen, 2008). Each group consisted of 20 animals per sex. No treatment related effects on clinical observations, food consumption, and functional observational or locomotor activity parameters were noted. There were no treatment-related macroscopic, organ weight or microscopic findings. Significantly lower body weight gains were noted in the 2,000 mg per kg bw per day group in males but not females. At the end of the dosing period, the body weight in males was 9.1% lower than the control group. Due to the small magnitude of difference from the control group value, the investigators did not consider this result to be adverse. The decrease was most likely due to the large proportion of the diet represented by the test material. The NOAEL was determined as ≥ 2,000 mg per kg bw per day.

A 6-month dietary toxicity study in Beagle dogs (4 per sex per group) was conducted to investigate the potential adverse effects of rebaudioside A (97.5% purity) at dosage levels of 0, 500, 1,000, or 2,000 mg per kg bw per day (Eapen, 2008). There were no unscheduled deaths during the course of the study. No treatment-related clinical observations were noted. Administration of rebaudioside A did not affect home cage, open field observations and functional observations and measurements. No differences in hematology findings, serum chemistry findings, or urinalysis findings between the groups were noted. Additionally, no treatment related gross necropsy observations, alterations in final body weight, alterations in organ weights, or histological changes were noted. The investigators concluded that no systemic toxicity of rebaudioside A was observed at dosage levels up to 2,000 mg per kg bw per day and the assigned NOAEL was ≥ 2,000 mg per kg bw per day.

In addition, a 90-day subchronic toxicity study was conducted in Sprague-Dawley rats using fermentation-derived Rebaudioside A, where no systemic or local toxicity was observed in rats dosed at 500 to 2,000 mg per kg bw per day. All test animals survived to scheduled necropsy (Rumelhard et al., 2016).

3. Mutagenicity Studies

In a set of *in vitro* and *in vivo* genotoxicity assays covering mutation, chromosome damage, and DNA strand breakage, rebaudioside A consistently and uniformly revealed negative results (Nakajima, 2000a, 2000b; Pezzuto et al., 1985; Sekihashi et al., 2002). These studies were critically reviewed by Brusick (2008). JECFA also reviewed an unpublished chromosome aberration assay of rebaudioside A in cultured mammalian cells (Nakajima, 2000a) and did not find increases in chromosome aberrations.

Additionally, FDA also reviewed three unpublished studies on rebaudioside A, including a bacterial mutagenicity study (Wagner & Van Dyke, 2006), a mouse lymphoma study (Clarke, 2006), and a mouse micronucleus study (Krsmanovic & Huston, 2006), submitted by Merisant as part of the GRAS Notification. All three studies demonstrated lack of mutagenic or genotoxic activity. Furthermore, L. D. Williams and Burdock (2009) also reported lack of genotoxicity in another set of published studies that included *in vitro* mutagenicity assays with *Salmonella, E. coli,* and mouse lymphoma cells. These investigators also reported lack of *in vitro* clastogenic effects in Chinese hamster V79 cells, and the absence of *in vivo* effects in a mouse micronucleus assay and a rat study for unscheduled DNA synthesis.

The recent evaluation of fermentation-derived rebaudioside A demonstrated a similar safety profile to plant-derived rebaudioside A. Rumelhard et al. (2016) reported that fermentation-derived rebaudioside A was not mutagenic in the bacterial reverse mutation assay, nor was it found to be clastogenic or aneugenic in the *in vitro* micronuleus assay. The similarity of the safety profile observed between plant-derived and fermentation-derived rebaudioside A further supports the applicability of the safety assessments to other steviol glycoside preparations.

The key mutagenicity testing results for rebaudioside A are summarized in Table L-1.

