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ORIGINAL SUBMISSION

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BY:.....

September 4, 2008

Robert Martin, Ph.D.
Deputy Director, Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety, CFSAN
U.S. Food and Drug Administration
4300 River Road, HFS-255
College Park, MD 20740-3835
301-436-1219

**RE: Notification of GRAS determination for Sucromalt**

Dear Dr. Martin,

*Per* your request, enclosed for OFAS review are three non-confidential copies of Cargill's GRAS Notification for the low-glycemic sweetener, sucromalt. These copies are meant to replace the three copies of this Notification that were delivered yesterday, all which had the word "CONFIDENTIAL" typed at the top and bottom of each page.

It is Cargill's understanding that you will compare this non-confidential version of the Notification to the redacted version that was included in yesterday's delivery and, if you agree that our redactions meet the requirements set forth under 21 CFR § 20.61,[1] you will redact the same text in one of the enclosed non-confidential copies; and it is that OFAS-

[1] Cargill's justification for claiming the redacted text confidential were outlined in a letter authored by Mr Greg Thompson of Cargill's Law Department, which was included in the package delivered to OFAS on Thursday, September 4, 2008

redacted copy, which will be sent to the Freedom of Information (FOI) office to fulfill any future FOI requests associated with sucromalt.

Please let me know, if you have any questions.

Sincerely,

Phillip L. Casterton, M.S., D.A.B.T.
Cargill, Incorporated
Manager, Regulatory and Scientific Affairs
Food Ingredients & Systems North America
15407 McGinty Road West, MS-163
Wayzata MN 55391

Enclosures

## GRAS EXPERT PANEL EVALUATION OF SUCROMALT

The undersigned, an independent panel of recognized experts (the "Expert Panel") qualified by scientific training and relevant national/international experience to evaluate the safety of food ingredients, was commissioned by Cargill, Incorporated, to determine whether the proposed use of sucromalt in various foods with no limitations other than current Good Manufacturing Practices (cGMP) would entail a reasonable certainty of no harm and could be considered Generally Recognized as Safe (GRAS) through scientific procedures. Sucromalt contains 35-45% fructose, 7-15% leucrose, ≤5% other mono- and disaccharides and 40-60% higher oligosaccharides It is derived from the reaction of nine parts sucrose and one part maltose catalyzed by alternansucrase, a food-processing enzyme derived from *Leuconostoc citreum*, or a recombinant strain of *Bacillis licheniformis*, both organisms which have well established safe strain lineages. The Expert Panel, individually and collectively, critically evaluated a compilation of published and unpublished sucromalt documentation supportive of a GRAS determination, as well as other documentation deemed applicable. The members of the Expert Panel conferred by telephone on several occasions and unanimously concluded that the intended uses of sucromalt, meeting appropriate food grade specification and manufactured and used consistent with cGMP are safe and suitable and Generally Recognized As Safe (GRAS) based on scientific procedures. The basis for these conclusions includes the safe strain lineage of organisms involved in enzyme production, negative Ames assay, 28-day rat study with NOAELs of 19,800 mg/kg/day (females) and 18,560 mg/kg/day (males), uncomplicated human digestion *via* well-established metabolic pathways without adverse effects, current safe consumption of constituents, and intake estimates much lower that current carbohydrate consumption.

| | |
|---|---|
| Joseph F. Borzelleca, Ph.D., Professor Emeritus, Fellow ATS<br>Virginia Commonwealth University School of Medicine | 08 May 2008<br>Date |
| Robert J Nicolosi, Ph.D., Professor of Nutrition<br>University of Massachusetts, Lowell | 07 May 08<br>Date |
| I. Glenn Sipes, Ph.D., Professor, Fellow ATS<br>Department of Pharmacology, University of Arizona | 06 May 08<br>Date |
| Michael W. Pariza, Ph.D.,<br>Michael W. Pariza Consulting LLC | 11 May 08<br>Date |

GRAS NOTIFICATION

## I. Claim of GRAS Status

### A. Claim of Exemption from the Requirement for Premarket Approval Requirements Pursuant to Proposed 21 CFR § 170.36(c)(1)

Sucromalt, for use as a nutritive carbohydrate, has been determined to be Generally Recognized As Safe, and therefore, exempt from the requirement of premarket approval, under the conditions of its intended use as described below. The basis for this finding is described in the following sections.

Signed,

_____ Date May 16, 2008

Phillip L. Casterton, M.S., D.A.B.T.
Cargill, Incorporated
Manager, Regulatory and Scientific Affairs
Food Ingredients & Systems North America
15407 McGinty Road West, MS-163
Wayzata MN 55391

## B. Name and Address of Notifier:

Phillip L. Casterton, M.S., D.A.B.T.
Cargill, Incorporated
Manager, Regulatory and Scientific Affairs
Food Ingredients & Systems North America
15407 McGinty Road West, MS-163
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## C. Common or usual name of the notified substance:

The common name of the substance of this notification is sucromalt

## D. Conditions of use:

Sucromalt is intended for use as a nutritive carbohydrate in baked goods, non-alcoholic beverages, alcoholic beverages, breakfast cereals, chewing gum, condiments and relishes, confectionary and frostings, frozen dairy, fruit ices, gelatins and puddings, hard candy, dairy products, non-dairy analogs, jams and jellies, milk, milk products, processed fruits and fruit juices, snack foods, soft candy, sweet sauce, and salad dressings at use levels of 1.5 to 85%. The resulting consumption of sucromalt from the use in these food categories is determined by the 2003-2004 Continuing Survey of Food Intake by Individuals (CSFII, 2006). Based on these determinations, the intended use of sucromalt in the above mentioned food categories resulted in an estimated mean *per capita* daily intake of 890 mg/kg/day and "users only" mean and 90$^{th}$ percentile estimates of 1090 mg/kg body weight/day and 2540 mg/kg body weight/day, respectively.

## E. Basis for GRAS Determination:

In accordance with 21 CFR § 170.30, the intended use of sucromalt has been determined to be generally recognized as safe (GRAS) based on scientific procedures. A comprehensive search of the scientific literature was also utilized for this review.

## F. Availability of Information:

The data and information that forms the basis for this GRAS determination will be provided to Food and Drug Administration upon request and are located at the office of:

Phillip L. Casterton, M.S., D.A.B.T.
Cargill, Incorporated
Manager, Regulatory and Scientific Affairs
Food Ingredients & Systems North America
15407 McGinty Road West, MS-163
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Phone: 952-742-4497
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## II. Detailed Information About the Identity of the Notified Substance:

### A. Chemical name

A mixture of carbohydrate produced from sucrose and maltose following enzymatic reaction.

### B. Trade Name:

The subject of this notification will be marketed as sucromalt

### C. Chemical Abstract Registry Number:

To the best of our knowledge, no Chemical Abstract Service Registry Number has been established for sucromalt.

### D. Chemical Formula:

Not applicable (a mixture of carbohydrates)

### E. Structure:

Not applicable (mixture)

### F. Molecular Weight

Not applicable (mixture)

### G. Physical Characteristics

Sucromalt is produced as a Clear to light yellow liquid with some cloud.

### H. Typical Composition and Specifications

Typical compositional analysis and specifications of sucromalt are presented in the following Tables.

**Typical composional analysis and specifications of sucromalt**[1]

| Parameter | Specification | Assay method |
|---|---|---|
| | **Carbohydrate profile** | |
| Fructose (%dry) | 35-45 | Cargill method LS-030-1 |
| Leucrose (%dry) | 7-15 | Cargill method LS-030-1 |
| DP2* (%dry) | ≤ | Cargill method LS-030-1 |
| Higher saccharides and polymer (%dry) | >40 | Cargill method LS-030-1 |
| | **Chemical/physical properties** | |
| Brix (%) | ≥0 | Cargill method DS LS-002-1 |
| Moisture (%) | ≤0 | Calculation |
| pH | 3.5 - 6 0 | Cargill method LS-006-1 |
| Sulfur dioxide (ppm) | 5 max | Cargill method LS-012-1 |
| Heavy metals (As, Cd, Hg, Pb) (ppm) | <1 | National food lab methods MN5012/MN5013 |
| | **Sensory characteristics** | |
| Appearance | Clear to light yellow liquid with some cloud | |
| Taste | Sweet, bland | Cargill method QAHM-SOP-GL0027 |
| Odor | Characteristic | Cargill method QAHM-SOP-GL0027 |
| | **Microbiological assays**** | |
| Total plate count | < 5000 cfu/g | CRA*** SMM IV-A |
| Yeast | < 100 cfu/g | CRA SMM III-A |
| Mold | < 100 cfu/g | CRA SMM III-A |

* = DP2 signifies saccharides with two degrees of polymerization; ** = Microbiological assays are done less frequently that are assays for carbohydrate profile, chemical/physical properties and sensory characteristics; *** = Corn Refiners Association; cfu = colony forming units

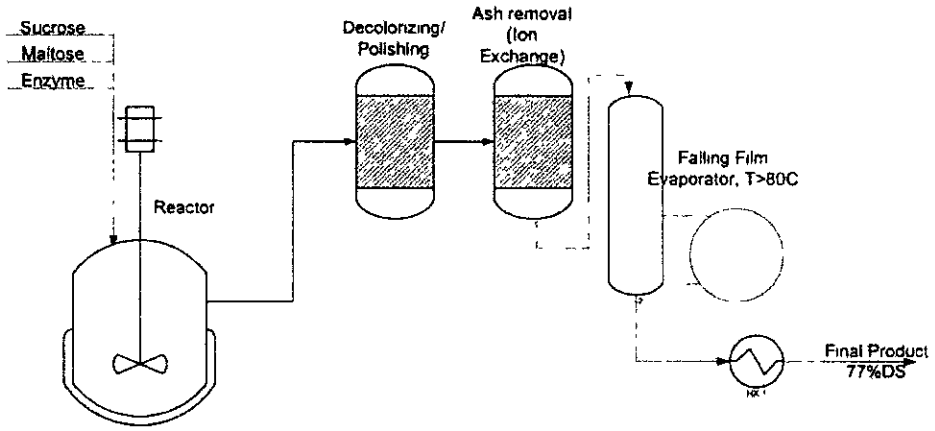
## I. Method of Manufacture

Manufacturing of sucromalt is accomplished by the addition of alternansucrase, a food-producing enzyme derived from production organisms with safe strain lineages, to a mixture of

[1] Note on the "CRA" methods under "Microbiological assays": The Corn Refiners Association is the national trade association for the U.S. corn refining (wet milling) industry. Members of that industry routinely use several methods developed and endorsed by CRA's technical committee. Cargill is currently developing data expected to validate that CRA-method results are similar to results obtained when using FDA's BAM (Bacteriological Analytical Manual) and/or methods recommended by AOAC (Association of Analytical Chemists). At the present time; however, CRA methods are used for the analysis of sucromalt for total plate count, yeast and mold. Cargill does not routinely run pathogen testing on sucromalt because its osmotic pressure, *percent* dissolved solids and low water content do not support pathogen growth, conditions that are similar to other commercially available corn syrups that are commonly consumed by humans every day. (Citations associated with pathogen testing are found in Footnote 20 of the attached GRAS dossier.) However, if specifically requested by a customer to run pathogen analysis, that testing will be completed.

approximately 8-11 parts food-grade sucrose and one part food grade maltose (or high-maltose corn syrup). The reaction-product sugars are both present as 40-50% aqueous mixtures prior to the addition of enzyme at 1-6 U *per* gram of combined sugar weight. The reaction is carried out at 37 to 47°C with mild mixing for several hours with pH maintained at 5.0-5.8 by acid or base addition. The aqueous sugar/enzyme mixture is sampled regularly to evaluate reaction progress and is considered finished when the amount of carbohydrate with two degrees of polymerization (*i.e.*, DP2) is less than 3% of the total carbohydrates, measured typically by high performance liquid chromatography. At that point, the sucromalt syrup is tested against the standard product specifications shown above. The syrup can be purified with decolorizing resin or activated carbon, or passed through cation and anion exchange resins to remove any protein, color, and ions. Combinations of these resins can be used based upon the ion load of the syrup. The purified syrup is evaporated under vacuum to obtain 75 to 80% dry solids. All processing aids are food-grade and are similar to those used in typical corn syrup production. A general schematic of sucromalt production is summarized in the following figure.

***Sucromalt Process Flow***




**Overview of the sucromalt production process**

Alternansucrase is sourced from one of two organisms, (1) a Cargill-developed strain of *Leuconostoc citreum*, and (2) a recombinant, *i.e.*, genetically modified, strain of *Bacillis licheniformis* developed by Novozymes, Inc. The production strain used to generate the Cargill-derived alternansucrase preparation was modified by classical bacterial mutagenesis techniques

and did not employ methods involving recombinant DNA. Alternansucrase derived from *B. licheniformis* has been determined as GRAS by scientific procedures.

The processing steps, the facility, and the controls used in the manufacturing of sucromalt conform to cGMP for human food in accordance with the applicable parts of 21 CFR, Part 110. Although the manufacturing process described above is thought to effectively eliminate alternansucrase from sucromalt, its absence has not been shown definitively. However, several items of evidence suggest that if alternansucrase were present in sucromalt, it would be at extremely low levels, if present at all. First, based on conservative estimates, the maximum residual protein in sucromalt that could be derived from alternansucrase is 6 ppm. Also, when finished sucromalt is combined with the sucrose-maltose reaction mixture under conditions of manufacturing, no additional sucromalt is produced; leading to the conclusion that active alternansucrase is absent from finished sucromalt. Additionally, the results of polymerase chain reaction (PCR) testing showed that the promoter gene sequence responsible for the production of alternansucrase was not present when *B licheniformis*-derived alternansucrase was analyzed by PCR.

### J. Intended Technical Effects

Sucromalt is intended for addition to a limited number of conventional foods as a low glycemic nutritive carbohydrate. It has a glycemic index that is approximately 65% that of high fructose corn syrup, likely due to slowly digestible α-(1,6)- and α-(1,3)-linked glucooligosaccharides. Sucromalt is intended for the general population at the levels identified in this document for baked goods, non-alcoholic beverages, alcoholic beverages, breakfast cereals, chewing gum, condiments and relishes, confectionary and frostings, frozen dairy, fruit ices, gelatins and puddings, hard candy, dairy products, non-dairy analogs, jams and jellies, milk, milk products, processed fruits and fruit juices, snack foods, soft candy, sweet sauce, salad dressings. We recognize that there are Standard of Identity requirements for some of these proposed foods and do not intend to refer to them by the commonly recognized names such as milk, chocolate or yogurt.

## III. Summary of the Basis for the Notifier's Determination that Sucromalt is GRAS

An independent panel of recognized experts (the "Expert Panel") qualified by scientific training and relevant national/international experience to evaluate the safety of food ingredients, was commissioned by Cargill, Incorporated to determine whether the proposed use of sucromalt in various foods with no limitations other than current Good Manufacturing Practices (cGMP) would entail a reasonable certainty of no harm and could be considered Generally Recognized as Safe (GRAS) through scientific procedures. Sucromalt contains 35-45% fructose, 7-15% leucrose, ≤5% other mono- and disaccharides and >40% higher oligosaccharides. It is derived from the reaction of nine parts sucrose and one part maltose catalyzed by alternansucrase, a food-processing enzyme derived from *Leuconostoc citreum*, or a recombinant strain of *Bacillis licheniformis*, both organisms which have well established safe strain lineages. The Expert Panel, individually and collectively, critically evaluated a compilation of published and unpublished sucromalt documentation supportive of a GRAS determination, as well as other documentation deemed applicable. The members of the Expert Panel conferred by telephone on several occasions and unanimously concluded that the intended uses of sucromalt, meeting appropriate food grade specification and manufactured and used consistent with cGMP are safe and suitable and Generally Recognized As Safe (GRAS) based on scientific procedures. The basis for these conclusions includes the safe strain lineage of organisms involved in enzyme production, negative Ames assay, 28-day rat study with NOAELs of 19,800 mg/kg/day (females) and 18,560 mg/kg/day (males), uncomplicated human digestion *via* well-established metabolic pathways without adverse effects, current safe consumption of constituents, and intake estimates much lower that current carbohydrate consumption

## IV. Basis for a Conclusion that Sucromalt is GRAS for its Intended Use.

The attached GRAS dossier served as the basis for the Expert Panel's collective opinion that sucromalt is GRAS for its intended uses. Expert Panelist signatures are included on pages 67 and 68.

# EVALUATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS

# OF

# SUCROMALT

**April 30, 2008**

Cargill, Incorporated
15407 McGinty Road W
Wayzata, MN 55391

000015

**Evaluation of the Generally Recognized as Safe (GRAS) Status of Sucromalt**

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## EXECUTIVE SUMMARY

Cargill, Incorporated (hereafter, Cargill) convened the undersigned independent panel of experts (hereafter, the Expert Panel),[1] qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, to determine the safety and Generally Recognized as Safe (GRAS) status, based on scientific procedures, of the proposed use of the sweetener, sucromalt. Sucromalt was previously self-affirmed as GRAS by scientific procedures in 2004 and 2005 for use in various food products at levels providing a daily intake of ≤50 gm/day, a level later amended to have no limitations other than cGMP (Cantox, 2004/2005). This dossier revises those earlier dossiers by including information on, (1) the enzyme used to manufacture sucromalt, (2) the organisms that produce that enzyme, (3) estimated human consumption, and (4) the historical safety literature on leucrose. Descriptions of additional human clinical studies are also included.

Sucromalt is a product of the alternansucrase-catalyzed reaction between eight to eleven parts sucrose and one part maltose; and is intended for use as a low glycemic carbohydrate in various food products. The raw materials used in the production of sucromalt are suitable for food manufacturing and are considered safe, either as such or as components of products obtained from starch by enzyme-catalyzed or acid-catalyzed hydrolysis. Sucromalt contains 35-45% fructose, 7-15% leucrose, ≤5% other mono- and disaccharides, and 40-60% higher oligosaccharides. Sucromalt has a glycemic index that is approximately 65% that of high fructose corn syrup, likely due to slowly digestible α-(1,3)- and α-(1,6)-linked glucooligosaccharides.

A comprehensive literature review was conducted by Cargill through September 2007 for safety and toxicity information on sucromalt as well as its reaction products and constituents, and is summarized in this dossier. The supporting documentation and this dossier were made available to the Expert Panel, who also evaluated other materials deemed appropriate. The support for the

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[1] Modeled after Section 201(s) of the Federal Food, Drug and Cosmetic Act, as amended. *Curriculum vitae* documenting the expertise of the Expert Panelists are attached.

safety of sucromalt was established by several factors, including historical dietary intake of its reaction products and its constituents, their digestion by normal metabolic pathways, the current GRAS status of sucrose, maltose and fructose, a negative Ames assay, a negative 28-day repeated dose rat toxicity study that resulted in no-observed-adverse-effect-levels (NOAEL) of 18,560 mg/kg/day in males and 19,800 mg/kg/day in females and several human clinical studies suggesting uncomplicated sucromalt metabolism without adverse effects. Following independent and collective critical evaluations, the Expert Panel unanimously agreed to the decision described herein.

## 1.0 INTRODUCTION

Published data supporting the safety of sucromalt were obtained from online sources and through literature searches conducted by Cargill at various times through September 2007 in medical, chemical, and toxicological databases such as Medline, RTECS® (Registry of Toxic Effects of Chemical Substances), Toxline, and ChemIDplus Advanced; Cargill provided unpublished data.

Cargill has developed sucromalt, a product that arises from reactions between sucrose[2] and maltose[3] utilizing alternansucrase, a glucosyltransferase enzyme[4] extract from (1) a strain of *Leuconostoc citreum*, or (2) a recombinant strain of *Bacillis licheniformis* developed by Novozymes, Inc. This reaction results in a product containing fructose and leucrose, as well as glucooligosaccharides containing α-(1,3), α-(1,4), and α-(1, 6) glycosidic linkages with a degree of polymerization of (DP) up to ≤2.[5] Sucromalt contains 35-45 % fructose, 7-15% leucrose, ≤5% other mono- and disaccharides (*i.e.*, primarily other isomers of sucrose such as

---

[2] Sucrose content in this reaction product: >95%.

[3] This reaction product will typically be a high purity maltose source (*i e.*, >93%), with glucose, maltotriose and higher oligosaccharides completing the substance (Personal communication with Dr. Anton Woo, Senior Research Scientist, Cargill Health and Nutrition, 13 November 2007.)

[4] In general, glucosyltransferase enzymes transfer glucose units from sucrose to acceptors such as maltose, maltotriose (a trisaccharide composed of three glucose units (ChemIDplus Advanced, 2007h)), and higher oligosaccharides in corn syrup, and release fructose into the reaction medium (Cote and Leathers, 2005).

[5] These oligosaccharides consist of an initial maltose unit linked to glucose with subsequent linkages being exclusively glucose-to-glucose. The initial maltose to glucose pairing occurs *via* an α-(1,4) glycosidic linkage; all subsequent glucose:glucose glycosidic linkages are in the α-(1,3) and/or α-(1, 6) configurations. (Personal communication with Dr. Anton Woo, Senior Research Scientist, Cargill Health and Nutrition, 11 January 2008.)

isomaltulose,[6] unreacted sucrose and maltose, and glucose) and 40-60% higher oligosaccharides.[7] Sucromalt has a glycemic index (GI) of approximately 53 (compared to glucose at 100) (GIL, 2005), which is 65% of the GI of high fructose corn syrup.

In this dossier, Cargill will establish that the proposed uses of sucromalt as a carbohydrate in various foods and beverages are safe and GRAS based on scientific procedures under 21 CFR § 170.30(b) with no use limitation other than current Good Manufacturing Practices (cGMP). This dossier is a compilation of published and unpublished data intended to support the determination by qualified experts that the proposed uses of sucromalt are safe and GRAS. There is multi-faceted support for the safety of sucromalt, including, (1) the well established dietary safety of its reaction products (sucrose and maltose) and its constituents (fructose, leucrose, and saccharides/oligosaccharides), (2) their long-time safe presence in the human diet, (3) their digestion by normal metabolic pathways, (4) the current GRAS status of sucrose (21 CFR §184.1854), maltose (in corn syrup, 21 CFR § 184.1865) and fructose (in high fructose corn syrup, 21 CFR § 184.1866) with no limitations other than cGMP, (5) a negative Ames assay, (6) a negative 28-day repeated-dose rat toxicity study that resulted in no-observed-adverse-effect-levels (NOAEL) of 18,560 mg/kg/day in males and 19,800 mg/kg/day in females; (7) clinical studies suggesting uncomplicated metabolism without reported adverse effects, and (8) human clinical investigations that showed no signs of malabsorption and no adverse effects.

## 2.0 BACKGROUND

### 2.1 History of use

Sucromalt is a proprietary carbohydrate substance developed in recent years exclusively by Cargill, Inc. As such, it has no long history of use.

### 2.2 Functionality in food

Sucromalt is intended to function as a nutritive carbohydrate in food.

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[6] 6-O-*alpha*-D-glucopyranosyl-D-fructose (ChemIDplus Advanced, 2007g), a disaccharide composed of glucose and fructose.
[7] All saccharides are expressed as *percentages* on a dry weight basis (Table 9).

## 2.3 Regulatory status

Sucromalt was previously self-affirmed as GRAS by scientific procedures in 2004 and 2005 for use in various food products at levels providing a daily intake of ≤50 gm/day, an intake that was later amended, again by scientific procedures, to have no limitations other than cGMP (Cantox, 2004/2005). This dossier revises those earlier dossiers by including information on, (1) the enzyme used to manufacture sucromalt, (2) the organisms that produce that enzyme, (3) estimated human consumption, and (4) the historical safety literature on leucrose. Descriptions of additional human clinical studies are also included.

Additionally, Novozymes recently determined that the alternansucrase derived from their *Bacillis licheniformis* by recombinant DNA techniques is GRAS as defined under 21 CFR § 170.30 (a) for use as a processing aid in the starch industry for the production of carbohydrates for use in food and beverages (Novozymes, 2007). This determination included an attestation to the safe strain lineage of the source *B. licheniformis* organism.

## 2.4 Intended use

Cargill's customers will use sucromalt as an alternative to other nutritive carbohydrates such as sucrose, corn syrup, high-fructose corn syrup, brown rice syrup, malt syrup, etc. in the foods and food categories identified in Appendix 2.

# 3.0 CARBOHYDRATES

## 3.1 Dietary carbohydrates

Carbohydrates can be divided into various categories based on the number of saccharide monomers. Monosaccharides such as glucose and fructose consist of a single saccharide unit. Disaccharides such as sucrose, maltose, and leucrose consist of two saccharides joined by a glycosidic linkage. Roberfroid and Slavin (2000) define oligosaccharides as substances containing 2 to 20 monosaccharides; however, common practice is to define these substances as containing 3-20 saccharide units.

The structures of the sucromalt reaction products (*i.e.*, sucrose and maltose) and its constituent saccharides (*i.e.*, glucose, fructose, and leucrose) are shown in Figure 1 and their chemical names

in Table 1. Sucrose and maltose are both disaccharides, sucrose being composed of glucose and fructose *via* an α-(1,2)-glycosidic linkage, whereas maltose is composed of two glucose molecules attached *via* an α-(1,4)-glycosidic linkage. The use of alternansucrase in the sucrose/maltose reaction effectively drives three key occurrences, (1) breaks the α-(1,2)-glycosidic linkage of sucrose to release fructose, (2) transfers glucose units sequentially by α-(1,3) and α-(1, 6) glycosidic linkages onto maltose, and (3) forms leucrose, which is joined by α-1,5)-glycosidic linkages. The fundamental structure of the oligosaccharides in sucromalt is maltose being linked with up to 12 or more units of glucose. When consumed, di- and oligosaccharides are typically hydrolyzed into their constituent monosaccharides, making them available for diffusion across the gut epithelium into the circulation where they undergo biotransformation processes that ultimately result in the generation of metabolic energy (Stryer, 1988).

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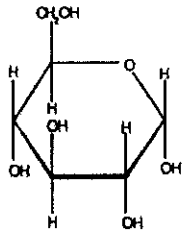
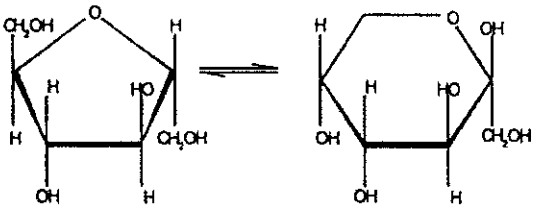
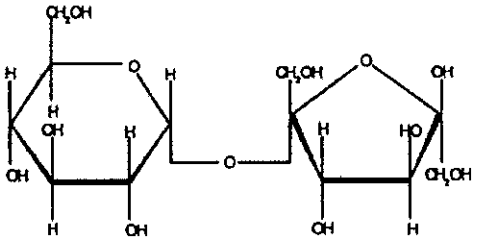


Glucose



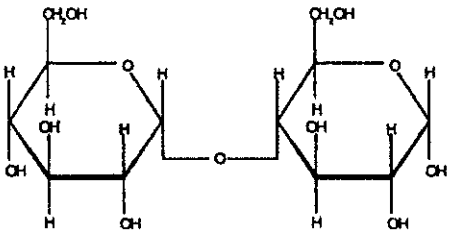
*Fructofuranose* *Fructopyranose*

Fructose



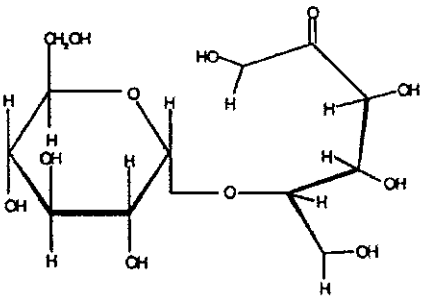
*Glucose* *Fructofuranose*

Sucrose
(α-(1,2)-glycosidic linkage)



*Glucose* *Glucose*

Maltose
(α-(1,4)-glycosidic linkage)



*Glucose* *Fructopyranose*

Leucrose
(α-1,5)-glycosidic linkage

**Figure 1. Molecular structures of mono- and disaccharides associated with sucromalt**

**Table 1. Common and synonyms associated with saccharides associated with sucromalt**

| Common name | CAS Number | Synonym | Reference |
|---|---|---|---|
| Glucose | 50-99-7 | *alpha*-D-Glucopyranose | ChemIDplus Advanced (2007a) |
| Sucrose | 57-50-1 | (*alpha*-D-Glucosido)-*beta*-D-fructofuranoside | ChemIDplus Advanced (2007b) |
| Maltose | 69-79-4 | 4-O-*alpha*-D-Glucopyranosyl-D-glucose | ChemIDplus Advanced (2007c) |
| Fructose | 57-48-7 | Levulose | ChemIDplus Advanced (2007d) |
| Leucrose | 7158-70-5 | D-Glucopyranosyl-*alpha*-(1-5)-D-fructopyranose | ChemIDplus Advanced (2007e) |

CAS = Chemical Abstracts Service

### 3.1.1 Carbohydrate requirements and intakes

Table 2 summarizes the Dietary Reference Intake (DRI) values for carbohydrates established by the National Academy of Sciences' Institute of Medicine (IOM, 2005). A Recommended Dietary Allowance (RDA) of 130 grams of carbohydrates *per* day, based on the average minimum amount of glucose utilized by the brain, was established for adults and children over one year old, and were equally applicable to males and females. Higher RDA values were established for pregnancy (175 g/day) and lactation (210 g/day). IOM (2005) further noted that these intake levels are typically exceeded to meet the energy needs while consuming acceptable intake levels for fat and protein. The median intake of carbohydrates is reported to be approximately 200 to 330 g/day for men and 180 and 230 g/day for women. No recommendations based on glycemic index were made by IOM due to a lack of sufficient evidence regarding its role in the prevention of chronic diseases in generally healthy individuals. For children ages 0-6 months, an Adequate Intake (AI) of 60 gm/day was established based on the average carbohydrate content in mother's milk; for children 7-12 months old, a higher AI of 95 gm/day was established based on the average intake of mother's milk and complementary foods (IOM, 2005).

IOM found insufficient evidence for establishing a Tolerable Upper Intake Level (UL) for an association between over consumption of sugars such as sucrose and fructose, and adverse effects such as dental caries, hyperactivity, cancer, risk of obesity, and risk of hyperlipidemia. However, IOM (2005) recommended a maximal intake level of 25% or less of energy from added sugars based on scientific literature suggesting that increased intake of sugars might result in decreased micronutrient intake. The relationship between dietary carbohydrate AI's and RDA's and the estimated daily intake of sucromalt is described in Section 6 (page 31).

**Table 2. Criteria and dietary reference intake values for carbohydrates by life stage group (IOM, 2005)**

| Life stage group | Criterion | EAR (gm/day) | | RDA (gm/day) | | AI |
|---|---|---|---|---|---|---|
| | | Male | Female | Male | Female | (gm/day) |
| 0 through 6 mo | Average content of human milk | | | | | 60 |
| 7 through 12 mo | Average intake from human milk plus complementary foods | | | | | 95 |
| 1 through 3 y | Extrapolation from adult data | 100 | 100 | 130 | 130 | |
| 4 through 8 y | Extrapolation from adult data | 100 | 100 | 130 | 130 | |
| 9 through 13 y | Extrapolation from adult data | 100 | 100 | 130 | 130 | |
| 14 through 18 y | Extrapolation from adult data | 100 | 100 | 130 | 130 | |
| >18 y | Brain glucose utilization | 100 | 100 | 130 | 130 | |
| Pregnancy, 14-18 y | Adolescent female EAR plus fetal brain glucose utilization | | 135 | | 175 | |
| Pregnancy, 19-50 y | Adult female EAR plus fetal brain glucose utilization | | 135 | | 175 | |
| Lactation, 14-18 y | Adolescent female EAR plus average human milk content of carbohydrate | | 160 | | 210 | |
| Lactation, 19-50 y | Adult female EAR plus average human milk content of carbohydrate | | 160 | | 210 | |

AI = Adequate Intake. The observed average or experimentally determined intake by a defined population or subgroup that appears to sustain a defined nutritional status, such as growth rate, normal circulating nutrient values, or other functional indicators of health. The AI is used if sufficient scientific evidence is not available to derive an EAR. For healthy infants receiving human milk, the AI is the mean intake; EAR = Estimated Average Requirement. The intake that meets the estimated nutrient needs of half of the individuals in a group; RDA = Recommended Dietary Allowance. The intake that meets the nutrient need of almost all (97-98 percent) of individuals in a group; mo = months; y = years

## 3.2 Glycemic Index (GI)

GI is a carbohydrate ranking system based on the effect of food on blood glucose levels, and defined by the area under the two hour blood glucose response curve (AUC). In effect, it compares carbohydrate digestibility in individual foods. Glucose (GI = 100) is the reference standard for GI determinations. Thus, rapidly digested carbohydrates have higher GI's than more slowly digested carbohydrates and consequently, lower GI foods will tend to have slower gut absorption rates. Lower GI foods are also thought to impart a lower insulin demand, better long-term blood glucose control and a reduction in blood lipids. This systematic classification of foods according to the glycemic responses they elicit can also supplement information on chemical composition presented in various food tables (Wolever *et al.*, 1991). IOM (2005) reports that there is a body of evidence indicating health advantages associated with a diet of low GI carbohydrates because their slowed digestion reduces the glycemic load of the total diet.

Thus, low GI and low glycemic load diets may improve human health, particularly in population subsets suffering from obesity, inactivity, and/or insulin resistance (IOM, 2005).

The reason behind the development of glycemic index was the knowledge that a food's glycemic effect could have applications in understanding the physiological effects of entire diets. However, unexpected GI values for different foods brought to light the importance of other factors such as the form of the food, the nature of the constituent starches, food processing, antinutrients and particle size. Wolever *et al.* (1991) described several aspects of GI-determination methodology that should be considered so that results of testing in different laboratories can be viewed as consistent and comparable. Included among those factors were portion size tested, choice of a standard food, the number of standard tests to be run, the blood sampling regimen, and calculations of AUC.

Cargill determined the GI of high fructose corn syrup and four different preparations of sucromalt using glucose as the control standard (GIL, 2005). The procedures closely followed the principles of GI testing identified by Wolever *et al.* (1991). Twenty healthy volunteers (12 female, 8 male; 37 ±3 years of age) participated. On each day of the study, the subjects, who had fasted for 10-14 hours, were weighed, had baseline blood taken *via* finger-prick and then consumed 50 grams of carbohydrate test substance. Additional blood samples were taken 15, 30, 45, 60, 90 and 120 minutes following exposure. Collected blood samples were analyzed for glucose with the results used to calculate AUC for the blood glucose responses. Peak rise was defined as the maximum concentration attained minus the baseline fasting value. GI was calculated by expressing each subject's test substance AUC as a *percentage* of his or her average response after consuming glucose. The results are presented in Table 3.

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**Table 3. Glycemic indices of four sucromalt samples and high fructose corn syrup (GIL, 2005)**

| Sample | Blood glucose response / AUC (0-2 hrs; mM x min/l) | Glycemic Index (GI)* |
|---|---|---|
| Glucose | 249 ± 17 | 100 |
| HFCS | 196 ± 12[a] | 81.1 ± 5.1[a] |
| Sucromalt sample #1[c] | 134 ± 10[b] | 54.0 ± 3.0[b] |
| Sucromalt sample #2[c] | 126 ± 9[b] | 52.7 ± 3.5[b] |
| Sucromalt sample #3[c] | 135 ± 9[b] | 55.9 ± 3.7[b] |
| Sucromalt sample #4[c] | 114 ± 10[b] | 48.7 ± 4.6[b] |
| Mean of samples 1-4 | 127 ± 10[b] | 52.8 ± 3.7[b] |

* GI compared to that of glucose, a = responses significantly less than those for glucose at $P<0.05$; b = responses significantly less than those for HFCS at $P<0.05$; c = sample met sucromalt specifications shown in Table 9; HFCS = High fructose corn syrup

The data in Table 3 demonstrate that sucromalt has a mean GI of 52.8 compared to a GI of 81.1 for high fructose corn syrup. Thus, sucromalt's GI is approximately 65% of the GI of high fructose corn syrup,[8] indicating much slower digestibility attributable to sucromalt.

### 3.3 Principal carbohydrate components of sucromalt

Sucromalt is derived from the reaction between sucrose and maltose catalyzed by alternansucrase. The resulting syrup contains approximately 35-45% fructose, 7-15% leucrose, ≤3% other disaccharides, and 40-60% higher oligosaccharides; all *percentages* are determined on a dry basis. Many of the oligosaccharides present in sucromalt are natural components of honey (Weston and Brocklebank, 1999).

#### 3.3.1 Fructose

Fructose is an important monosaccharide that occurs naturally in a large number of fruits (see Appendix 1) and in honey (Weston and Brocklebank, 1999). Fructose occurs in both the furanose (5-carbon ring) and pyranose (6-carbon ring) forms (see Figure 1).

#### 3.3.2 Leucrose

Leucrose is a structural isomer of sucrose that differs only in the type of linkage that connects the glucose and fructose (pyranose form) moieties. Whereas glucose and fructose are connected

[8] 52.8/81.1 = 0.651 or approximately 65%.

*via* an α-(1,2)-glycosidic linkage in sucrose (ChemIDplus Advanced, 2007b), these two monosaccharides are connected *via* an α-(1,5)-glycosidic linkage in leucrose (ChemIDplus Advanced, 2007e). Thus, leucrose is also referred to as a bond isomer of sucrose (Elias *et al.*, 1996). Leucrose is a minor component of honey (Watanabe and Aso, 1960). Ziesenitz *et al.* (1989) report that leucrose was a rare substance up until the late 1980's despite published methods for its chemical synthesis having been in existence from 1978.

3.3.3 Other oligosaccharides

During sucromalt production, alternansucrase catalyzes the release of fructose and glucose from sucrose, and transfer of the released glucose to maltose, which leads to the formation of glucose-based oligosaccharides attached by α-(1,3) and α-(1,6) glycosidic linkages. The resulting syrup contains approximately 50% glucose oligomers (Cargill, 2005b).

Studies in ileostomized human subjects indicate that the oligosaccharides in sucromalt are slowly, but almost fully (*i.e.*, 95.4%) absorbed in the small intestine (NutriScience, 2004). Consequentially, this slower absorption rate would be expected to result in a slower increase in serum glucose.

Sucromalt may also contain very small amounts of nondigestible oligosaccharides. However, in general, nondigestible oligosaccharides are not of toxicological concern because they are composed of naturally occurring mono- or disaccharides that, if not hydrolyzed by normal physiological pathways, are fermented by endogenous bacteria to produce short-chain fatty acids. Nondigestible oligosaccharides may be classified as dietary fibers that are generally recognized as safe (FDA, 2006) even though they may cause gastrointestinal discomfort in some individuals due to their high fermentation rate and osmotic properties. Such effects; however, are common to all nondigested or fermented dietary substrates (Roberfroid and Slavin, 2000).