Table L-1. Mutagenicity & Genotoxicity Studies on Rebaudioside A

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
Bacterial Mutagenicity	5 *Salmonella* strains with & without exogenous metabolic activation system	Reb A	99.5	1.5, 5.0, 15, 50, 150, 500, 1,500 & 5,000 μg per plate	No mutagenic response	Wagner and Van Dyke (2006)
Bacterial Mutagenicity	4 *Salmonella* strains & 1 *E. coli* strain with & without exogenous metabolic activation system	Reb A	95.6	Up to 5,000 μg per plate	No mutagenic response	L. D. Williams and Burdock (2009)
Bacterial Mutagenicity	4 *Salmonella* strains & 1 *E. coli* strain with and without exogenous metabolic activation system	Fermentation-derived Reb A	≥ 95%	Up to 5,000 μg per plate	No mutagenic response	Rumelhard et al. (2016)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence & presence of exogenous metabolic activation system	Reb A	99.5	Cloning conc. of 500, 1,000, 2,000, 3,000, 4,000 & 5,000 μg/mL	No mutagenic or clastogenic response	Clarke (2006)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence & presence of exogenous metabolic activation system	Reb A	95.6	Up to 5,000 μg/mL	No mutagenic or clastogenic response	L. D. Williams and Burdock (2009)
Human Lymphocytes	Human lymphocytes in absence & presence of exogenous activation system	Fermentation-derived Reb A	≥ 95%	Up to 5,000 μg/mL	Not clastogenic or aneugenic	Rumelhard et al. (2016)
Chromosome Aberration	Human lymphocytes in absence & presence of exogenous metabolic activation system	Reb A	95.6	Up to 5,000 μg/mL	No mutagenic or clastogenic response	L. D. Williams and Burdock (2009)
Mouse Micronucleus	Micronucleus study in groups of 5 male & 5 female ICR mice	Reb A	99.5	500, 1,000 & 2,000 mg/kg bw	No increase in micronuclei formation	Krsmanovic and Huston (2006)
Mouse Micronucleus	Micronucleus study in groups of 5 male & 5 female NMRI mice	Reb A	95.6	Up to 750 mg/kg bw	No increase in micronuclei formation	L. D. Williams and Burdock (2009)
Unscheduled DNA Synthesis	Unscheduled DNA synthesis in one group of 4 Wistar rats	Reb A	95.6	Up to 2,000 mg/kg bw	No increase in unscheduled DNA synthesis	L. D. Williams and Burdock (2009)
DNA damage	Male BDF1 mouse stomach, colon, liver	Stevia extract	Stevio-side,	250 – 2,000 mg/kg bw	Negative[a]	Sekihashi et al. (2002)

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
(comet assay)			52%; Reb A, 22%			
Chromosomal aberration	CHL/IU Chinese hamster lung fibroblasts	Reb A	NS	1.2 - 55 mg/mL	Negative[b]	Nakajima (2000a)
Micronucleus formation	BDF1 mouse bone marrow	Reb A	NS	500-2,000 mg/kg bw/ day for 2 days	Negative[c]	Nakajima (2000b)
Forward mutation	*S. typhimurium* TM677	Reb A	NS	10 mg/plate	Negative[b]	Pezzuto et al. (1985)

NS = Not specified.

[a] Sacrificed at 3 hours and 24 hours.

[b] With or without metabolic activation (source not specified in original monograph).

[c] Sacrificed at 30 hours after 2nd administration.

4. Reproductive & Developmental Toxicity Studies

In a two-generation reproductive toxicity study, rebaudioside A (97% purity) at 0, 7,500, 12,500, and 25,000 ppm was administered in diet to male and female Han Wistar rats (Curry et al., 2008). Administration of rebaudioside A was not associated with any signs of clinical toxicity or adverse effects on body weight, body weight gain, or food consumption. Similarly, administration of rebaudioside A did not affect reproductive performance parameters including mating performance, fertility, gestation lengths, estrous cycles, or sperm motility, concentration, or morphology in either the F_0 or F_1 generations. The survival and general condition of the F_1 and F_2 offspring, their pre-weaning reflex development, overall body weight gains, and the timing of sexual maturation, were not adversely affected by rebaudioside A treatment. The NOAEL for reproductive effects was 25,000 ppm, and the NOAEL for the survival, development, and general condition of the offspring also was considered to be 25,000 ppm, or 2,048 to 2273 mg per kg bw per day (the highest dose tested).

The results from two unpublished studies with rebaudioside A (Sloter, 2008a, 2008b) further support the above described findings from published studies. In a two-generation dietary reproduction study, four groups of male and female Crl:CD(SD) rats (30 per sex per group) were fed either basal diet or the diet containing rebaudioside A (purity 95.7%) for at least 70 consecutive days prior to mating (Sloter, 2008a). For the F_0 and F_1 generations, rebaudioside A doses were 0, 500, 1,000, and 2,000 mg per kg per day. At initiation of study, F_0 animals were approximately 7 weeks of age. The test diet was offered to the offspring selected to become the F_1 generation following weaning [beginning on postnatal day (PND) 21]. The F_0 and F_1 males continued to receive rebaudioside A throughout mating, continuing through the day of euthanasia. The F_0 and F_1 females continued to receive rebaudioside A throughout mating, gestation and lactation until day of euthanasia. The authors concluded that there were no effects on reproduction in males or females as evaluated by estrus cycles, mating, fertility, conception or copulation indices, number of days between pairing and coitus, gestation length, and spermatogenic endpoints. Both for parental

systemic and reproductive toxicity, a dose level ≥ 2,000 mg per kg bw per day (highest dose administered) was assigned to be the NOAEL.