## 4.0 BASIS FOR GRAS DETERMINATION

In accordance with Volume 62, Number 74 of the April 17, 1997 Federal Register, Pages 18937 to 18964 (Proposed Rules, 21 CFR Parts 170, *et al.*), Cargill establishes herein that the use of sucromalt as a low glycemic carbohydrate in the described foods and beverages, at levels providing an intake not to exceed cGMP, would entail a reasonable certainty of no harm and can

be considered safe and GRAS through scientific procedures. Table 4 presents the applicable sections, subsections, and specific requirements outlined in 21 CFR § 170.36(c) of the Proposed Rule. A GRAS determination would exempt the use of sucromalt in various food products from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

**Table 4. Subset of requirements for GRAS determination by scientific procedures**

| 21 CFR Section | Specific Requirements |
|---|---|
| 170.36(c)(2): Identity and Specifications | Notice must include detailed information about the identity of the…substance, including chemical name, structural formula, quantitative composition, method of manufacture, characteristic properties, specifications, *etc.* |
| 170.36(c)(3): Self-Limiting Levels of Use | Notice must include any self-limiting levels of use for the substance. |
| 170.36(c)(4)(i.)(A): Technical Evidence of Safety | Notice must include a detailed summary of the basis for determination that use of the substance is GRAS by scientific procedures. Summary should include a comprehensive discussion of, and citations to, generally available and accepted scientific data, information, methods, or principles used to establish safety, as well as consideration of probable consumption and cumulative effect of the substance in the diet. |
| 170.36(c)(4)(i)(C): Basis for Concluding Expert consensus | Notice summary of a scientific procedure GRAS determination must include the basis for concluding that there is a consensus among qualified experts that there is reasonable certainty that the substance is not harmful under the intended conditions of use. |

## 5.0 MANUFACTURING AND PRODUCT SPECIFICATIONS

### 5.1 Alternansucrase

Alternansucrase is a glucosyltransferase type enzyme (termed a glucansucrase) that belongs to the glycoside hydrolase family 70, a part of the larger α-amylase family,[9] many of which are used in food processing. There are approximately 60 different glucansucrases in the glycoside hydrolase family 70 that have been classified (Vujicic-Zagar, 2007). Family 70 glucansucrases can synthesize a variety of glycosidic linkages. The most studied enzyme in this family, dextransucrase, synthesizes primarily α-(1,6) glycosidic linkages but also a small amount of α-(1,3) glycosidic linkages. Alternansucrase differs from dextransucrase in that it synthesizes an alternating sequence of α-(1,3) and α-(1,6) linked glucose units using sucrose as the donor

[9] CAZy database - Carbohydrate-Active Enzymes database. www.cazy.org/ (Site visited 24 October 2007.)

substrate. The alternansucrase and dextransucrase genes are organized into four main sections, (1) signal peptide, (2) variable region, (3) catalytic domain, and (4) glucan binding domain. Although alternansucrase has not historically been used commercially, dextransucrase has been utilized to produce dextrans for pharmaceutical and food applications for many years (European Commission, 2000).

The alternansucrase that is used to manufacture sucromalt is sourced from one of two organisms, (1) a Cargill-developed strain of *Leuconostoc citreum*, and (2) a recombinant, *i.e.*, genetically modified, strain of *Bacillis licheniformis* developed by Novozymes, Inc. The production strain used to generate the Cargill-derived alternansucrase preparation was modified by classical bacterial mutagenesis techniques such as exposure to ultraviolet (UV) irradiation and chemical treatment, and did not employ methods involving recombinant DNA. The modifications accomplished by Cargill are further described below in section 5.1.1.1. Although details of the *B. licheniformis* derivation have not been made available to Cargill, Novozymes (2007) recently determined that alternansucrase derived from their *B. licheniformis* is GRAS by scientific procedures. As such, that derivation is expected to follow the same scientific principles of scientific development as described herein.

Alternansucrase uses sucrose as a substrate and catalyzes the formation of the polymer alternan, a substance similar to dextran (produced by dextransucrase) except for the presence of alternating α-(1,3) linkages between the linear chain α-(1,6) linkages. Alternansucrase as well as dextransucrase are capable of adding branch points of differing linkages to the linear chain and can catalyze the formation of shorter oligosaccharides in the presence of sucrose and additional carbohydrate or other free-hydroxyl containing molecules (*i.e.*, acceptors). These reactions form the basis for the industrial uses of alternansucrase in the production of low glycemic carbohydrate syrups for use in foods.

The identification characteristics of alternansucrase are presented in Table 5.

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**Table 5. Identity of alternansucrase**

| Parameter | Description |
|---|---|
| Classification | Alternansucrase (common name) |
| IUB nomenclature (systematic name) | Sucrose:1,6(1,3)-α-D-glucan 6(3)-α-D-glucosyltransferase |
| IUB Number | 2.4.1.140 |
| CAS Number | 100630-46-4 |
| Mode of action | Alternately transfers a D-glucosyl residue from sucrose to the 3 or 6 position of a non-reducing terminal residue of a D-glucan, resulting in glucans with alternating α-1,3 and α-1,6 linkages |

CAS = Chemical Abstracts Service, IUB = International Union of Biochemistry

### 5.1.1 *Leuconostoc spp.* production strains

*Leuconostoc* species are ubiquitous Gram-positive lactic acid bacteria that are widespread in the natural environment and frequently isolated from food. They have been studied extensively and utilized commercially for more than 50 years in food manufacture, *e.g.*, dairy, bread dough, meat, pickle and sauerkraut fermentations (Server-Busson *et al.*, 1999; Hemme and Foucaud-Scheunemann, 2004; JGI, 2007); they are also used in the production of dextrans (*i.e.*, high molecular weight polysaccharides) for blood plasma extender applications (Rodrigues *et al.*, 2003). A *Leuconostoc* species (*L. mesenteroides)* that is phylogenetically very similar to *L. citreum* (Leisner *et al.*, 2005) is also approved by the U.S. Food and Drug Administration (FDA) for use in producing dextrans, which are considered generally recognized as safe (GRAS) indirect food substances (21 CFR § 186.1275).

*Leuconostoc* species strains are not considered to be pathogens in the classical sense, *i.e.*, organisms able to cross or evade noncompromised host barriers or to produce enterotoxins or other toxins associated with human disease. However, there are a few isolated reports associating these bacteria with illness in compromised human subjects. Although Hemme and Foucaud-Scheunemann (2004) and Adams (1999) have reported that *Leuconostoc* species strains can infect immunocompromised patients, thus possibly being considered "opportunistic", they are not proficient at doing this (Falkow, 1997). In addition, the number of cases of *Leuconostoc* species infections reported in the literature is relatively low (*i.e.*, less than 100 since 1985) and most of these infections were in adults with weakened immunity or underlying diseases (Hemme and Foucaud-Scheunemann, 2004).

To the contrary, rather than contributing to human illness, many *Leuconostoc* species (including *L. citreum*) produce bacteriocin peptides that can kill or inhibit the growth of some human pathogens, *e.g.*, *Listeria monocytogenes* (Hechard *et al.*, 1993; Francis and O'Beirne, 1998).

*Leuconostoc* species are also not known to produce antibiotics, however they may generate antimicrobial substances such as organic acids and bacteriocins (Hemme and Foucaud-Scheunemann, 2004; Stiles, 1994). An antibiotic activity assay performed according to the guidelines in JECFA (2006a, 2006b) indicated that an alternansucrase preparation from *L. citreum* did not produce antibiotics as indicated by the absence of zones of inhibitions on lawns of six test bacteria (TRAC Microbiology, 2007).

5.1.1.1 *Leuconostoc citreum* mutant strain taxonomy

The production organism used to generate Cargill's alternansucrase preparation is *L citreum* NRRL B-30894, a mutant strain derived from a natural isolate of *L. citreum* NRRL B-1355. The United States Department of Agriculture (USDA) recognizes *L. citreum* as the correct genus/species associated with strain NRRL B-1355;[10] however, the historical scientific literature associated with this organism (*e.g.*, Jeanes, 1952; Jeanes *et al.*, 1954) calls it *L. mesenteroides* NRRL B-1355, a point recognized recently by Kok-Jacon (2007). This slight controversy compelled Cargill to employ well-established and accepted 16S rRNA sequencing techniques to demonstrate that the *Leuconostoc* strain being used to produce alternansucrase is indeed correctly named as *L. citreum* NRRL B-1355 (Cargill, 2007a). The results of Cargill's investigation, coupled with USDA's recognition that strain NRRL B-1355 belongs to *L. citreum*, firmly establishes the relationship of taxonomic equality between Cargill's production strain and its parents, a correlation that is not only fundamental to a finding that *L. citreum* possesses safe strain lineage, but also is a factor contributing to a GRAS finding for sucromalt.

The currently used strain, NRRL B-30894, is an alternansucrase-overproducing mutant that expresses enzyme when grown on glucose. Strain NRRL B-30894 is derived from the natural isolate NRRL B-1355, which was originally sequestered by the USDA from an orange juice

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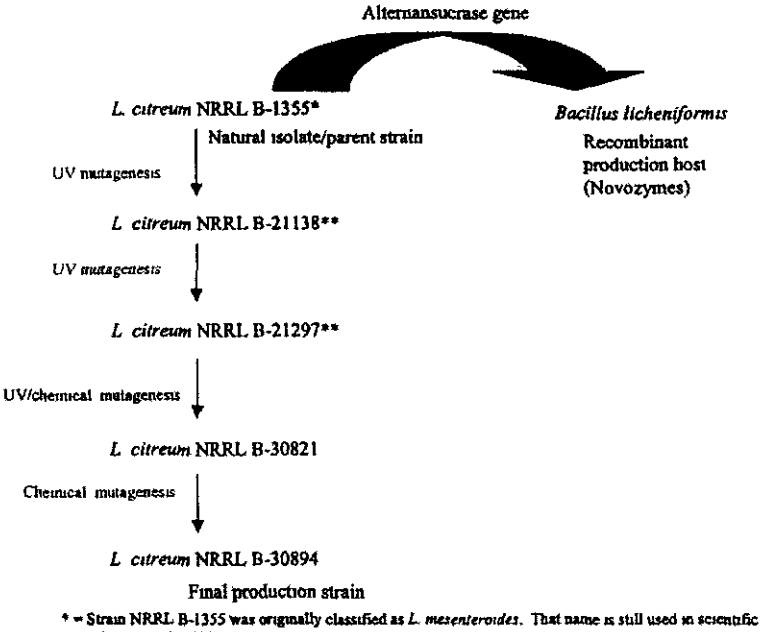
[10] See http://nrrl.ncaur.usda.gov/cgi-bin/usda/process html. *L. citreum* NRRL B-1355 was originally isolated in 1878. (Site visited 31 March 2008.)

production plant prior to 1952 (Jeanes, 1952). Later, the USDA mutated this natural isolate twice *via* UV irradiation in an attempt to enhance alternansucrase production and diminish dextransucrase production (Leathers *et al.*, 1997). The first mutant strain, which was patented by USDA, was designated NRRL N-21138. USDA mutated this strain a second time and the resulting USDA strain was designated as NRRL B-21297. Cargill researchers further mutated strain NRRL B-21297 by a combination of UV and chemical means to generate an intermediate, glucose-constitutive alternansucrase-producing strain, designated as NRRL B-30821. This strain synthesized alternansucrase in glucose medium at an approximately 25% higher titer than the parent NRRL B-1355 strain. Chemical mutagenesis was then applied to strain NRRL B-30821 to generate the final production strain, NRRL B-30894, an organism that produces ~ 30% more alternansucrase than did NRRL B-30821, when grown on glucose. Figure 2 demonstrates this sequence of mutations from the parent strain to the final alternansucrase production strain as well as showing that the alternansucrase gene that was introduced into Novozymes' *B. licheniformis* recombinant production host was also derived from the parent strain, NRRL B-1355.




* = Strain NRRL B-1355 was originally classified as *L. mesenteroides*. That name is still used in scientific literature (Kok-Jacon *et al.*, 2007)

** = Strains NRRL B-21138 and NRRL B-21297 are sequential mutants of the parent strain, NRRL B-1355, both generated by the USDA (Leathers *et al*, 1997) Only strain NRRL B-21297 was improved/typed by Cargill

**Figure 2. Sequence of bacterial mutagenesis resulting in *L. citreum* strain NRRL B-30894 with high alternansucrase production capacity**

To ensure that these mutant strains were not taxonomically different from the parent strain, Cargill (2007a) conducted the above-mentioned 16S ribosomal RNA sequence analyses on the parent and mutant strains. Table 6 displays the results from the analyses, which demonstrates that mutagenesis of strain NRRL B-1355 and subsequent strains did not alter the genotype of the mutants such that the identity at the species level of the microorganisms was changed. Inclusive of the parent NRRL B-1355 strain, all strains were identified as *L. citreum* and displayed a 0.28 – 1.78% difference in 16S rRNA sequence from the *L. citreum* sequence in the commercial database that was used.[11] Note that since Cargill never worked with strain NRRL B-21138, and although it appears in Figure 2, it was not included in this analysis and thus, does not appear in Table 6.

**Table 6. *Leuconostoc* parent and mutant strain properties and identification.**

| | Properties* | Identification | % Difference | Confidence Level |
|---|---|---|---|---|
| NRRL B-1355 | Natural isolate | *L citreum* | 1.78 | Species |
| NRRL B-21297 | AS overproducing | *L. citreum* | 0.28 | Species |
| NRRL B-30821 | Glucose-constitutive | *L citreum* | 0.28 | Species |
| NRRL B-30894 | AS overproducing | *L. citreum* | 0.28 | Species |

Mutants retained the previously obtained properties of the parent (*e.g.*, strain 30894 is also glucose-constitutive, in addition to producing more alternansucrase).

Both *L. mesenteroides* and *L. citreum* are frequently isolated in relatively high numbers from traditionally fermented food products that do not involve the use of starter cultures, and are assumed to be important in flavor and texture development (Kelly *et al.*, 1995; Valerio *et al.*, 2004). Both species also have long histories of safe use in food manufacture (Server-Busson *et al.*, 1999; Hemme and Foucaud-Scheunemann, 2004), for example the use of *L. citreum* as a starter culture in the production of certain cheeses (Sanchez *et al.*, 2006) and kimchi (a fermented cabbage product that is popular in Korea) (Choi *et al.*, 2003).

According to the FDA, changes in the taxonomic placement of an organism should not affect the ability to identify toxicity, pathogenicity, or use in the production of food or enzymes by the

[11] All sequencing work was carried out by Accugenix, Inc., an organization expert in microbial identifications and characterizations (http://www.accugenix.com).

organism of interest.[12] Hence, a re-classification of an organism does not necessarily make it unsafe for food use and information relevant to the organism prior to its re-classification is still valid. While Cargill's taxonomy work resulted in a re-classification of the strain of interest, the safety assessment should rest upon the properties of the parent strain, because the mutants have been shown to be taxonomically equivalent to the parent (Pariza and Foster, 1983; Pariza and Johnson, 2001).

Regarding this latter point, there is ample evidence supporting the safety of the parent strain, *L. citreum* NRRL B-1355, including, (1) its isolation by USDA in 1952 (*i.e.*, prior to enactment of the FD&C Act of 1958) (Jeanes, 1952; Jeanes *et al.*, 1954), (2) the historical use of *L. mesenteroides* and/or *L. citreum* in the manufacturing of foods and drug products (Server-Busson *et al.*, 1999; Hemme and Foucaud-Scheunemann, 2004; JGI, 2007; Rodrigues *et al.*, 2003; Kelly *et al.*, 1995; Valerio *et al.*, 2004; Sanchez *et al.*, 2006; Choi *et al.*, 2003), (3) its phylogenetic similarity to *L. mesenteroides,* which is GRAS-approved (Leisner *et al.*, 2005), and (4) the nonpathogenic nature of *Leuconostoc spp.* (Falkow, 1997). Thus, *L. citreum* NRRL B-1355 can be considered a safe organism and, because of the demonstrated direct strain lineage to *L. citreum* NRRL B-30894, this latter strain can also be considered safe.

5.1.1.2 Alternansucrase gene sequence

The alternansucrase genes from mutant strains NRRL B-30821 and NRRL B-30894 were sequenced from genomic DNA using polymerase chain reaction primers derived from the publicly available wild-type sequence (strain NRRL B-1355).[13] From sequence alignments, no differences exist among the three genes under comparison, in the coding portion, or in the 600 upstream and 430 downstream regions of the gene. Thus, mutagenesis of the alternansucrase-producing strain did not change the alternansucrase enzyme itself, at least regarding its primary sequence.

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[12] U. S. Food and Drug Administration. 58 FR. 27197-27199. May 7, 1993.
[13] http://www.ncbi.nlm.nih.gov/Genbank/ (Site visited 07 August 2007.)

5.1.1.3 Fermentation profile and morphology

*L. citreum* grows on glucose *via* heterolactic fermentation and the ratio of organic products produced is dependent upon the fermentation conditions. Under standard fermentation conditions for the production of enzyme, the mutant and parent *L. citreum* strains resulted in similar lactic acid and ethanol yields (Table 7). Consequently, under enzyme production conditions, there does not appear to be evidence of altered physiology of the mutant *L. citreum* strains compared to the parent strain. In addition, no morphological differences in the microbial cells are observed microscopically or in colony morphology.

**Table 7. Lactic acid and ethanol yields from glucose of mutant strains**

| Yields* | Mutant strains | | | |
|---|---|---|---|---|
| | NRRL B-1355 | NRRL B-21297 | NRRL B-30821 | NRRL B-30894 |
| $Y_{lactic}$ | 48% | 46% | 45% | 46% |
| $Y_{ethanol}$ | 24% | 24% | 23% | 22% |

* = yields calculated from fermentations with initial glucose values near 4%

5.1.2 Alternansucrase production

The alternansucrase fermentation process is accomplished by a liquid fermentation of a pure culture strain of *L. citreum* in a growth medium containing food-grade glucose, yeast extract, and salts at 25-30 °C for up to 30 hours. Agitation of the culture is provided and the pH is controlled near neutral with a base solution suitable for food manufacturing, such as sodium hydroxide. All equipment is operated, sanitized and maintained in order to prevent contamination by foreign microorganisms. Physical, chemical and microbial control measures are in place during the fermentation, and microbial analysis is performed to ensure absence of foreign microorganisms.

Each fermentation batch is initiated with a lyophilized culture of *L. citreum*. Cultures are checked for purity and the enzyme titer is evaluated prior to use in production. Samples are regularly taken during the course of production fermentations and are examined microscopically after plating on nutrient agar plates (incubated for 24-48 hours). In addition, growth parameters such as optical density and glucose utilization are monitored throughout the process to ensure consistency among batches. Fermentations are considered contaminated if two fermentation samples show the presence of non-production microorganisms by phase contrast microscopy or

by plate growth. All contaminated fermentations are rejected. The fermentation is considered complete when the glucose provided in the media is consumed.

The recovery process commences immediately following the fermentation process. Because alternansucrase is naturally secreted by *L. citreum*, the enzyme is recovered by removing the cells from the culture broth and by concentrating the enzyme *via* a series of filtration steps. Cell removal is accomplished by centrifugation or by microfiltration. Once cells have been removed, additional microfiltration is performed to remove cells and other culture debris. Then the cell-free broth is concentrated through an ultrafiltration unit and is polished through a series of descending pore size microfilters (smallest pore size 0.2 micron). The product is loaded into clean containers (drums, totes, etc.) in a concentrated, liquid form. Final products are examined according to the specifications outlined in Table 8.

All raw materials used in the fermentation and recovery processes conform to *Food Chemicals Codex* (FCC) (NAS, 2004) specifications and are standard ingredients used in the enzyme industry (Anonymous, 1994). The manufacturing of alternansucrase is conducted under cGMP and all locations follow the USDA's guidelines for HACCP (Hazard Analysis and Critical Control Point) processes.[14] Figure 3 provides an overview of the enzyme preparation method.

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[14] HACCP guidelines are available from the USDA website at http://foodsafety.nal.usda.gov/nal_display/index.php?info_center=16&tax_level=1&tax_subject=177. (Site visited 10 January 2008.)

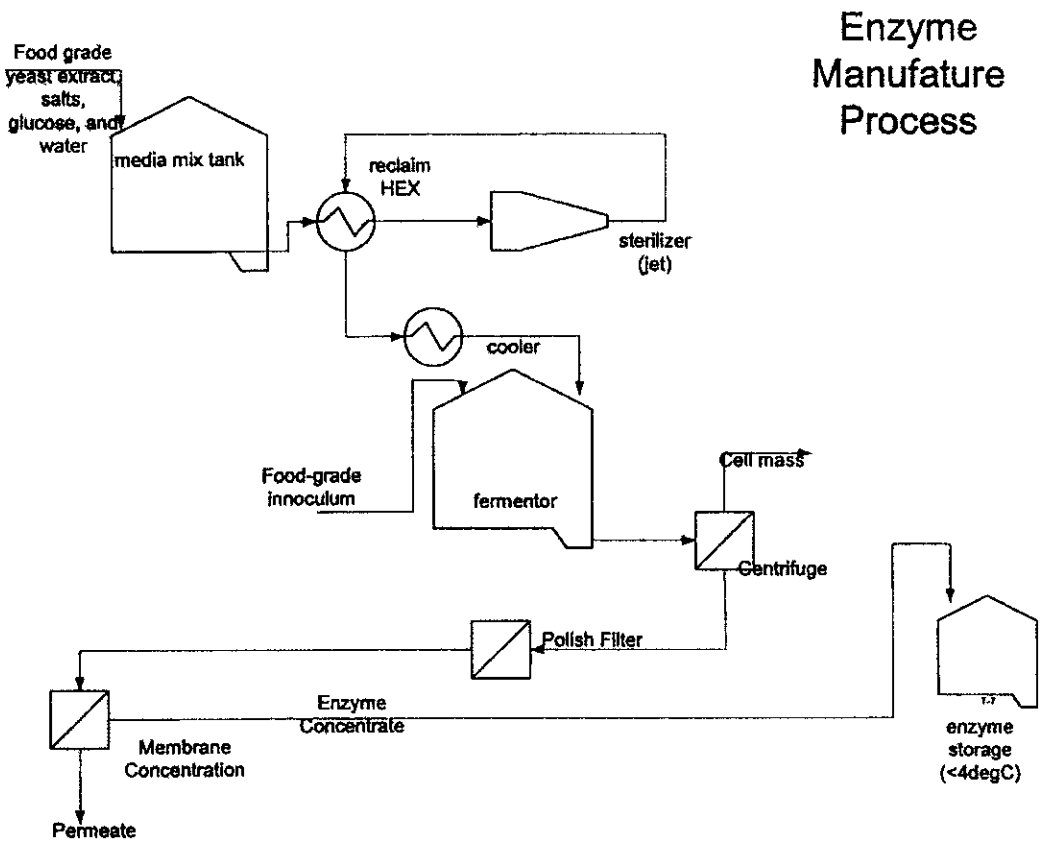


**Figure 3. Method of alternansucrase preparation**

5.1.3 Alternansucrase composition and specifications

Alternansucrase preparations are typically composed of water (88-97%), carbohydrate (<5%), ash (<3%), fat (<0.2%) and protein (<3%) and conform to specifications outlined for enzyme preparations in the FCC (NAS, 2004) and JECFA (2006a). Cargill's specifications for alternansucrase are presented in Table 8.

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**Table 8. Cargill specifications for alternansucrase**

| Parameter | Limit | Reference |
|---|---|---|
| Enzyme activity* | 20-100 T-1 U/ml | Cargill internal method LS-068-1[a] |
| Heavy metals | < 40 ppm | MN5013, 5012 (The National Food Lab)[b] |
| Lead | < 5 ppm | MN5013 (The National Food Lab)[b] |
| Arsenic | < 3 ppm | MN5013 (The National Food Lab)[b] |
| Total viable count/g | ≤10,000 | AOAC Method 966.23 |
| Yeast and mold/g | < 100 | BAM |
| Total coliform/g | ≤30 | BAM |
| Enteropathogenic *E. coli*/25 g | Negative by test | BAM |
| *Salmonella*/25 g | Negative by test | AOAC Method 2003.09 |
| Antibiotic activity | Negative by test | JECFA (2006a); Appendix A |
| Mycotoxins | Negative by test | Methods 000031-2, 000157-2, 000164-0 and 000266-1,Romer Labs[c] |

* = Typical levels are 40-60 T-1 U/ml, with one unit being equal to the release of 1 μM of fructose from sucrose *per* minute at 37°C, a = Determination of enzyme activity by T-1 acceptor assay. January 5, 2006; b = The National Food Lab Internal Methods for heavy metals by ICP-MS (*i.e.*, inductively coupled plasma mass spectroscopy); c = HPLC (high performance liquid chromatography) and TLC (thin layer chromatography) methods of Romer Labs (Union MO); AOAC = Association of Official Analytical Chemists; BAM = FDA Bacteriological Analytical Manual, 8th Ed, Chapters 3, 4 and 5, 1995

With the exceptions of enzyme activity and total viable count *per* gram of sucromalt, alternansucrase from Novozymes, Inc. has the same specifications as shown in Table 8. Novozymes, Inc. measures enzyme activity using the 2,4-dinitrosalicyclic acid (DNS) reducing sugar assay and will provide minimally 150 DNS units/g (Bernfield, 1955).[15] In addition, the total viable count specification for Novozymes' alternansucrase preparations is ≤50,000 *per* gram.

5.1.4 Use of alternansucrase

5.1.4.1 Mode of action

Alternansucrase alternatively transfers a D-glucosyl residue from sucrose to the 3 or 6 position of a non-reducing terminal residue of a D-glucan, resulting in glucans with alternating α-1,3 and α-1,6 linkages and free fructose.

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[15] Historically, T-1 activity units are approximately five times higher than DNS units. (Personal communication with Dr. Anton Woo, Senior Research Scientist, Cargill Health and Nutrition, 09 October 2007.)

5.1.4.2 Use levels

The recommended amount of alternansucrase to be used for each gram of sugar to be converted is 1-6 U.[16] Alternansucrase is used in accordance with cGMP during the normal production at minimum concentrations to achieve the desired effect.

5.1.4.3 Enzyme residues in the final sucromalt product

Although no formal specification exists for total residual protein in sucromalt syrup, Cargill has established an informal maximum specification of <62.5 ppm (<10 ppm Nitrogen).[17] Of that total residual protein, Cargill estimates that no more than 6 ppm can be actual enzyme protein.[18] If sucromalt were incorporated into final foods at 85% (*i.e.*, the maximum anticipated use level of sucromalt; see Table 10), the maximum residual protein derived from alternansucrase would be <5.1 ppm (*i.e.*, 6 x 0.85). However, this maximum would occur only in sweet sauces with lower maximum levels in all other food categories.

5.2 Sucromalt

5.2.1 Sucromalt production

Food-grade sucrose and food-grade maltose or high-maltose corn syrup are mixed at a specified ratio (optimally 8-11 to 1) and at concentrations between 40-50% before enzyme addition. The pH of the reaction is adjusted to 5.0-5.8 and may be maintained in this range by acid or base addition if necessary. The aqueous enzyme reaction is carried out at 37 °C to 47 °C with mild mixing for several hours. The reaction is sampled regularly to evaluate reaction progress and is considered finished when the amount of carbohydrate with two degrees of polymerization (*i.e.*, DP2) is less than 3% of the total carbohydrates, as measured, typically by an HPLC method. At that point, the sucromalt product is tested against the specifications shown in Table 9. The resulting syrup may be heated to inactivate the enzyme; however, it has been shown that the downstream processing deactivates the remaining enzyme. The syrup can be purified with

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[16] This use level applies equally to alternansucrase derived from either *L. citreum* or *B lichenıformis* The amount of alternansucrase used is only a factor of the desired speed on the reaction, not its efficiency.
[17] Cargill internal method LS-029-1. (Total nitrogen in sweeteners is measured by complete combustion of the sample in oxygen at high temperature.)
[18] This assumption is based on a 1 U/ml enzyme titer at 160 U/mg of specific activity. (Personal communication with Dr. Anton Woo, Senior Research Scientist, Cargill Health and Nutrition, 15 January 2008.)

decolorizing resin or activated carbon, or passed through cation and anion exchange resins to remove any protein, color, and ions. Combinations of these resins can be used based upon the ion load of the syrup. The purified syrup is evaporated under vacuum to obtain 75 to 80% dry solids. All processing aids are food-grade and are similar to those used in typical corn syrup production. Figure 4 provides an overview of sucromalt production.

***Sucromalt Process Flow***

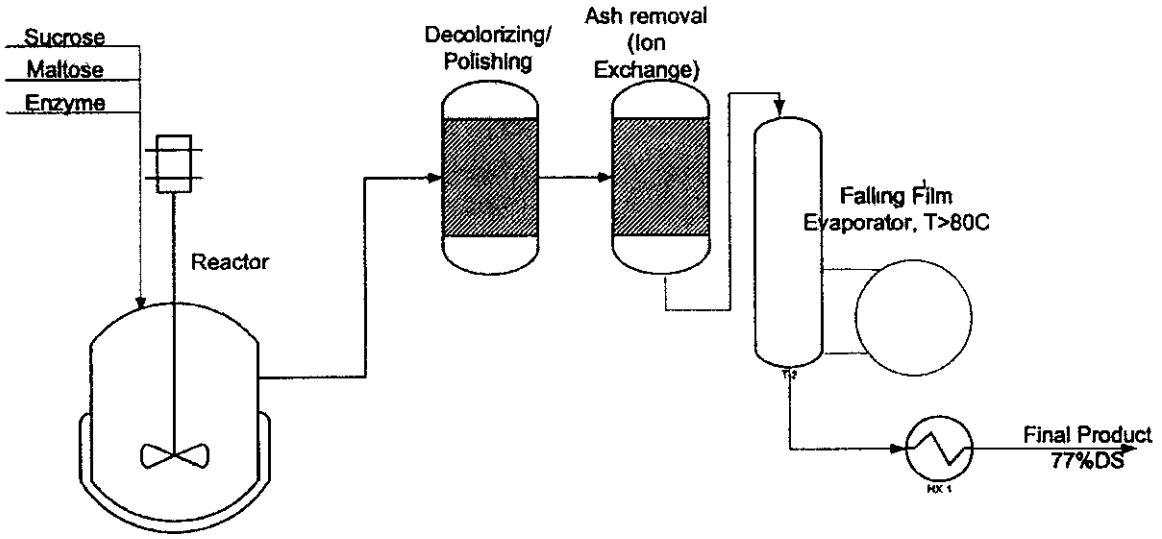


**Figure 4. Overview of the sucromalt production process**

5.2.2 Process controls

The processing steps, the facility, and the controls used in the manufacturing of sucromalt conform to cGMP for human food in accordance with the applicable parts of 21 CFR, Part 110. As a food manufacturer, Cargill plants are regulated by the FDA and are subject to FDA inspections. Cargill performs microbiological testing on select lots of sucromalt in accordance with the Cargill business unit HACCP (Hazard Analysis and Critical Control Points) plan. Testing includes assays for mesophilic bacteria, yeast, and mold, and is performed monthly. Pesticides and heavy metal analyses are performed annually.

5.2.3 Control of alternansucrase in sucromalt

Although the manufacturing process described above is thought to effectively eliminate alternansucrase from sucromalt, its absence has not been shown definitively. However, several items of evidence suggest that if alternansucrase were present in sucromalt, it would be at extremely low levels, if present at all. First, the calculations described in section 5.1.4.3 indicate that the maximum residual protein in sucromalt that could be derived from alternansucrase is 6 ppm. Also, when finished sucromalt is combined with the sucrose-maltose reaction mixture under conditions of manufacturing, no additional sucromalt is produced; leading to the conclusion that active alternansucrase is absent from finished sucromalt. Another item of supportive documentation is provided by the results of polymerase chain reaction (PCR) testing conducted by Novozymes. This testing showed that for the promoter gene sequence responsible for the production of alternansucrase was not present when *B. licheniformis*-derived alternansucrase was analyzed by PCR.

5.2.4 Sucromalt chemical and physical characteristics and stability

Sucromalt is non-crystallizing and provides sweetness and body in various food products. A typical analysis of sucromalt, including carbohydrate profile,[19] chemical/physical properties and sensory characteristics, is shown in Table 9. The carbohydrate Analytical data generated in Cargill's Research and Development lab from five separate non-consecutive sucromalt batches, are also shown in this table and demonstrate that all batches were within the limits of a typical analysis, an illustration of consistent sucromalt manufacturing.[20] Regarding stability, sucromalt

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[19] There are no addition constituents in sucromalt other than carbohydrates. Thus, the carbohydrate profile represents sucromalt in its entirety.

[20] Table 9 represents specifications that will be met for all manufactured batches; however, additional specification testing may be completed quarterly or yearly, or they may be run *per* specific customer requirements, *e.g.*, % sulfated ash, conductivity (µOhms), heavy metals (Arsenic, Cadmium, Mercury, Lead; ppm), pesticide residues and microbiology. Regarding microbiology, less frequent testing evaluates standard plate counts (specification: < 5000 cfu/g) as well as counts of yeast and mold (each with a specification of < 100 cfu/g). Those microbiology studies are run *via* methods developed by the Corn Refiners Association (CRA), a national trade association representing the U.S. corn refining (wet milling) industry. Members of that industry routinely use methods developed and endorsed by CRA's technical committee. Cargill is currently developing data expected to validate that CRA-method results are similar to results obtained when using FDA's BAM (Bacteriological Analytical Manual) and/or methods recommended by AOAC (Association of Analytical Chemists). At the present time; however, CRA methods are used for the analysis of sucromalt for total plate count, yeast and mold. Cargill does not routinely run pathogen testing on sucromalt because its osmotic pressure, *percent* dissolved solids and low water content do not support pathogen growth, conditions that are similar to other commercially available corn syrups that are commonly

has been shown stable from pH 2 to 8 at temperatures up to 121°C (retort condition). When stored for six months at 32.2°C or 37.8°C, sucromalt is stable in terms of microbial counts, carbohydrate profile, pH and dry solids; and sucromalt-containing food products, *i.e.*, lemon sorbet (pH>4.6), chocolate meal replacement drink (pH<4.6), and chocolate mint bar (pH>4.6), have also been demonstrated stable for at least 6 months when stored at 4°C. In addition, carbonated energy drink (pH<4.6) was stable in an accelerated shelf-life study *via* storage at 40°C.

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consumed by humans every day (Cargill, 2007b; CRA, 2000). However, if specifically requested by a customer to run pathogen analysis, that testing will be completed.

**Table 9. Typical analysis of sucromalt, including carbohydrate profile, chemical/physical properties and sensory characteristics of sucromalt**

| Parameters | Specification | Analytical results (batch designations) | | | | |
|---|---|---|---|---|---|---|
| | | DYSXM042407A* | 433PO701* | 433PO702* | H7M537** | H7A124** |
| | | | Carbohydrate profile | | | |
| Fructose (%dry) (Cargill method LS-030-1)[d] | 35-45 | 39 | 39 | 41 | 39 | 39 |
| DP2 (%dry)[a] (Cargill method LS-030-1)[d] | ≤5 | 3 | 3 | 3 | 4 | 3 |
| Leucrose (%dry) (Cargill method LS-030-1)[d] | 7-15 | 11 | 9 | 9 | 13 | 12 |
| Higher saccharides and polymer (%dry)[b] (Cargill method LS-030-1)[d] | >40 | 48 | 49 | 48 | 46 | 46 |
| | | | Chemical/physical properties | | | |
| Brix (%)[c] (Cargill method DS LS-002-1)[e] | ≥70 | 78 | 78 | 79 | 79 | 77 |
| Moisture (%) (*via* calculation)[f] | ≤30 | 22 | 22 | 21 | 21 | 23 |
| pH (Cargill method LS-006-1)[g] | 3.5-6.0 | 4 | 4 | 4 | 4 | 5 |
| Sulfur dioxide (ppm) (Cargill method LS-012-1)[h] | 5 max | 1.4 | <1 | <1 | <1 | 1.30 |
| | | | Sensory characteristics | | | |
| Appearance | Clear to light yellow liquid with some cloud | Clear | Clear | Clear | Clear | Clear |
| Taste (Cargill method QAHM-SOP-GL0027)[i] | Sweet, bland | Sweet | Sweet | Sweet | Sweet | Sweet |
| Odor (Cargill method QAHM-SOP-GL0027)[i] | Characteristic | Characteristic | Characteristic | Characteristic | Characteristic | Characteristic |

* = these batches were manufactured with alternansucrase derived from Novozymes' *Bacillis licheniformis* organism; ** = these batches were manufactured with alternansucrase derived from the Cargill-developed *Leuconostoc citreum* organism; DP = degrees of polymerization; a = DP2 may encompass monosaccharide (*i e.*, unreacted glucose) and disaccharides (*e g.*, unreacted sucrose and maltose and possibly sucrose isomers such as isomaltulose); b = higher saccharides and polymer encompasses saccharides with ≥DP (*i.e.*, DP3+); c = Brix signifies sucromalt content; Cargill internal method descriptions: d = "Saccharides by liquid chromatography "; e = "% Dry substance and Baume of starch hydrolysate and sweeteners by refractometer."; f = moisture is not an analyzed endpoint but simply calculated as (100% – dry substances, which are composed of total solids, sulfated ash and carbohydrates); g = "pH of brewing syrups."; h = "$SO_2$ in finished sweeteners (10ppm or less)."; i = "Organoleptic evaluation of food grade products."

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## 6.0 ESTIMATED DAILY INTAKE

To determine the hypothetical maximum daily human intake of sucromalt as an added food ingredient, a consumption analysis database[21] was used to calculate (1) mean *per capita*, (2) mean "eaters-only", and (3) 90th percentile "eaters-only" daily sucromalt consumption when it is added to the foods identified in Appendix 2, which represent the food categories identified in Table 10 (Burdock Group, 2008).

In a 2003-2004 nationwide survey conducted over two days, participants answered comprehensive and detailed questions about their consumption of food during the previous 24 hours, and the results were used to code individual foods and portion sizes that were consumed. The number of participants on Day 1 of the survey was 9034; there were 8354 participants on survey Day 2 (Total = 17,388). These survey results were weighted to place more strength on foods that were consumed by more individuals and then were extrapolated to the entire U.S. population. The collected data represents consumption of foods specified by the study participants only, not necessarily those foods consumed by the entire U.S. population. Thus, the "eaters-only" analysis represents the amount of sucromalt in foods consumed only by the study participants. In contrast, the *per capita* analysis represents the amount of sucromalt consumed in foods, weighted by foods most consumed and then extrapolated to the entire U.S. population. The foods identified in Appendix 2 were selected by Cargill as representing those in which sucromalt can be used as an ingredient to replace other nutritive carbohydrates (*e.g.*, cane and beet sugars, high fructose corn syrup, glucose syrups and dextrose) that are currently consumed in the U.S., and includes the food categories in which it is possible that sucromalt will be used. Those categories are presented in Table 10, along with the range of expected sucromalt *percentages* in each.

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[21] U.S. Department of Agriculture, Agriculture Research Service (2006) What We Eat In America, NHANES 2003-2004: Documentation and Data Files. Available: http://www.ars.usda.gov/ba/bhnrc/fsrg and http://www.cdc.gov/nchs/about/major/nhanes/nhanes2003-2004/nhanes03-04.htm.

**Table 10. Food categories in which sucromalt is intended to be used**

| Requested Food Categories | Concentration* |
|---|---|
| Baked Goods | 1.5-50% |
| Beverages, Non-alcoholic | 2-45% |
| Beverages, Alcoholic | 5-15% |
| Breakfast Cereals | 5-30% |
| Chewing Gum | 15% |
| Condiments and Relishes | 5-25% |
| Confectionary and Frostings | 20-40% |
| Frozen Dairy | 9-20% |
| Fruit Ices | 12.5-35% |
| Gelatins and Puddings | 8-18% |
| Hard Candy | 10-30% |
| Dairy Products (and non-dairy analogs) | 5-8% |
| Jams and Jellies | 12-60% |
| Milk | 3% |
| Milk Products | 3-15% |
| Processed Fruits and Fruit Juices | 5-20% |
| Snack Foods | 5-10% |
| Soft Candy | 5-40% |
| Sweet Sauce | 8-85% |
| Salad Dressings | 8-20% |

* = food categories are those shown in Appendix 2.