In an embryo/fetal developmental toxicity study in rats (Sloter, 2008b), effects of rebaudioside A administered *via* gavage were investigated. Rebaudioside A administration did not affect intrauterine growth and survival, and there were no test article-related fetal malformations or developmental variations at any dosage level. In the absence of maternal or developmental toxicity, a dose level ≥ 2,000 mg per kg bw per day (highest dose administered) was considered to be the NOAEL for maternal and embryo/fetal developmental toxicity.

5. Clinical Studies on Rebaudioside A

In a four week randomized, double-blind, placebo controlled trial, hemodynamic effects of rebaudioside A, at a dose of 1,000 mg per day rebaudioside A (97% purity) or placebo in 100 individuals with normal and low-normal systolic blood pressure (SBP) and diastolic blood pressure (DBP), were investigated (Maki, Curry, Carakostas, et al., 2008). Subjects were predominantly female (76% rebaudioside A and 82% placebo) with a mean age of ~41 (range 18 to 73) years. At baseline, mean resting, seated SBP/DBP was 110.0/70.3 mm Hg and 110.7/71.2 mm Hg for the rebaudioside A and placebo groups, respectively. Compared with placebo, administration of rebaudioside A did not significantly alter resting, seated SBP, DBP, mean arterial pressure (MAP), heart rate (HR) or 24-hour ambulatory blood pressure responses. The investigators concluded that consumption of 1,000 mg per day of rebaudioside A produced no clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure.

In another trial, effects of 16 weeks of consumption of 1,000 mg per person per day rebaudioside A (97% purity, n = 60) were compared to placebo (n = 62) in men and women (33-75 years of age) with type 2 diabetes mellitus (Maki, Curry, Reeves, et al., 2008). Changes in glycosylated hemoglobin levels did not differ significantly between the rebaudioside A (0.11 ± 0.06%, mean ± standard error) and placebo (0.09 ± 0.05%; p = 0.355) groups. Similarly, no significant ($p > 0.05$ for all) changes from baseline for rebaudioside A and placebo, respectively, in fasting glucose (7.5 ± 3.7 mg per dL and 11.2 ± 4.5 mg per dL), insulin (1.0 ± 0.64 µU per mL and 3.3 ± 1.5 µU per mL), and Cpeptide (0.13 ± 0.09 ng per mL and 0.42 ± 0.14 ng per mL) were noted. No treatment related changes in blood pressure, body weight, and fasting lipids were noted. Rebaudioside A was well-tolerated, and records of hypoglycemic episodes showed no excess versus placebo. Based on these results, the investigators suggested that chronic use of 1,000 mg per person per day rebaudioside A does not alter glucose homeostasis or blood pressure in individuals with type 2 diabetes mellitus.

6. Safety of Rebaudioside A

There have been a significant number of studies regarding the safety and toxicity of rebaudioside A, including many that have been published since the two initial GRAS notifications were submitted to FDA by Cargill (GRN 253) and Merisant (GRN 252). These, and some other unpublished

studies, formed the basis of the two initial GRAS notifications to FDA by Cargill (GRN 253) and Merisant (GRN 252). Prior to this, a limited number of toxicology studies specifically on rebaudioside A were conducted. Even before these new studies were completed, and as noted in the previous section, JECFA concluded that 7 (which was later expanded to 9) common steviol glycosides are deemed to be safe for use as sweetener preparations when present in any combination, as long as a combined purity of 95% or more was established.

Since a majority of the previous pharmacokinetic research was conducted with steviol glycosides, the presumed strategy adopted for the more recent research on rebaudioside A was to conduct a limited number of well-designed and executed toxicology studies on rebaudioside A itself, and to demonstrate that rebaudioside A is handled pharmacokinetically similarly to stevioside in rats and humans. This approach appears to have been undertaken to justify the JECFA-generated ADI without having to conduct a chronic study in rats with rebaudioside A. Additionally, the Merisant group conducted three mutagenicity assays on rebaudioside A that FDA generally considers to be most predictive for carcinogenicity potential. The Cargill group conducted two clinical studies to assure that rebaudioside A does not have potentially problematic pharmacological effects on blood glucose and blood pressure.