For the consumption estimate, Cargill provided the maximum anticipated sucromalt use concentrations for the foods in each category. (See Appendix 2 for those maximum concentrations in each food.) The overall results of the consumption analysis are shown in Table 11 and the age-group breakdown of mean, weighted, eaters-only consumption is shown in Table 12.[22]

**Table 11. Estimated consumption of sucromalt from food categories specified in Appendix 2**

| | mg/day | mg/kg/day* |
|---|---|---|
| Mean *per capita* | 52,920 | 890** |
| Mean, weighted, eaters-only*** | 64,850 | 1090*** |
| 90th Percentile, weighted, eaters-only*** | 147,600 | 2540*** |

* = these values are rounded off to the nearest 10 from the exact estimates shown in APPENDIX 3, ** = body weight in this calculation is based on the general assumption of the average person in the U.S. population weighing 60 kg; *** = the body weights used in these calculations were based on respondent ages, derived from Portier *et al.* (2007); *** = estimates are based on individuals in the U.S. that consume the foods identified in Table 10, *i e*, eaters-only In the 2003-2004 survey, 466,778,866 individuals consumed the targeted foods

[22] The software used to create the consumption estimates in Table 12 provides the age-group breakdown only for the estimated mean, weighted, eaters-only consumption. Overall, 48.7% of "eaters" were male and 51.3% were female; however, no additional breakdown by sex was provided. (Personal communication with Dr. Ray Matulka, Assistant Director of Toxicology, Burdock Group, 18 October 2007.)

**Table 12. Estimated mean, weighted eaters-only consumption of sucromalt broken down by age groups.**[23]

| Age groups | | | Consumption | |
|---|---|---|---|---|
| Age range (yrs) | Average weight (kg)* | # of eaters | mg/day** | mg/kg/day** |
| 1-2 | 11.4 | 1022 | 31,300 | 2750 |
| 3-5 | 18.2 | 947 | 48,150 | 2660 |
| 6-11 | 33.5 | 1708 | 71,430 | 2130 |
| 12-19 | 64.7 | 4035 | 88,990 | 1370 |
| 20-29 | 77.6 | 1456 | 93,580 | 1200 |
| 30-39 | 81.4 | 1326 | 74,840 | 920 |
| 40-49 | 82.5 | 1317 | 63,340 | 770 |
| 50-59 | 82.9 | 981 | 49,720 | 600 |
| 60-69 | 81.5 | 1267 | 36,950 | 460 |
| 70+ | 73.7 | 1766 | 33,090 | 450 |
| | | $\Sigma$=15,822 | | |

* = body weights in this column are derived from Portier *et al.* (2007); **= these values are rounded off to the nearest 10 from the exact estimates shown in APPENDIX 4.

For dietary sugars in general, the mean *per capita* intake can be estimated from agricultural commodity consumption tables published by the U.S. Department of Agriculture (USDA). In 2005, USDA reported that the *per capita* consumption of caloric carbohydrates – this includes total cane and beet sugar, high fructose corn syrup, glucose syrups and dextrose - was 140.2 lbs/yr.[24] Converting the units from lbs/yr to mg/kg/day yields a *per capita* yearly consumption for these caloric carbohydrates of 2933 mg/kg/day; this value is comparable to the estimated mean *per capita* intake from Table 11 of 890 mg/kg/day. Thus, the estimated mean *per capita* daily intake of sucromalt, which is intended to replace those various carbohydrates in certain foods, represents approximately 30% of their estimated total *per capita* yearly intake, *i.e.*, 890/2933, or 30.3%.

It should also be noted that the consumption estimates in Table 11 are quite conservative and encompass all of the foods to which sucromalt could be added. However, because the cost of sucromalt is expected to be greater than the carbohydrates it can replace, it is most likely that sucromalt will find actual use as a replacement carbohydrate in less than half of the food categories shown in Table 10.

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[23]The sum of eaters identified in Table 12 (*i.e.*, 15,822) is less than the total number of survey participants (*i.e.*, 17,388) because only that many participants reported eating the foods identified in Appendix 2.

[24] United States Department of Agriculture, Economic Research Service. Food availability (*per capita*) data system. (http://www.ers.usda.gov/data/foodconsumption/FoodAvailSpreadsheets.htm#sweets. Site visited 18 October 2007.)

# 7.0 SAFETY

## 7.1 Overview

The digestion and absorption characteristics of sucromalt components and breakdown products are well understood and are described in the published scientific literature as well as in standard biochemistry textbooks. These historical data are further supported by the results of nonclinical and clinical studies employing sucromalt. The results of *in vitro* and *in vivo* digestibility studies indicate that sucromalt is well digested. Sucromalt exhibited no mutagenic potential in the Ames bacterial mutagenicity assay. Similarly, no signs of toxicity were evident following 28 days of dietary sucromalt administration to female rats at 19,800 mg/kg/day and to male rats at 18,560 mg/kg/day. No signs of malabsorption (measured *via* breath hydrogen) were observed in a clinical investigation in which healthy subjects received single doses of 50 g or 80 g sucromalt.

## 7.2 Carbohydrate kinetics

Monosaccharides, disaccharides and oligosaccharides are natural carbohydrate components of the human diet and are important sources of metabolic energy. The mechanisms by which carbohydrates are digested, absorbed, and metabolized are well understood. Following ingestion, the monosaccharides glucose and fructose readily absorb across the intestinal epithelium into the circulation by passive diffusion and/or active transport mechanisms. Once in the blood, they are distributed throughout the body for cellular metabolism *via* glycolysis (Figure 5) followed by eventual conversion to metabolic energy in the citric acid cycle (Stryer, 1988). Disaccharides and oligosaccharides require conversion by hydrolysis to monosaccharides prior to absorption. With respect to sucromalt, fructose will be directly available for absorption and metabolism while leucrose and higher oligosaccharides must first be hydrolyzed to their constituent monosaccharides. The nature of those substances dictates that the monosaccharides will be predominated by glucose with a relatively small amount of fructose generated (from leucrose). Early work by Larner and Gillespie (1956) reported on the enzymatic hydrolysis of nigerose (*i.e.*, an α-1,3-linked glucose-to-glucose disaccharide)[25] by a human intestinal extract. A few years later, Dahlqvist (1962) used homogenates of human jejunal and ileal mucosa to demonstrate

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[25] Online medical dictionary. http://cancerweb.ncl.ac.uk/cgi-bin/omd?nigerose. (Site visited 11 April 2008.)

specificity of eleven different human intestinal disaccharidases for known disaccharides, including maltase (for maltose, the α-1,4-linked glucose-to-glucose disaccharide in sucromalt) and isomaltase (for isomaltose, the α-1,6-linked glucose-to-glucose disaccharide in sucromalt). Thus, it has been over 50 years since the scientific literature first demonstrated the presence of hydrolytic enzymes in humans that are specific for the glycosidic linkages found in sucromalt oligosaccharides. Evidence for the enzymatic cleavage of leucrose is found in cariogenic research as well as in studies targeting mammalian metabolism. In a cariogenicity-associated study, Gülzow and Polihronu (1990) demonstrated the breakdown of leucrose's α-1,5-glucose-to-fructose linkage in human saliva. Those findings supported the earlier work of Ziesenitz *et al* (1989), in which the enzymatic cleavage of leucrose was observed using carbohydrase complexes isolated from human jejunal mucosa. A more detailed summary of the carbohydrase-related work of Ziesenitz *et al.* (1989) is found later in this dossier in section 7.3.2.1 (Page 40). The works of Gray *et al.* (1979) and Brunner *et al.* (1979), offered evidence suggesting that the sucromalt-linkage-specific disaccharidases may be associated with the active sites on a sucrase-isomaltase complex contained in the intestinal brush border membrane. Gray *et al.* (1979) showed that the glucose units in an α-dextrin hexasaccharide are removed by the composite sucrase-isomaltase, sequentially yielding single glucose units and saccharides of one less glucose unit. Because the glucooligosaccharides in sucromalt have the same type of α-1,4- and α-1,6-glucose-to-glucose linkages as are in the α-dextrin hexasaccharide evaluated by Gray *et al.* (1979), it is most likely that the process of sequential glucose unit removal also occurs following ingestion of sucromalt. Further support for this or a very similar mechanism is found in several studies summarized in later sections of this dossier where the metabolisms of leucrose and sucromalt are both shown to yield blood glucose curves that are effectively identical to the blood glucose curves of sucrose. The further metabolism of liberated glucose and fructose eventually yields pyruvate, which is oxidatively decarboxylated to form acetyl CoA, leading to condensation with oxaloacetate to form citrate in the citric acid cycle and the eventual yield of metabolic energy (Stryer, 1988). It is possible, albeit unlikely, that small amounts of glucooligosaccharides will move into the large intestine before being hydrolyzed. In that scenario, they could be used as substrates by intestinal microbes or eliminated in the feces. However, human studies summarized in later sections demonstrate that if this occurs, it would be without any detectable adverse effect.

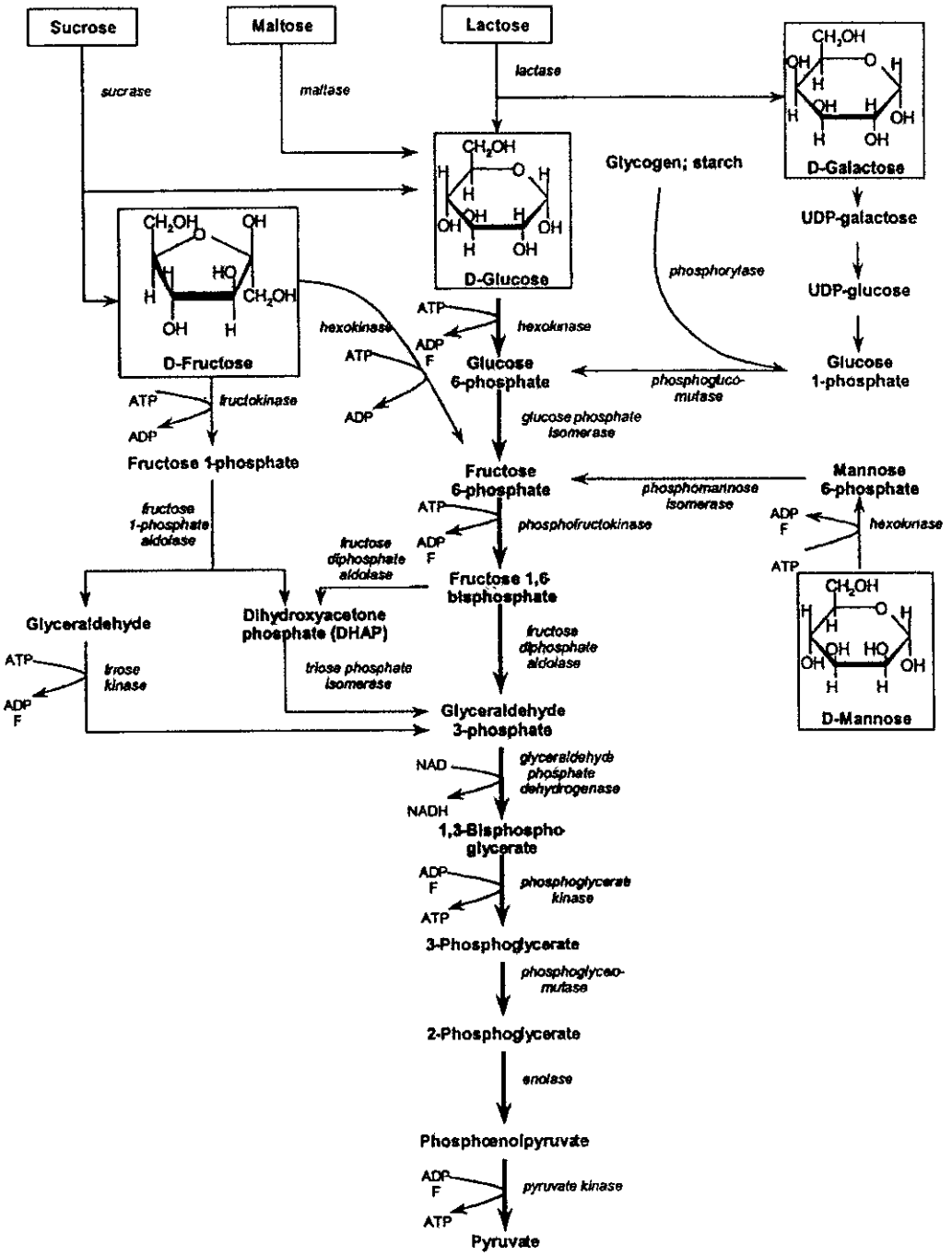


**Figure 5. Glycolysis pathway (BioCarta;[26] King[27])**

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[26] Feeder Pathways for Glycolysis. Available on-line through: http://www.biocarta.com/index.asp.
[27] King, M.W. Metabolism of Major Non-Glucose Sugars and Glycolysis. The Medical Biochemistry Page. Indiana State University School of Medicine. Available on-line through: http://www.indstate.edu/thcme/mwking/home.html.

7.3 Carbohydrate safety

7.3.1 Fructose

Because of its everyday consumption from natural sources and *via* high-fructose corn syrup (at 42 to 55%),[28] the general safety of fructose consumption among humans is well accepted. In healthy individuals, ingested fructose is readily absorbed by the intestinal mucosa through normal physiological pathways. Fructose obtained from the hydrolysis of sucrose or directly from the diet undergoes hepatic conversion to fructose 1-phosphate *via* fructokinase. In other tissues, fructose is converted to fructose 6-phosphate by hexokinase. Although the pathways differ slightly, fructose 1-phosphate and fructose 6-phosphate are ultimately converted to glyceraldehyde 3-phosphate, an important intermediary metabolite in glycolysis (Stryer, 1988).

However, fructose malabsorption and intolerance can occur in individuals with certain hereditary metabolism disorders caused by a deficiency or lack of fructose 1-phosphoaldolase (aldolase) in liver, kidney, and/or small intestine. Individuals with this affliction develop hypoglycemia, nausea, vomiting, irritability, and tremor after fructose ingestion and prolonged fructose exposure may lead to jaundice, hepatomegaly, hepatic failure, convulsion, coma and possibly even death. Another inherited condition that might result in fructose intolerance is a deficiency of fructose 1,6-bisphosphatase, which catalyzes the conversion of fructose 1,6-phosphate to fructose 6-phosphate. This disorder is characterized by episodes of fasting hypoglycemia, ketosis, and lactic acidosis; it can be fatal in newborns (Ravich *et al.*1983; Kneepkens *et al*, 1984; Truswell *et al.*, 1988; Riby *et al.*, 1993). Yet another fructose-associated infirmity is essential fructosuria, a benign metabolic disorder occurring in approximately 1 out of 130,000 individuals that results from a genetic defect of fructokinase. Aside from abnormally high urinary fructose, the condition is generally asymptomatic and may go undetected (The Merck Manual, 1999). Although these disorders occur in a small subset of the human population, those individuals would be considered a risk group for sucromalt consumption. However, sucromalt would not represent a risk beyond that associated with high-fructose syrups already on the market.

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[28] GRAS-affirmed by FDA for direct addition to food under 21 CFR §184.1866,

Regarding carbohydrate malabsorption in general, lowered digestive tolerance of an ingested carbohydrate can result in its malabsorption and this can be viewed as applicable to fructose. However, in multiple studies, fructose has been ingested by humans alone or in combination with glucose with results showing a direct correlation between amount of ingested glucose and greater fructose absorption capacity, and in the absence of glucose, many subjects could not completely absorb fructose at doses of 20-50 grams, amounts commonly contained in products sweetened with high fructose corn syrup or crystalline fructose (Ravich *et al.*, 1983; Kneepkens *et al.*, 1984; Rumessen and Gudmand-Høyer, 1986, 1988; Truswell *et al.*, 1988; Riby *et al.*, 1993). Rumessen and Gudmand-Høyer (1986) and Smith *et al.* (1995) have shown that glucose actually facilitates fructose transport across the small intestine brush border membrane *via* a glucose-dependent facilitated transport mechanism, thereby increasing the rate of fructose absorption. Based on these studies, it is then logical to propose that during sucromalt digestion, when both glucose and fructose are released for simultaneous gut absorption, there will be no expectation that the fructose moiety of sucromalt will be malabsorbed. This expectation is fully supported by the results of clinical studies reported below.

7.3.2 Leucrose

Ziesenitz *et al.* (1989) conducted a nutritional assessment of leucrose that included the evaluation of, (1) *in vitro* carbohydrase activity, (2) growth and metabolism in rats, (3) the effects associated with intravenous (*i v.*) leucrose (and sucrose) injection in rats, and (4) blood glucose responses in humans. This study was undertaken to determine whether leucrose's (α-1,5) glucose-to-fructose glycosidic linkage (see Figure 1) might impart nutritional properties that are different from its monosaccharide constituents. Later, Elias *et al.* (1996) presented a comprehensive safety profile of leucrose in which the results from a wide array of studies were reported, including those from Ziesenitz *et al.* (1989). The findings summarized in Elias *et al.* (1996) included results from (1) metabolism studies in humans; (2) pharmacokinetic studies in rats, dogs and humans; (3) subchronic studies in rats and dogs; (4) embryotoxicity studies in rats and rabbits; and (5) four genotoxicity studies. The reported findings of Ziesenitz *et al.* (1989) are summarized in the following four sections. Ensuing after those sections are summaries of the findings reported by Elias *et al.* (1996).

7.3.2.1 Carbohydrase activity on leucrose

In the *in vitro* portion of their leucrose evaluation, Ziesenitz *et al.* (1989) examined the enzymatic cleavage of leucrose, leucritol (*i.e.*, hydrogenated leucrose), sucrose and maltose using a mixture of carbohydrase complexes prepared from human jejunal mucosa.[29] As endpoints for enzymatic cleavage, the authors used $K_m$ (mM/l) as the rate of cleavage, which was associated with affinity,[30] and $V_{max}$ (mM/(min x mg protein)) as the maximum metabolic rate.[31] The results are shown in Table 13 and demonstrate that the enzyme mixture had affinity for the test substances and participated in their respective metabolisms.

**Table 13. Cleavage of leucrose, leucritol, sucrose and maltose by a complex of carbohydrase from human jejunal mucosa**

| | Activity by human carbohydrase complex | |
|---|---|---|
| Sugar substrate | Cleavage rate (mM/l) | Maximal metabolic rate (μM/min x mg protein) |
| Maltose | 5.9 | 609 |
| Sucrose | 17 | 304 |
| Leucrose | 12 | 190 |
| Leucritol | 25 | 21 |

$K_m$ = rate of glycosidic linkage cleavage; $V_{max}$ = maximum metabolic rate

7.3.2.2 Effects on growth and metabolism in rats

Ziesenitz *et al.* (1989) also examined the effect of leucrose on the growth of male Sprague-Dawley rats compared to growth effects of sucrose and cornstarch. Groups of six rats each were fed a basal diet supplemented with 25% (by weight) leucrose, sucrose, or cornstarch for six weeks; the energy contents of each diet were nearly identical (18.68-18.96 kJ/g dry matter). Body weight was measured in all animals before and after exposure, and three times each week during exposure, for calculation of protein efficiency ratio (grams of weight gain/grams of protein ingested) and energy efficiency ratio (grams of weight gain/energy intake in mJoules). Following the exposure phase, the weights of liver, kidneys, empty stomach, small intestine and

[29] Ziesenitz *et a.*, (1989) also examined the cleavage properties of a yeast α-glucosidase preparation; however, the results are not applicable to human risk assessment and are not presented in this dossier.
[30] A lower cleavage rate correlates with higher affinity because less enzyme is required to cleave the glycosidic linkage.
[31] It is recognized that $K_m$ is typically considered the Michaelis constant, which is related to substance concentrations and expressed in molar quantities (*e.g.*, mM or μM) (Stryer, 1988). Ziesenitz *et al.* (1989) did not explain why $K_m$ was chosen to represent cleavage rate.

caecum (with and without contents) were determined. The results of this investigation showed no effects on body weight gains, protein or energy efficiency ratios, and relative kidney, liver and small intestine weights. Empty stomach and full caecum weights were different between the groups fed leucrose and cornstarch ($P \leq 0.05$) but there were no differences in these parameters when diets containing leucrose and sucrose were compared (Ziesenitz *et al.*, 1989).

7.3.2.3 Effects of *i.v.* leucrose and sucrose administration on rats

In a third segment, Ziesenitz *et al.* (1989) administered intravenous (*i.v.*) doses of aqueous leucrose (1 g/2 ml water), aqueous sucrose (1 g/2 ml water) or saline (control, 2 ml) to groups of six anesthetized female Cara rats. The test substances were infused over a 15-minute period before the rats were transferred to metabolism cages for collection of urine and feces over four days. Based on assumptions of, (1) an extracellular compartment of 42 ml/250 g body weight of rat, (2) a rat weight of 255 g, and (3) injection of one g leucrose or sucrose, Ziesenitz *et al.* (1989) estimated that the leucrose or sucrose concentrations in the intracellular water after 15 minutes of administration would have been approximately 30 mM/l. During that time, food/water consumption and body weights were measured. Urine was analyzed for concentrations of glucose, sucrose, leucrose, sucrose and constituent monosaccharides, while only fecal weight was measured. There were no treatment-related effects on body weight, urine volume, or fecal weight and neither glucose nor fructose was identified in urine. The amounts of leucrose and sucrose excreted in urine within 24 hours were approximately equal, *i.e* , 681 ± 353 mg and 675 ± 275 mg, respectively;[32] thus suggesting a common metabolic process.[33] The ratio of water to food consumption was not different between groups before injection but following injection; trends toward increased water consumption and decreased food consumption were noted for both the leucrose- and sucrose-injected groups (Ziesenitz *et al.*, 1989).

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[32] Urinary leucrose and sucrose were determined by high performance liquid chromatology and/or gas chromatography (Ziesenitz *et al.*, 1989).

[33] Because leucrose and sucrose were administered intravenously rather than enterally, these measurements constituted test substances that had not been hydrolyzed/cleaved. Thus, they were unchanged (Ziesenitz *et al.*, 1989).

7.3.2.4 Human blood glucose following leucrose intake

Ziesenitz *et al.* (1989) also fed 100 grams of either leucrose or sucrose (both in 400 ml water) to twelve fasted healthy male and female volunteers (6/sex) and collected blood samples for analysis at 0.25, 0.75, 1, 1.5, 2, 2.5, 3, 4 and 5 hours following consumption. Analytical parameters included plasma glucose, fructose, insulin and C-peptide. No symptoms of intestinal discomfort were reported. Peak insulin and C-peptide levels were not significantly different between sucrose and leucrose; however, peak plasma glucose tended to be lower following leucrose ingestion compared to sucrose ingestion. Peak values of blood fructose were significantly lower ($P = 0.0066$) with its elimination half-life ($t_{½}$) being longer ($P = 0.0238$) after leucrose consumption compared to sucrose consumption.

Based on their overall study results, Ziesenitz *et al.* (1989) concluded that digestion of leucrose in the small intestine is comparable to that of sucrose, and that both are generally complete in healthy persons. Leucrose appeared to be well digested and tolerated by both humans and rats. In addition, although the intravenous study did not address metabolic utilization *per se*, it helps to support the safety of leucrose because no obvious adverse reactions were observed in any organ following exposure to approximately 30 mM leucrose or sucrose *per* liter of intracellular water.

7.3.2.5 Metabolism studies in humans

Elias *et al.* (1996) reported the results of two unpublished human metabolism studies of leucrose conducted in 1988 and 1989 at Biodesign Institute in Freiburg, Germany. In the 1988 study, seven male and three female subjects (19-30 years and 26-28 yrs, respectively) consumed diets containing either 265 g leucrose or 250 g sucrose as the only carbohydrate source.[34] Each diet was consumed for seven consecutive days, separated by a single seven-day rest period. On days 1 and 7 of each feeding period, blood glucose and fructose were measured eleven times; blood insulin concentrations were also measured eleven times but only on day 7. The results of these measurements were used to compare maximum blood concentration and AUCs of glucose, fructose and insulin between the leucrose and sucrose treatments. The results of this investigation revealed no differences between treatments.

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[34] A greater amount of leucrose was fed to compensate for 6% that was bound-up in water.

In the 1989 Biodesign Institute study described by Elias *et al.* (1996), twelve subjects (ages 37-73) with Type II diabetes consumed single oral doses of leucrose (53 and 106 g) and sucrose (50 and 100 g). Blood was drawn at 0, 0.5, 1, 1.5, 2, and 4 hours following exposure and examined for glucose, fructose, insulin and C-peptide concentrations. The results showed slower rates of blood glucose and fructose increase after leucrose consumption compared to sucrose consumption, which was reflected in statistically significant lower peak blood insulin following leucrose consumption. No additional experimental details were provided (Elias *et al.*, 1996).

7.3.2.6 Pharmacokinetic studies in rats, dogs and humans

Elias *et al.* (1996) also summarized the findings of an unpublished rat pharmacokinetics study of leucrose (rats strain unspecified). In an oral bioavailability study, twelve males were each injected *i.v.* with 10 mg/kg bwt leucrose and nine males were each administered leucrose *via* gavage at 1000 mg/kg bwt. At unspecified intervals, blood was drawn from the rats in these two groups for analysis of plasma leucrose. A maximum plasma leucrose concentration of 1.3 mg/l was reached after one hour, a finding that led to a calculated absolute bioavailability of 0.11%. The plasma half-life following *i.v.* injection was 16.1 minutes with the volume of distribution being 0.2 l/kg. In another phase of this investigation, groups of twelve males were administered the same *i.v.* and gavage doses of leucrose followed by urine collection at unspecified time points over 24 hours for measurement of urinary leucrose. This experiment showed a 24-hour urinary excretion of 0.08% following the oral dose whereas urinary excretion was 74% following the *i.v.* dose. The results of these blood and urine investigations shows that, despite high oral doses, only minute amounts of leucrose entered the systemic circulation, thereby suggesting gastrointestinal breakdown into its constituent monosaccharide units followed by complete conversion to metabolic energy *via* glycolysis and the citric acid cycle (Elias *et al.*, 1996).

Elias *et al.* (1996) additionally summarized a human crossover study where six subjects (age and sex distribution unspecified) consumed 70 g leucrose and 67 g sucrose in single doses with urine collected during three intervals, 0-6 hrs, 6-12 hrs and 12-24 hrs. Within 24 hours, up to 57% of leucrose and 47% sucrose had been excreted in the urine, with approximately 94% of leucrose and 57% of sucrose excreted within the first six hour urine collection interval. Although the method of measurement was not specified, it is unlikely to have involved direct measurements of these two disaccharides because an additional statement mentioned that the calculated totals of

unchanged leucrose and sucrose were approximately 0.05-0.06% of the administered doses (Elias *et al.*, 1996).

7.3.2.7 Subchronic studies in rats and dogs

Thirteen-week dietary studies using beagle dogs and the Tif: RALf (SPF) strain of Sprague-Dawley rat also failed to identify any toxic or other adverse biological effects attributable to leucrose exposure. In the rat study, leucrose was fed to groups of 40 rats (20/sex) at dietary concentrations of 0, 5, 10 and 20%, with a 20% sucrose diet serving as a positive control and an 18% wheat starch diet serving as a negative control.[35] Measurements of food consumption resulted in mean leucrose exposures of 0, 1000, 2100 and 4200 mg/kg/day for males and 900, 1800 and 3000 mg/kg/day in females. At the end of 13 weeks, 15 rats/sex in each exposure group were killed and necropsied with the remaining 5 rats/sex/group being held for an additional 4-week recovery period while consuming a normal diet. The parameters examined in the rat study included food and water intake, hematology and clinical chemistry endpoints, gross organ abnormalities, weights of eleven organs, and histopathology of 48 organs. Plasma leucrose ranged from 0.3-0.6 μg/ml, values that were characterized as minimal. Urinary leucrose concentrations were 27-51 μg/ml; described as dose dependent but not dose-proportional.[36] Because there were no observed effects on any of the examined endpoints, the male and female NOAELs for leucrose in this study were concluded to be the highest administered doses, *i.e.*, 4200 and 3000 mg/kg/day, respectively. The mean male and female rat intakes of the sucrose positive control were 4200 and 2700 mg/kg/day, respectively (Elias *et al.*, 1996).[37]

In the dog study reported by Elias *et al.* (1996), the effects of dietary leucrose were examined at concentrations of 0, 5, 10 and 20%. In the low- and mid-concentration groups (both containing three dogs *per* sex), the reported leucrose doses were 2500 and 5000 mg/kg/day. In the high-concentration group, which contained four dogs *per* sex, the leucrose dose was reported as

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[35] This 18% wheat starch diet was considered by the authors to be isocaloric to the 20% leucrose and sucrose diets.
[36] Elias *et al.* (1996) mentioned that these urinary leucrose concentrations were similar to human urinary leucrose concentrations of 35-141 μg/ml following single 70 g doses. Although the source of these human urinary leucrose concentrations was not specified, it is probable they arose from the human study described in the previous section where six humans consumed single 70 g quantities of leucrose.
[37] The parameters examined in the rat study included food and water intake, hematology and clinical chemistry endpoints, gross organ abnormalities, weights of eleven organs, and histopathology of 48 organs. In the dog study, the examined parameters included clinical signs, food and water intake.

10,000 mg/kg/day. The positive and negative control groups also contained four dogs *per* sex; the former consuming 10,000 mg/kg/day of sucrose (from a 20% sucrose diet) and the latter consuming 9000 mg/kg/day of wheat starch (from a 18% wheat starch diet). After 13 weeks, all dogs from the low- and mid-dose groups were killed and necropsied as were three of the four dogs *per* sex in the high-dose and two control groups. The remaining dogs consumed a normal diet during a four-week recovery period. In this study, the examined parameters included clinical signs (including vomiting and diarrhea), food intake, body weights, hematology and clinical chemistry endpoints, blood pressure, electrocardiogram, gross organ abnormalities, weights of eleven organs, and histopathology of 40 organs. Plasma leucrose was measured after one and 13 weeks of exposure. The resulting values ranged from 0.7-4.3 µg/ml, which were reported as low, dose-dependent and comparable at both time points. Mean plasma sucrose concentrations in control dogs were higher at 8-10 µg/ml. Urinary leucrose concentrations were 161-661 µg/ml; also described as dose dependent and comparable at both time points, although greater than urinary leucrose seen in rats and humans at comparable doses. Mean urinary sucrose in control dogs was 919 µg/ml after one week and 184 µg/ml after 13 weeks; however, the one-week value was reported as having a very high standard deviation and thus, not statistically different from the 13-week value. Because there were no observed adverse effects in the dog study, a NOAEL of 5000 mg/kg/day was reported for both sexes.

Because there were no observed effects of leucrose on organ weights or the gastric mucosa in either of species tested, Elias *et al.* (1996) reported that longer-term toxicology studies were not expected to demonstrate a significant human health hazard of leucrose. Thus, no chronic studies were conducted on leucrose.

7.3.2.8 Reproductive toxicity

Elias *et al.* (1996) reported that special studies of the potential effects of leucrose on fertility and reproduction in rats were not conducted due to the results of metabolism studies and the findings from the 13-week rat study. In this latter study, at the highest doses, weights of ovaries, testes and pituitary glands were unchanged and the histopathology of both male and female reproductive systems was normal.

7.3.2.9 Embryotoxicity studies in rats and rabbits

Elias *et al* (1996) also reported on the potential embryotoxic effects of leucrose in Sprague-Dawley rats (Tif: RALf (SPF) strain) and Himalayan rabbits. In the rat study, groups of 24 pregnant females were administered gavage doses of 0, 300, 900 and 2700 mg/kg bwt on gestational days (GD) 6-15. The negative control group was administered gavage doses of a 0.8% aqueous hydroxypropyl methylcellulose gel and a positive control group was gavaged with 2700 mg/kg bwt sucrose on the same GDs as the treated and negative control groups. On GD 20, dams were killed following delivery of fetuses by caesarean section. All treated dams tolerated treatment well. Food intake was not affected at any dose; however, high-dose dams had a moderate but significant (P level unspecified) depression in body weight gain during GD 6-20 compared to untreated control dams. No differences were observed between treatments and controls for resorption rates, fetal weights and placental weights. There were also no treatment-related effects on fetal malformations or variations. These findings led Elias *et al.* (1996) to conclude that leucrose is not embryotoxic or teratogenic in rats.

In the rabbit study, the same gavage doses of leucrose and control substances were administered to groups of 12 pregnant rabbits during GD 6-18. On GD 29, dams were killed after the fetuses were delivered by caesarean section; fetuses were observed for viability during 24 additional hours while in a 37°C incubator. Treatment was well-tolerated by dams with no effects on food intake or body weight gain. Based on there being no observed treatment-related effects on fetal numbers, body weights, placental weights, sex distribution, viability, resorption rates, postimplantation losses, variation rates or malformations, Elias *et al.* (1996) concluded that leucrose was not embryotoxic or teratogenic to rabbits.

Elias *et al.* (1996) further reported that they did not conduct a human peri-postnatal toxicity study of leucrose because biologically relevant amounts of unchanged leucrose cannot reach mother's milk following ingestion.

7.3.2.10 Genotoxicity studies

7.3.2.10.1 Ames assay

Elias *et al.* (1996) reported the findings of an Ames reverse mutation assay using the standard plate incorporation procedure. *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and

TA1537 were exposed to leucrose concentrations of 0, 156, 312, 625, 1250, 2500 and 5000 µg/plate in the absence and presence of metabolic S-9 activation prepared from Aroclor 1254-induced rat liver microsomes; exposures in the presence of S-9 were accomplished at both 4% and 10% S-9 concentrations. The negative control solvent was not specified. With one exception, there were no significant increases in the number of revertants compared to controls. That exception was an observed significant increase (Dunnett's test) in revertants in tester strain TA1537 at 2500 µg/plate in the presence of 10% S-9. Because (1) a similar increase in revertants was not seen at the same dose in the presence of 4% S-9, (2) regression analysis did not show a dose-response, and (3) all other genotoxicity assays (summarized below) were negative, this isolated finding was not considered to be indicative of an overall positive Ames assay for leucrose (Elias *et al.*, 1996).

7.3.2.10.2 Mouse micronucleus assay

The results of a mouse micronucleus assay on leucrose were also reported by Elias *et al.* (1996). A range-finding assay evaluated gavage leucrose doses at 0, 250, 1000, 2000 and 4000 mg/kg bwt in groups of six Swiss-Webster mice (3/sex). The negative control solvent was not specified. Significant clinical adverse signs were not observed. At 72 hours post-exposure, there were no significant depressions in the ratio of polychromatic erythrocytes to red blood cells (PCE:RBC) in (1) at least 200 RBC collected from bone marrow, and (2) at least 1000 RBC collected from peripheral blood. In the definitive assay, groups of 30 mice (15/sex) were administered single gavage doses of 0, 1000, 2000 and 4000 mg/kg bwt. Male mice in a positive control group (number of animals not specified) were gavaged with 300 mg/kg bwt of urethane. After dosing, bone marrow was collected at 24, 48 and 72 hours from five mice/sex/group and examined for cytotoxicity. Approximately 2000 PCE from the collected bone marrow were examined for the presence of micronuclei. In the positive control mice, the expected significant increase in micronucleated PCE was shown but in treated animals, there were no significant decreases in PCE:RBC ratio and no significant increases in PCE with micronuclei. Thus, Elias *et al.* (1996) concluded that leucrose was not mutagenic in the mouse micronucleus assay.

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7.3.2.10.3 Mouse lymphoma assay

The mouse lymphoma assay was another tool used by Elias *et al.* (1996) to evaluate the potential genotoxicity of leucrose. In a preliminary procedure, cultures of L5178Y murine lymphoma cells were exposed to leucrose at 0, 39, 78, 156, 313, 625, 1250, 2500 and 5000 μg/ml in the presence and absence of S-9 metabolic activation. The negative control solvent was not specified. Using standard protocols for evaluating the frequency of mutation at the thymidine kinase (tk) locus of the exposed cells, there were no observed indications of leucrose-induced cytotoxicity. The definitive assay evaluated leucrose concentrations of 0, 600, 1000, 1800, 3000 and 5000 μg/ml, also in the presence and absence of S-9. All positive control substances (*i.e.*, ethylmethanesulfonate, methylmethanesulfonate and 3-methylcholanthrene) induced the expected significant increases in mutants at the tk locus; however, the results for leucrose were negative and Elias *et al.* (1996) concluded that leucrose was not mutagenic in the mouse lymphoma assay.

7.3.2.10.4 Chromosome aberration assay

The final genotoxicity evaluation reported by Elias *et al.* (1996) was a chromosome aberration assay in Chinese hamster ovary (CHO) cells. In the preliminary assay, CHO cells were exposed to leucrose at concentrations of 0, 42, 126, 380, 1140 and 3420 μg/ml with and without S-9 (exposure time and negative control solvent not specified) and evaluated for chromosomal damage by standard procedures. In the definitive assay, leucrose concentrations of 0, 855, 1710 and 3420 μg/ml were evaluated, also with and without S-9. In the absence of S-9, there were no significant increases in the frequency of cells with structural chromosomal aberrations; however those effects were noted in the presence of S-9 at 855 and 1710 μg/ml. The highest concentration of 3420 μg/ml did not induce the effects observed at the two lower concentrations. Because there was no demonstrated dose-response associated with these findings, the assay was repeated. In the second assay, no effects were observed at any leucrose concentration regardless of whether S-9 was present. As with the other genotoxicity assays summarized above, the positive control substances (methylmethanesulfonate and cyclophosphamide) induced the expected positive responses in both the initial and repeated assays. Based on these findings, Elias *et al.* (1996) concluded that leucrose was negative in this assay.

## 7.4 Sucromalt safety

A series of *in vitro* and *in vivo* (animal and human) studies were conducted to assess the safety of sucromalt (sucrose:maltose = 9:1).[38] *In vitro* studies included an investigation of sucromalt digestibility and an Ames bacterial reverse mutation assay while the *in vivo* animal studies included a 7-day dose-range-finding study in rats, and a 28-day oral toxicity study in rats. Human clinical investigations included an evaluation of sucromalt effects on (1) human plasma glucose, insulin response, and breath hydrogen, (2) gastrointestinal symptoms in triathletes during heavy exercise, and (3) postprandial glycemia and insulinemia. These studies are described below.

### 7.4.1 *In vitro* studies

#### 7.4.1.1 Digestibility

The digestibility of sucromalt made from various sucrose:maltose molar ratios was assessed by an *in vitro* digestibility assay (Cargill, 2005a).[39] The tested sucrose to maltose ratios included 1:1, 4:1, 8:1, 9:1 and 10:1. Aqueous sucromalt solutions (4%) were mixed with rat intestinal powder and incubated at 37 °C with samples taken from the incubated mixture at 0, 4 and 8 hours. The amounts of glucose released by the rat intestinal enzymes were measured by HPLC and expressed as *percent* of total available/theoretical glucose as an indication of expected *in vivo* digestion. The results of this assay are summarized in Table 14. The *in vitro* digestion rate of sucromalt decreased as the ratio of sucrose to maltose increased; however, little difference occurred in digestion rate of sucromalt made with sucrose to maltose ratios of 8:1, 9:1 and 10:1.

**Table 14. *In vitro* digestibility of sucromalt with varying sucrose: maltose ratios at baseline, 4 hours, and 8 hours (Cargill, 2005a)**

| | *Percent* (theoretical) glucose concentration released | | |
|---|---|---|---|
| Sucrose: maltose ratio | Baseline | 4 hours | 8 hours |
| 1:1 | 2 | 79 | 81 |
| 4:1 | 2 | 38 | 47 |
| 8:1 | 3 | 28 | 33 |
| 9:1 | 3 | 29 | 31 |
| 10:1 | 4 | 30 | 32 |

[38] All of these studies were accomplished using sucromalt that met the specifications presented in Table 9.
[39] This study used a method developed for internal Cargill use as a screen for digestibility. As such, there is no attempt in this dossier to tie the results to a human risk assessment conclusion.