In a review article, M. C. Carakostas et al. (2008) summarized the most recent Cargill research program findings on rebaudioside A, as follows:

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

The consumption estimates described by JECFA, A. G. Renwick (2008), and the GRN 252 and GRN 253 Expert Panels very conservatively represent a potential high user of Rebaudioside A if this non-nutritive sweetener becomes widely available in food.

Regarding the available aggregate safety information, multiple qualified entities have concluded that JECFA has critically and extensively evaluated the use of steviol glycosides in foods and agrees that, at the present time, the ADI for steviol glycosides of adequate purity, as defined by JECFA specifications, has been properly determined to be 4 mg per kg bw per person as steviol equivalents, which corresponds to 12 mg per kg bw per day for rebaudioside A, on a dry weight basis. Unwanted pharmacological effects are not likely to occur at this level and, moreover, high consumers of rebaudioside A are not likely to exceed this level. Therefore, the JECFA-derived ADI was adopted as a safe exposure for rebaudioside A and the corresponding food uses meeting the specifications within the limits determined by this esteemed international body of food safety experts can be considered to be generally recognized as safe (GRAS).

JECFA---which is composed of dozens of scientists that are internationally known experts on food ingredient safety---has established ADIs for food ingredients over the last 40 years. Both Merisant and Cargill took rather rigorous scientific approaches to demonstrate the safety of rebaudioside A. The studies were equally well conducted. The safety profiles compiled by Merisant and Cargill differ somewhat, yet the results are complementary and are mutually reinforcing of rebaudioside A safety.

The studies conducted by Cargill provided significant insight into the pharmacokinetics of rebaudioside A, while demonstrating clinical safety of rebaudioside A regarding lack of effects on blood pressure and glucose metabolism that could result from doses expected from use in food. The Merisant notification augmented genotoxicity data in three systems recognized by FDA as good predictors of carcinogenic potential. Two of these assays were conducted in mouse systems. Additional mutagenicity and genotoxicity studies have been published on rebaudioside A (L. D. Williams & Burdock, 2009). Merisant added a subchronic study in dogs and a teratology study in rats. Both Cargill and Merisant relied on the JECFA ADI for steviol glycosides as determined largely by published chronic studies in rat. Both groups justified the use of the ADI on pharmacokinetic arguments showing the similarity of stevioside and rebaudioside A metabolism and excretion.

Appendix M Studies on Principal Metabolite: Steviol

Studies on Principal Metabolite: Steviol

In a number of studies, steviol, the principal mammalian metabolite of stevioside, has been investigated for its safety. The results of these studies are summarized below.

Acute Toxicity Studies

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

Developmental Toxicity Studies

Groups of 20 pregnant golden hamsters were given steviol (purity, 90%) at doses of 0, 250, 500, 750, or 1,000 mg per kg bw per day (only 12 animals at the highest dose) by gavage in corn oil on days 6 - 10 of gestation. A significant decrease in body weight gain and increased mortality (1/20, 7/20, and 5/12) were observed at the three highest doses, and the number of live fetuses per litter and mean fetal weight decreased in parallel. Histopathological examination of the maternal kidneys showed a dose-dependent increase in the severity of effects on the convoluted tubules (dilatation, hyaline droplets). However, no dose-dependent teratogenic effects were seen. The NOEL was 250 mg per kg bw per day for both maternal and developmental toxicity (Wasuntarawat et al., 1998).

Mutagenicity & Genotoxicity Studies

In a number of studies mutagenicity and genotoxicity of steviol has been investigated. These studies reviewed by JECFA are summarized in Table M-1.

Table M-1. Mutagenicity & Genotoxicity Studies on Steviol

	In Vivo/In Vitro	System	Test Sample Purity	Author Conclusion	Results and Remarks
Sekihashi et al. (2002)[a]	*In Vivo/In Vitro*	Comet Assay	Not reported	Negative	In *in vitro* study, steviol at 62.5, 125, 250 and 500 µg/ml did not damage DNA of TK6 and WTK1 cells in presence or absence of S9 mix. In *in vivo* study, mice sacrificed 3 or 24 hours after one-time oral administration of 250, 500, 1,000 or 2,000 mg/kg of steviol. Stomach, colon, kidneys, testis and liver DNA not damaged. An identical *in vivo* experiment with stevia extract performed, which also gave negative results.
Oh et al. (1999)[b]	*In Vivo?*	Cell Mutation and DNA damage	Not reported	Negative	Steviol gave negative results for cell mutation and DNA damage in cultured cells.
Matsui et al. (1996)[c]	*In Vivo?*	Mutagenicity and Chromosome aberration (Chinese hamster lung fibroblasts)	Not reported	Positive	Gene mutation and chromosomal aberration found in Chinese hamster lung fibroblasts after metabolic activation of steviol. In hamsters, several metabolites of stevioside found that have not been found in rats or humans. Therefore, experimental relevance should be questioned when hamsters are used.
Terai, Ren, Mori, Yamaguchi, and Hayashi (2002)[a]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Positive	Steviol found to be mutagenic in Aroclor induced rat liver S9 fraction. 15-oxo-steviol found to be mutagenic at 10% level of steviol. Specific mutagenicity of lactone derivative in presence of S9 mixture 10x lower than that of derivative without S9 mixture.
P Temcharoen, Pimbua, Glinsukon, Rojanapo, and Apibal (1998)[c]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Positive	Mutagenic effects of steviol and/or metabolites found in *S.typhimurium* TM677 by tranversions, transitions, duplications, and deletions at the guanine phosphoribosyltransferase (gpt) gene. Magnitude of increase of these mutations over the control not reported.
Klongpanichpak et al. (1997)[c]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Negative	Steviol and stevioside inactive in TA strains of *S. typhimurium, E. coli WP2, uvrA/PKM101* and rec assay using *B. subtilis* even when microsomal activated fraction present. Magnitude of increase of these mutations over the control not reported.
Matsui et al. (1996)[a]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Negative	Testing of Southern Blot technique with probe for gpt gene DNA of *E. coli*. The chromosomal DNA of TM677 and steviol-induced TM677

	In Vivo/*In Vitro*	System	Test Sample Purity	Author Conclusion	Results and Remarks
					mutants digested by restriction enzymes and probed. No significant differences found in fragment length between wild-type and mutant DNA.
Matsui et al. (1996)[a]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Both	Steviol weakly positive in umu test, either with or without metabolic activation. Steviol negative in reverse mutation and other bacterial assays even in presence of S9 activation.
Procinska, Bridges, and Hanson (1991)[c]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Negative	The direct mutagenic activity of 15-oxo-steviol was refuted.
Compadre, Hussain, Nanayakkara, Pezzuto, and Kinghorn (1988)[a]	*In Vitro*	Bacterial Mutagenicity, Mass Spec	Not Reported	Positive	Mass spectral analysis of steviol and analogues under conditions known to produce a mutagenic response. 15-oxo-steviol, a product of the metabolite, 15-alpha-hydroxysteviol was found to be direct-acting mutagen. Magnitude of increase over control in assay not discussed.
Pezzuto et al. (1985)[d]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Positive	Using *S. typhimurium* TM677 strain, steviol found to be highly mutagenic in presence of 9000 x g supernatant from livers of Aroclor 1254-pretreated rats. This mutagenicity dependent on pretreatment of rats with Aroclor and NADPH addition, as unmetabolized steviol was inactive. None of other metabolites tested was mutagenic. Authors concluded that structural features of requisite importance for the expression of mutagenic activity may include a hydroxy group at position 13 and an unsaturated bond joining the carbon atoms at positions 16 and 17.
Punya Temcharoen et al. (2000)[c]	*In Vivo*	Micronucleus (rat)	90%	Negative	Very high doses (8 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals.
Punya Temcharoen et al. (2000)[c]	*In Vivo*	Micronucleus (mouse)	90%	Negative	Very high doses (8 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals.
Matsui et al. (1996)[a]	*In Vivo*	Micronucleus (mouse)	Not Reported	Negative	Steviol did not increase number of micronuclei observed in this study.
Punya Temcharoen et al. (2000)[c]	*In Vivo*	Micronucleus (hamster)	90%	Negative	Very high doses (4 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals.

[a] Abstract only. [b] As reported in WHO (2006). [c] As reviewed by J. M. C. Geuns (2003). [d] Full article.

END

SUBMISSION END