7.4.1.2. Mutagenicity

The mutagenic potential of sucromalt[40] was assessed in the Ames bacterial reverse mutation assay using *Salmonella typhimurium* tester strains TA97a, TA98, TA100, TA102, and TA1535 in the presence and absence of Sprague-Dawley rat-derived S9 metabolic activation.[41] Based on a range-finding study that evaluated test concentrations of 0.05, 0.158, 0.5, 1.58 and 5 mg/plate, the final sucromalt concentrations evaluated in the definitive study were 0.5, 1.58 and 5 mg/plate. Under these test conditions, sucromalt did not induce mutagenicity in any of the strains tested (Carlson *et al.* (2008).

7.4.2 Animal studies

7.4.2.1 Seven-day study

A 7-day, range-finding toxicity study was conducted (WIL Research Laboratories, Inc., 2003a) as a prelude to a 28-day repeated dose toxicity study of sucromalt that is described in detail later in this section (Eapen *et al*, 2007). Sucromalt was administered to Crl:CD®(SD)IGS BR rats (4 animals/sex/dose) *via* addition to a certified rodent diet[42] for seven consecutive days at 0, 100,000 ppm (10%), 150,000 ppm (15%), and 200,000 ppm (20%). Animals were housed individually in wire-mesh cages suspended above cage board and observed twice daily for mortality and moribund condition; clinical examinations (*e.g.*, body scabbing, fecal softness) were performed each day.[43] Detailed physical examinations were conducted prior to randomization and again prior to scheduled sacrifice at study end. Individual body weights and food consumption were recorded twice weekly; there was no evidence that sucromalt was unpalatable. After the seventh day of dietary exposure, all animals were weighed, their food consumption measured and they were euthanized. There were no deaths during the study and there was no evidence of toxicity. In all treatment groups (both sexes), there were statistically significant increases in food consumption; however, those increases were evident only when

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[40] The specific sucromalt test substance was prepared by reaction of nine parts sucrose to one part high maltose corn syrup, *i.e.*, "sucromalt 9:1" and met the specifications presented in Table 9.
[41] This study followed the guidelines of OECD 471 (Bacterial reverse mutation test).
[42] PMI Nutritional International LLC Certified Rodent LabDiet® 5002 (meal). Complete composition available at http://www.labdiet.com/indexlabdiethome.htm.
[43] Because this was a range-finding study, the effects of the various sucromalt concentrations on clinical chemistry were not determined. See Eapen *et al.* (2007) below for a description of clinical chemistry and other endpoints evaluated in the ensuing 28-day study.

consumption was evaluated during days 0-4 and 4-7. When mean food consumptions were evaluated from days 1-7, there were no significant differences in either sex, a finding supportive of the observation that there were was no treatment-related effects on body weight. The mean sucromalt consumption for all exposure groups are presented in Table 15. Based on the results of this study, *i.e.*, a 7-day dietary no-observed-adverse-effect level (NOAEL) of 200,000 ppm (or approximately 20,000 mg/kg/day), sucromalt concentrations of 0, 50,000, 100,000 and 200,000 ppm were selected for the subsequent 28-day study (Eapen *et al.*, 2007).

**Table 15. Calculated sucromalt consumption during 7-day toxicity study in rats**

| | Exposure Group | | | |
|---|---|---|---|---|
| | 0 ppm | 100,000 ppm | 150,000 ppm | 200,000 ppm |
| | | *MALES* | | |
| Days 0 to 4 Mean consumption (mg/kg/day)* | 0.00 | 10,160 | 14,740 | 21,240 |
| Days 4 to 7 Mean consumption (mg/kg/day) | 0 00 | 9240 | 13,340 | 18,890 |
| Grand mean | 0.00 | 9700 | 14,040 | 20,060 |
| | | *FEMALES* | | |
| Days 0 to 4 Mean consumption (mg/kg/day) | 0.00 | 9840 | 15,630 | 20,090 |
| Days 4 to 7 Mean consumption (mg/kg/day) | 0.00 | 9150 | 14,140 | 18,140 |
| Grand Mean | 0.00 | 9490 | 14,890 | 19,120 |

* = Mean consumption calculated from individual consumptions

7.4.2.2 28-day Study

A 28-day toxicity study was reported by Eapen *et al.* (2007) in which Crl:CD®(SD)IGS BR rats (10 animals/sex/dose) were administered sucromalt at dietary concentrations of 0, 50,000 ppm (5%), 100,000 ppm (10%), and 200,000 ppm (20%) over 28 days; the rats were all approximately six weeks old at the beginning of the study.[44, 45] This study included an evaluation

[44] The diet used in this study was identical to the diet used in the preceding seven-day range-finding study (section 7.4.2.1.). See footnote 42 on page 49.

[45] The test material used in this study met the standards of composition established for the sucromalt commercial product, with the exception of total solids, which were 70% because it was difficult to blend the syrup with the rest of the diet at 80%.

of potential neurotoxicity by functional observation battery (FOB)[46] as well as an assessment of locomotor activity *via* an evaluation of fine motor skills and ambulatory motor activity. This study was consistent with OECD Guidelines 407 and Redbook 2 and was GLP-compliant. Animals were housed individually in wire-mesh cages suspended above cage board and were observed twice daily for mortality and moribund condition; clinical examinations were conducted daily and detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded weekly. FOB and locomotor data were recorded for 5 animals/sex/group during study week 3, and ophthalmic examinations were performed during weeks 1 and 3 by a board certified veterinary ophthalmologist. All animals were euthanized after four weeks of treatment and clinical pathology evaluations (hematology,[47] serum chemistry,[48] and urinalysis)[49] were performed. Complete necropsies were conducted on all test animals and included examination of external surfaces, all orifices, and the cranial, thoracic, abdominal and pelvic cavities, including viscera. At necropsy, organs/tissues were collected for weighing and histopathologic examination,[50] which was conducted on tissues collected from all animals in the control and high-dose groups (Eapen *et al.*, 2007).

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[46] FOB parameters included observations in the home cage (posture, convulsions/tremors and feces consistency), in handling (ease of removal from cage), lacrimation/blood tears, piloerection, palpebral closure, eye prominence, red/crusty deposits), in the open (mobility, rearing, convulsions/tremors, grooming, bizarre/stereotypic behavior), sensory parameters (approach response, startle response, pupil response, forelimb extension, air righting reflex), neuromuscular parameters (hindlimb extensor strength, hindlimb foot splay) and physiological observations (catalepsy, body temperature).

[47] Hematolysis parameters included total leukocyte count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count, reticulocyte count (*percent* and absolute), and *percent* and absolute leukocyte count (including neutrophils, lymphocytes, monocytes, eosinophils and basophils).

[48] Serum chemistry parameters included albumin, total protein, globulin, albumin/globulin ratio, total bilirubin, urea nitrogen, creatinine, alkaline phosphatase (AP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), *gamma*-glutamyltransferase (GGT), glucose, total cholesterol, calcium, chloride, phosphorous, potassium, sodium and triglycerides.

[49] Urinalysis parameters included volume, urobilinogen, color, appearance, pH, protein, glucose, ketones/bilirubin, occult blood, leukocytes and nitrates.

[50] The organs and tissues that were collected included adrenals, aorta, bone with marrow from femur and sternum, bone marrow smear, brain (cerebrum levels 1 and 2, cerebellum with medulla and pons), gastrointestinal tract (esophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum), heart, kidneys, liver, lungs (with bronchi), lymph nodes (mandibular and mesenteric), mammary gland (females), nasal cavities, ovaries (with oviducts), pancreas, peripheral nerve (sciatic), pituitary, prostate, salivary glands (mandibular), skeletal muscle (rectus femoris), skin, spinal cord (cervical, thoracic, lumbar), spleen, thymus, thyroid and parathyroid glands, tongue, trachea, urinary bladder, uterus with vagina, and other gross lesions.

Mean food consumption was significantly greater at some or all weeks in low-dose females, and in both sexes in the mid- and high-dose groups (P<0.05 or P<0.01). These increases in food consumption resulted in higher body weight gains during week 3 for males and during all four weeks for females. However, body weights for males and females were only slightly higher (2% to 4%) than control group animals during study weeks 3 and/or 4, and these values were not considered by the authors to be toxicologically or biologically relevant and they were not statistically significant. The mean sucromalt consumption among males and females is presented in Table 16.

**Table 16. Calculated daily sucromalt consumption during 28-day toxicity study in rats**

| | Exposure Group | | | |
|---|---|---|---|---|
| | 0 ppm | 50,000 ppm | 100,000 ppm | 200,000 ppm |
| | | *MALES* | | |
| Weeks 0-1 | | | | |
| Mean consumption (mg/kg/day)* | 0 | 5290 | 10,550 | 22,450 |
| Weeks 1-2 | | | | |
| Mean consumption (mg/kg/day)* | 0 | 4440 | 8780 | 19,050 |
| Weeks 2-3 | | | | |
| Mean consumption (mg/kg/day)* | 0 | 3990 | 8060 | 17,110 |
| Weeks 3-4 | | | | |
| Mean consumption (mg/kg/day)* | 0 | 3630 | 7300 | 15,640 |
| Grand mean | 0 | 4340 | 8670 | 18,560 |
| | | *FEMALES* | | |
| Weeks 0-1 | | | | |
| Mean consumption (mg/kg/day)* | 0 | 5300 | 10,060 | 22,450 |
| Weeks 1-2 | | | | |
| Mean consumption (mg/kg/day)* | 0 | 4890 | 10,030 | 20,220 |
| Weeks 2-3 | | | | |
| Mean consumption (mg/kg/day)* | 0 | 4480 | 9230 | 18,950 |
| Weeks 3-4 | | | | |
| Mean consumption (mg/kg/day)* | 0 | 4060 | 8250 | 17,570 |
| Grand Mean | 0 | 4680 | 9390 | 19,800 |

* = Mean consumption calculated from individual consumptions

All animals survived to the scheduled necropsy. There were no treatment-related clinical observations, and no effects on FOB, locomotor activity or urinalysis parameters. There were also no ophthalmic lesions indicative of toxicity, and no macroscopic, microscopic, or organ weight changes. The only observed statistically significant findings were in the clinical

chemistry values for alanine aminotransferase (ALT) and aspartate aminotransferase (AST); and in the hematological values for mean corpuscular volume (MCV).

In males exposed to the lowest concentration of sucromalt, both ALT and AST were slightly elevated compared to untreated control males ($P<0.05$, Dunnett's test). Table 17 presents the individual male rat ALT and AST data for all dietary concentration and shows the approximate equal variation with the individual rats in each treatment group (WIL Research Laboratories, Inc. , 2003b). Although statistically significant, the low-dose findings do not indicate a treatment-related hepatotoxic effect of sucromalt because (1) they do not follow a dose-dependent trend, (2) they did not occur in both sexes, (3) they are not accompanied by adverse liver histopathological findings, and (4) the values are all within the historical control range for the same rat strain.[51] These latter two points are important because the magnitudes of difference between the control and significant ALT and AST (*i.e.*, 30 compared to 39 U/l for ALT, a 0.3-fold increase; and 72 compared to 90 U/l for AST, a 0.25-fold increase) are well below the >3 to 5-fold increases in these two enzymes that are generally considered by FDA to be adverse (FDA, 2001), particularly when the observed slight elevations occurred in the absence of liver histopathology.

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[51] The mean ALT value for historical control male Crl:CD®(SD)IGS BR rats at WIL Research Laboratories, Inc. is 40 U/l (SD = 7.3; n = 411; range = 25-60) and the mean AST values for those animals is 95 U/l (SD=17.1; n = 411; range = 67-154). Range of study dates = December, 2002 to February, 2007. (WIL Research Laboratories, Inc. (2007)

**Table 17. Individual rat data for ALT and AST for control and low-dose males that consumed the treatments of sucromalt (WIL Research Laboratories, Inc., 2003b)**

| | Alanineaminotransferase (ALT; U/l) Dietary sucromalt exposure (mg/kg/day) | | | | Aspartateaminotransferase (AST; U/l) Dietary sucromalt exposure (mg/kg/day) | | | |
|---|---|---|---|---|---|---|---|---|
| Male rat # | 0 | 4340 | 8670 | 18,560 | 0 | 4340 | 8670 | 18,560 |
| 1 | 26 | 53 | 30 | 34 | 72 | 94 | 74 | 72 |
| 2 | 33 | 41 | 34 | 56 | 71 | 76 | 80 | 104 |
| 3 | 27 | 30 | 26 | 30 | 72 | 92 | 60 | 73 |
| 4 | 33 | 33 | 36 | 29 | 70 | 74 | 77 | 68 |
| 5 | 24 | 57 | 35 | 34 | 61 | 106 | 81 | 80 |
| 6 | 23 | 32 | 23 | 27 | 63 | 67 | 75 | 78 |
| 7 | 27 | 39 | 33 | 47 | 85 | 107 | 75 | 89 |
| 8 | 25 | 34 | 32 | 35 | 62 | 78 | 75 | 121 |
| 9 | 38 | 41 | 28 | 33 | 81 | 87 | 78 | 73 |
| 10 | 40 | 32 | 34 | 41 | 86 | 114 | 82 | 88 |
| Mean | 30 | 39* | 31 | 37 | 72 | 90* | 76 | 85 |
| SD | 6.0 | 9.2 | 4.3 | 9.0 | 9.1 | 15.9 | 6.2 | 16 7 |
| # of animals | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

* = P<0.05; SD = standard deviation; U/l = International Units *per* liter.

For MCV (*i.e.*, the average volume of individual red blood cells, RBC), a minimal, yet statistically significant, decrease was noted in high-dose males compared to control males. This microcytosis finding is unlikely to be associated with a toxic hematological effect attributable to sucromalt ingestion. As evidence, the individual animal data for MCV is presented in Table 18 (WIL Research Laboratories, Inc., 2003b). A broad examination of the MCV values from control and treated animals shows little variability and no dose-response relationship. The range of MCV values noted among high-dose males (53.5-57.5 fl), all treated males (53.4-58.5 fl) and control males (54.0-58.5 fl) were all minimal and well within the range of historical MCV values (52.5-66.2 fl)[52] for control male Crl:CD®(SD)IGS BR rats. Furthermore, a similar difference was not observed in high-dose females despite their greater exposure to sucromalt relative to high-dose males. In addition, no statistically significant changes occurred in any of the other RBC-related hematological parameters measured in this study. This included RBC and reticulocyte counts, hemoglobin, hematocrit, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. Because the decrease in MCV from high-dose males lacked correlating changes in

[52] The mean MCV value for historical control male Crl:CD®(SD)IGS BR rats at WIL Research Laboratories, Inc. is 58.7 fl (SD = 3.13; n = 273; range = 52.5-66.2). Range of study dates = June, 2004 to February, 2007. (WIL Research Laboratories, Inc. (2007)

other red cell parameters, was of small magnitude and consistent with control values, did not occur in a dose-related manner, and did not occur in females that had a higher sucromalt exposure, the significant MCV decrease in high-dose males is not considered a sign of sucromalt hematotoxicity.

**Table 18. Individual MCV values for all test animals at all exposure levels (WIL Research Laboratories, Inc., 2003b)**

| | Mean corpuscular volume (MCV; fl) MALES Dietary sucromalt exposure (mg/kg/day) | | | | | Mean corpuscular volume (MCV; fl) FEMALES Dietary sucromalt exposure (mg/kg/day) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Male rat # | 0 | 4340 | 8670 | 18,560 | Female rat # | 0 | 4680 | 9390 | 19,800 |
| 1 | 56.2 | 55.1 | 55.5 | 54.9 | 1 | 57.6 | 56.6 | 55.7 | 54.0 |
| 2 | 55.2 | 55.8 | 57.5 | 55.3 | 2 | 56.1 | 55.7 | 55.3 | 53.5 |
| 3 | 58.1 | 57.1 | 57.1 | 55.7 | 3 | 56.5 | 53.6 | 56.5 | 52.6 |
| 4 | 58.0 | 54.5 | 56.8 | 57.5 | 4 | 56.2 | 58.1 | 56.0 | 55.0 |
| 5 | 57.7 | 55.0 | 58.0 | 55.5 | 5 | 54.8 | 56.7 | 58.0 | 56.8 |
| 6 | 56.7 | 55.0 | 57.8 | 55.8 | 6 | 54.1 | 55.3 | 54 3 | 55.2 |
| 7 | 58.0 | 53.5 | 55.0 | 53.4 | 7 | 55.7 | 53.8 | 56.5 | 57.6 |
| 8 | 56.1 | 55.0 | 56.1 | 53.5 | 8 | 55.4 | 53.9 | 57.4 | 56.7 |
| 9 | 54.0 | 58.5 | 57.5 | 53.8 | 9 | 56.2 | 54.9 | 55.7 | 58.2 |
| 10 | 58.5 | 56.1 | 56.5 | 56.2 | 10 | 56.6 | 56.6 | 55.6 | 54.2 |
| Range | 54.0-58.5 | 53.5-58.5 | 55.0-58.0 | 53.4-57.5 | Range | 54.1-57.6 | 53.6-58.1 | 54.3-58.0 | 52.6-58.2 |
| Mean | 56.9 | 55.6 | 56.8 | 55.2* | Mean | 55.9 | 55.5 | 56.1 | 55.4 |
| SD | 1.47 | 1.41 | 1.00 | 1.30 | SD | 0.98 | 1.50 | 1.06 | 1.87 |

* = $P<0.05$; fl = femtoliter (*i e.*, $10^{-15}$ liter); SD = standard deviation.

Based on the results of this study, the no-observed-adverse-effect levels (NOAEL) in rats administered sucromalt in the diet for 28 consecutive days were 18,560 mg/kg/day for males and 19,800 mg/kg/day for females, the highest doses tested. (Eapen *et al.*, 2007).

### 7.4.3 Human studies

#### 7.4.3.1 Digestibility in ileostimized patients

The human digestibility of sucromalt was investigated in two male and two female ileostimized patients; their characteristics are presented in Table 19.[53]

[53] In an earlier oral glucose tolerance study of sucromalt, the glucose and insulin responses to sucromalt were significantly lower than those for high fructose corn syrup; implying a lower GI. Thus, it could not be determined whether sucromalt ingestion results in an incomplete absorption in the small intestine, thereby resulting in a lower peak and AUC (area under the dose-response curve), or resulting in a lower postprandial response after complete absorption. Using ileostimized patients is, therefore, very useful because ileostomy effluent can be collected and the recovery of sucromalt can be measured so that *percent* absorption in the small intestine can be calculated.

**Table 19. Main characteristics of bioavailability study participants (NutriScience, 2004)***

| Subject | Sex | Age (years) | Height (m) | Weight (kg) | BMI (kg/m$^2$) | Systolic BP (mm Hg) | Diastolic BP (mm Hg) |
|---|---|---|---|---|---|---|---|
| 1 | F | 57 | 1.59 | 65.1 | 25.7 | 120 | 67 |
| 2 | M | 68 | 1.83 | 85.1 | 25.2 | 112 | 71 |
| 3 | M | 49 | 1.68 | 76.3 | 27.0 | 122 | 79 |
| 4 | F | 54 | 1.68 | 75.5 | 26.6 | 149 | 72 |
| Mean | | 57 | 1.70 | 75.5 | 26.1 | 126 | 72 |

* = The study participants met the following criteria: steady post-operative state, age 20-70 years, blood pressure 100/60-150/90, body mass index 20-27 kg/m$^2$, absence of glucose or protein in urine, and chronic ileostoma. (Indication for large bowel resection was ulcerative colitis (UC)) with the operation occurring at least one year prior and preferably three years prior. Crohn's Disease (CD) patients were eligible but had to be in remission for three years. For UC and CD patient eligibility, the minimum amount of ileum removed during resection could be no more than 7.5%.); BMI – body mass index; BP = blood pressure; Hg = mercury; kg = kilograms, m = meters.

All participants had fasted for at least ten hours prior to study commencement. On the morning on the study, subjects consumed two portions of a semi-liquid breakfast, each portion containing 50 g sucromalt syrup (sucrose:maltose = 9:1, 81.56% dry solids) dissolved in 200 g low fat fruit yogurt; consumption of the two portions was separated by one hour. Over the first six hours, the subjects did not consume food; however, drinking water and tea were allowed. After six hours, the subjects consumed a lunch with the amounts of each food in the lunch, and their dietary fiber contents, recorded. Ileostomy effluent was collected and frozen at hours 2, 4, 6, 8, 10, and 12 following consumption of the test sucromalt solution. During analysis, the weights of all frozen samples were determined prior to pooling of samples into Pool 1 (baseline samples); Pool 2 (samples from 2, 4, and 6 hours), and Pool 3 (samples from 8, 10, and 12 hours). The pooled samples were analyzed by HPLC for fructose content before acid hydrolysis, and for glucose and maltose content after acid hydrolysis. The levels of fructose, glucose, and maltose, measured as *percent* of ileostomy effluent weight, are shown in Table 20 (NutriScience, 2004).[54]

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[54] The fructose content was analyzed before acid hydrolysis because a substantial amount of the nondigested fructose present in the test product was altered into 5-(hydroxymethyl)-2-furaldehyde and hydroxyacetylfuran. Moreover, the co-elution of an unknown compound with fructose made direct quantification impossible. Acid hydrolysis was performed in order to hydrolyze nondigested oligosaccharides present in the test product into their respective mono- and disaccharides (glucose and maltose), thus facilitating quantification. Treatment with 0.24 mol HCl for 4 hours was sufficient to obtain complete hydrolysis of sucromalt.

**Table 20. Determination of carbohydrate content in freeze-dried ileostomy effluents (NutriScience, 2004)**

| Pooled sample # | Subject # | Maltose (% of effluent weight) | Glucose (% of effluent weight) | Fructose (% of effluent weight) | Total carbohydrates after hydrolysis (% of effluent weight) |
|---|---|---|---|---|---|
| 1 (Hour 0) | 1 | 0.67 | 8.62 | 3.13 | 12.42 |
| | 2 | 0.72 | 9.36 | 0.90 | 10.98 |
| | 3 | 0.77 | 7.68 | 0.97 | 9.42 |
| | 4 | 0.88 | 6.94 | 1.80 | 9.62 |
| 2 (Hours 2, 4 and 6) | 1 | 2 35 | 29.64 | 1.96 | 33.95 |
| | 2 | 3.01 | 37.10 | 2.89 | 43.00 |
| | 3 | 5 16 | 35.81 | 3 82 | 44.79 |
| | 4 | 3.00 | 46.65 | 3.70 | 53.35 |
| 3 (Hours 8, 10 and 12) | 1 | 2.21 | 27.13 | 3.00 | 32.34 |
| | 2 | 1.68 | 20.24 | 1.67 | 23.59 |
| | 3 | 1.53 | 19.34 | 0.00 | 20.87 |
| | 4 | 1.36 | 23.51 | 1.56 | 26.43 |

The levels of carbohydrates recovered in ileostoma fluid were compared to the levels of carbohydrates ingested to determine absorption and excretion. Absorption (%) was calculated as (amount ingested – amounts recovered) / amount ingested X 100. Excretion was calculated as (amounts recovered/amount ingested) X 100. The results of these calculations are provided in Table 21 and indicate that sucromalt is well digested.

**Table 21. Excretion and absorption of sucromalt (NutriScience, 2004)**

| Subject | Excretion in ileostomy effluent (grams) | Fiber content in lunch (grams) | Excretion corrected for dietary fiber (grams) | % Excretion | % Absorption |
|---|---|---|---|---|---|
| 1 | 17.4 | 10.6 | 6.8 | 8.3 | 91.7 |
| 2 | 10.7 | 9.5 | 1.2 | 1.5 | 98.5 |
| 3 | 10.1 | 7.1 | 3 | 3.7 | 96.3 |
| 4 | 11.2 | 7.1 | 4.1 | 5.1 | 94.9 |
| Mean | 12.4 | 8.58 | 3.8 | 4.6 | 95.4 |

### 7.4.3.2 Absorption in normal subjects

Using a randomized, crossover, double-blind design in each of two separate studies,[55] Grysman *et al.* (2007) examined the postprandial effects of ingested sucromalt, high-fructose corn syrup

[55] These studies were conducted at two separate facilities. The first study occurred at G.I. Testing, Inc. (Toronto, Ontario, Canada; http://www.gitesting.com/) a firm specializing in glycemic index testing. The second study was

(HFCS) and an inulin mixture[56] on breath hydrogen[57] and glucose, insulin, c-peptide,[58] and glucagon-like peptide 1 (GLP-1)[59] in blood. These test substances were mixed with water and consumed by groups of ten healthy fasted humans; none of the subjects participated in both studies. In the first study, seven male and three female subjects (27.6 ± 1.6 years old; body mass index (BMI) = 23.6 ± 1.1 kg/m$^2$) consumed 50 g of the test substances; 80 g of test substances were consumed in the second study. The compositions of the test substances appear in Table 22.

**Table 22. Saccharide compositions of HFCS, sucromalt and inulin studied in Grysman *et al.* (2007)**

| | Study 1* | | Study 2** | | |
|---|---|---|---|---|---|
| Ingredient | HFCS | Sucromalt | HFCS | Sucromalt | "Inulin" |
| Fructose (gm) | 21 | 21 | 34 | 33 | 35 |
| Glucose (gm) | 29 | 0 | 46 | 0 | 21 |
| Glucose oligosaccharides (gm) | 0 | 25 | 0 | 40 | 0 |
| Leucrose (gm) | 0 | 4 | 0 | 7 | 0 |
| Inulin (gm) | -- | -- | 0 | 0 | 24 |
| Total (gm) | 50 | 50 | 80 | 80 | 80 |

* = the study participants were seven males and three females, age 27.6 ± 1.6 years with an average Body Mass Index (BMI) of 23.6 ± 1.1 kg/m$^2$; ** = the study participants were five males and five females, age 25.2 ± 1 7 years with an average BMI of 23.7 ± 5.2 kg/m$^2$ ; HFCS = high-fructose corn syrup (42% fructose and 58% glucose);

In the first study, baseline breath and blood samples were taken immediately before subjects consumed the 50 g of test substances in 250 ml water (plus 250 ml of water only); additional samples were taken at 0.25, 0.5, 0.75, 1, 1.5, 2, 3 and 4 hours following test substance intake. Expired air was collected in 10 ml evacuated glass tubes for examination of breath hydrogen and blood was collected in fluoro-acetate tubes for determination of plasma glucose and insulin. (The

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conducted at St. Michael's Hospital (Toronto, Ontario, Canada; http://www.stmichaelshospital.com/), which is affiliated with the University of Toronto.

[56] Because humans do not absorb inulin, a mixture of inulin, glucose and fructose (see Table 22) was used to represent a model of incomplete carbohydrate absorption (Grysman *et al.*, 2007).

[57] The theory behind evaluating this endpoint is that some hydrogen gas that is normally produced by intestinal bacterial fermentation will be absorbed across the gut epithelium and transported to the respiratory tract for exhalation. Thus, if a substance is poorly absorbed yet can still be used as a substrate for gut fermentation, its absorption properties relative to other substances can be approximated by measuring and contrasting the amounts of exhaled hydrogen associated with each ingested substance. Substances that are poorly absorbed (*e.g.*, inulin) will elicit greater amounts of breath hydrogen than will substances that are well absorbed (*e.g.*, HFCS and sucromalt).

[58] C-peptide is a 30-amino-acid chain in proinsulin that connects the A and B chains of insulin; during conversion of proinsulin to insulin, c-peptide is removed. (Stedman's Online Medical Dictionary, 27th Edition, 2007; http://www.stedmans.com/section.cfm/45. Site visited 05 March 2008.)

[59] GLP-1 is a gastrointestinal hormone that slows gastric emptying and stimulates insulin secretion. (Stedman's Online Medical Dictionary, 27th Edition, 2007; http://www.stedmans.com/section.cfm/45. Site visited 02 January 2008)

blood collection method employed in Study 1 necessitated that glucose and insulin could only be measured in plasma.)[60]

The primary purpose of the second study was to examine the absorption characteristics of the test substances. In this study, baseline breath and blood samples were also taken immediately before test substance intake. Five male and five female subjects (25.2 ± 1.7 years old; BMI = 23.7 ± 5.2 $kg/m^2$). Subjects consumed the 80 g of test substances in 360 ml of water (plus 500 ml of water only). Expired air and blood were collected at the same time points as in Study 1 but the blood collection apparatus differed. In this study, blood for analysis of glucose, insulin, c-peptide and FFA was collected in Vacutainer® serum separator tubes containing clot activator and polymer gel silica activator. (This collection procedure allowed for endpoint analysis in serum; however, it eliminated the ability to determine the target analytes in plasma.)[61] For GLP-1, blood was collected in 6 ml Vacutainer® tubes containing potassium EDTA, which allowed the analysis to be done in plasma. The results of these various measurements, in incremental areas under the dose-response curve, are presented in Table 23.

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[60] Personal communication with Dr. T.M.S. Wolever, University of Toronto. 03 March 2008.
[61] Personal communication with Dr. T.M.S. Wolever, University of Toronto. 03 March 2008.

**Table 23. Incremental areas under the curve (AUC) after test substance consumption.**

| Parameter | AUC time (hr) | Study 1 HFCS | Study 1 Sucromalt | Study 2 HFCS | Study 2 Sucromalt | Study 2 "Inulin" |
|---|---|---|---|---|---|---|
| Plasma glucose (mmol x min $l^{-1}$) | 0-2 | 117 ± 14[a] | 63 ± 11[b] | -- | -- | -- |
| | 0-4 | 119 ± 14[a] | 81 ± 25[b] | -- | -- | -- |
| Plasma insulin (mmol x min $l^{-1}$) | 0-2 | 16.9 ± 3.9[a] | 8.4 ± 1.7[b] | -- | -- | -- |
| | 0-4 | 17.1 ± 3.9[a] | 8.7 ± 1 7[b] | -- | -- | -- |
| Serum glucose (mmol x min $l^{-1}$) | 0-2 | -- | -- | 192 ± 26 | 172 ± 17 | 130 ± 21 |
| | 0-4 | -- | -- | 193 ± 26 | 187 ± 23 | 147 ± 30 |
| Serum insulin (nmol x min $l^{-1}$) | 0-2 | -- | -- | 41.9 ± 4.6[a] | 31.1 ± 4.8[b] | 23.9 ± 3.1[b] |
| | 0-4 | -- | -- | 43.1 ± 4.7[a] | 34.8 ± 5.1[a] | 24.8 ± 3.1[b] |
| Serum c-peptide ((nmol x min $l^{-1}$) | 0-2 | -- | -- | 219 ± 34 | 180 ± 30 | 135 ± 20 |
| | 0-4 | -- | -- | 262 ± 49 | 221 ± 35 | 159 ± 22 |
| Plasma GLP-1 (pmol x min $l^{-1}$) | 0-2 | -- | -- | 334 ± 95[a, b] | 606 ± 119[a] | 173 ± 63[b] |
| | 0-4 | -- | -- | 447 ± 123[b] | 861 ± 163[a] | 230 ± 96[b] |
| Breath hydrogen (ppm/min) | 0-2 | 233 ± 136 | 262 ± 171 | 1410 ± 998[b] | 413 ± 336[b] | 4270 ± 948[a] |
| | 0-4 | 347 ± 165 | 541 ± 325 | 2950 ± 1330[b] | 717 ± 577[b] | 12,600 ± 1490[a] |

[a, b] = means with differing superscripts are significantly different from each other, $P<0.05$; GLP-1 = glucagon-like peptide-1; HFCS = 42% high-fructose corn syrup

The results from Study 1 demonstrate that despite similar gastrointestinal absorption (measured by breath hydrogen), lower glycemic and insulinemic responses occurred after sucromalt consumption compared to HFCS, findings that are supported by the blood glucose and glycemic index measurements shown in Table 3 (GIL, 2005). The glucose and insulin results from Study 2 are less convincing of that relationship. The only observed significant difference in Study 2 occurred for serum insulin during the first two hours of sampling, a finding perhaps due to measurements being made in serum rather than in plasma as in Study 1. The observation that serum c-peptide levels were effectively the same indicates its poor suitability as a marker for differences in gut absorption, at least for the three subject test substances examined in the study. Regarding breath hydrogen measurements in Study 2, the apparent numerical disparity between HFCS and sucromalt suggests that their absorptions were different; however, with standard error values factored in, there were no statistically significant differences in breath hydrogen values between the two treatments. Equally important was the finding that breath hydrogen values after

consumption of the inulin mixture were always much greater than either HFCS or sucromalt, thereby indicating that neither carbohydrate was malabsorbed in this study.

GLP-1 is a potent insulinotropic substance that is synthesized by intestinal L cells and in rats and humans; the greatest number of L cells are located in the distal ileal epithelium (Brubaker, 1991). Krause *et al.* (1982) demonstrated that slower digestion occurs for carbohydrates traversing furthest down the gut before being absorbed and Juntunen *et al.* (2003) further showed that these more distal gut absorptions stimulate a late rise in plasma GLP-1. With this as background, the highest GLP-1 values would be expected to result following consumption of foods that have the slowest digestion and this is clearly demonstrated by the GLP-1 values presented in Table 23.. Based on the results of Study 2, the investigators concluded that the reduced glucose and insulin responses brought about by sucromalt cannot be explained by malabsorption and are most likely associated with either digestive/absorption rate differences or postabsorption processing by the body (Grysman *et al.*, 2007).

7.4.3.3 Gastrointestinal effects in triathletes

The effects of four different sport drinks containing sucromalt, isomaltulose, sucrose or a isomaltulose/trehalose[62] mixture on gastrointestinal well being were evaluated in triathletes during 90 minutes of running and also during 120 minutes of cycling.(NutriScience, 2005). In the running phase, 42 healthy subjects (36 males, 6 females; ages 21-68, mean 44 ± 12 yrs; bwt: 74.5 ± 10.6 kg; BMI: 23.9 ± 2.4) participated and in the cycling phase, 46 healthy subjects (44 males, 2 females; ages 21-68, mean 42 ± 14 yrs; bwt: 72.5 ± 9.8 kg; BMI: 23.5 ± 2.7) participated. Nineteen subjects participated in both trials. The constituents of the various test sport drinks consumed by runners and cyclists are shown in Table 24. The control sport drink was the one containing sucrose.

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[62] Trehalose is a disaccharide made of two glucose units with an α-1,1 glycosidic linkage (ChemIDplus Advanced, 2007f; Cerestar, 2007),

**Table 24. Constituents of sport drinks consumed by triathletes (NutriScience, 2005)**

| Constituents | "Sucromalt" % | "Sucrose" % | "Isomaltulose" % | "Isomaltulose/trehalose" % |
|---|---|---|---|---|
| Sucromalt | 12 | 0 | 0 | 0 |
| Sucrose | 0 | 9 | 0 | 0 |
| Isomaltulose | 0 | 0 | 10 | 6 |
| Trehalose | 0 | 0 | 0 | 4 |
| Water | 88 | 91 | 90 | 90 |
| Total | 100 | 100 | 100 | 100 |

Citric acid at 0.1% and orange flavor at 0.15% were included in all mixtures

During the running phase, a total of 600 ml was consumed by each participant, 150 ml at each of four approximately equally spaced times during a run of approximately 90 minutes. In the cycling phase, a total of 800 ml was consumed by each participant, in increments of 150 ml, 325 ml and 325 ml. Following each exercise, subjects answered three questionnaires; the first questionnaire dealt with the effects of the test substances on taste, aftertaste, sweetness, sourness, bitterness, mouth feel, freshness and its ability to quench thirst; the second questionnaire dealt with physiological experiences during the exercise, and included bloated feeling, stomachache, stomach cramps, vomiting, intestinal cramps and flatulence; the third addressed experiences after exercising and also included questions on gastrointestinal activity including diarrhea, burping, heartburn or mucus formation. The questionnaire scores were analyzed for differences compared to the sucrose control using statistical methods that included a Wilcoxon Signed Rank test, a Friedman test, and a Mann Whitney test. Although there were differences noted among the test substances regarding taste and sweetness during and after both running and cycling, there were no differences in gastrointestinal complaints compared to the sucrose control drink and there were no adverse effect complaints associated with any test substance.

The intent of this study was to simply identify if any test sugar produced discomfort. Thus, the test report did not specify the exact amounts of test sugars consumed by runners and cyclists; however, these amounts can be estimated based on the *percentages* of constituents in each solution consumed (Table 24), which are approximately equal to 10 g/l. Those estimated amounts of consumed test sugars are presented in Table 25 (NutriScience, 2005).

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**Table 25. Calculated amounts of test sugars consumed by runners and cyclists (NutriScience, 2005)**

| | Runners | | | Cyclists | | |
|---|---|---|---|---|---|---|
| | Amount consumed (g/day)* | Average bwt (kg) | Daily consumption (mg/kg/day) | Amount consumed (g/day) | Average bwt (kg) | Daily consumption (mg/kg/day) |
| Sucromalt (12%) | 72 | 74.5 | 0.001 | 96 | 72.5 | 0.0013 |
| Sucrose (9%) | 54 | 74.5 | 0.0007 | 72 | 72.5 | 0.001 |
| Isomaltulose (10%) | 60 | 74.5 | 0.0008 | 80 | 72.5 | 0.001 |
| Isomaltulose (6%) | 36 | 74 5 | 0.0005 | 42 | 72.5 | 0.0006 |
| Trehalose (4%) | 24 | 74.5 | 0.0003 | 32 | 72.5 | 0.0004 |

* = amounts consumed calculated by multiplying the percentages of sugars in each test drink by 10 and then multiplying that number by the number of liters consumed, *i.e.*, 0.6 l by runners and 0.8 l by cyclists; bwt = body weight.

### 7.4.3.4 Effects on postprandial glycemia and insulinemia

The comparative effects of sucromalt, isomaltulose, trehalose, glucose, and sucrose on human postprandial blood metabolites and hormones as well as on substrate oxidation rates were evaluated in10 overweight but otherwise healthy men and women (two females, eight males, ages 22-55 years, 31.1 ± 4.1 yrs; wt: 89.7 ± 4.1 kg; BMI: 27.7 ± 0.8 kg/m$^2$) (Cerestar, 2007). Following an overnight fast, subjects consumed the test substances at 8:00 AM and again at 1:00 PM as part of a standardized lunch[63] that provided 50% of measured 24-hour resting energy expenditure. The test substances were administered randomly, one *per* day, as aqueous mixtures with a carbohydrate load of 75 anhydrous equivalents in 400 ml of water. Substrate utilization and energy expenditure were measured by indirect calorimetry[64] before and for three hours after each morning and lunchtime exposure to the test substances. Expired air was collected hourly for the calculation of exogenous carbohydrate oxidation and blood was collected at post-exposure hours 0.5, 1, 1.5, 2 and 2.5 for determination of circulating metabolites and hormones, including

[63] The macronutrient composition of the standardized lunches was 55% carbohydrate, 15% fat and 30% protein, with 25% of the carbohydrate portion being in the form of the test carbohydrate ingested at 8:00 the same morning

[64] Measured using instrumentation manufactured by Omnical, a company that manufactures reaction microcalorimeters and calorimetric reaction systems for the pharmaceutical and chemical industries to enable early process screening, process development, and reactive hazards assessment. The technology is based on heatflow microcalorimetry, miniaturization, and parallel experimentation that allow rapid process evaluation at small scales. (http://www.omnicaltech.com/. Site visited 09 November 2007.)

glucose, insulin, free fatty acids and triacylglycerol, and the satiety peptides ghrelin[65] and GLP-1. The effects of sucromalt compared to sucrose are presented in Table 26. Parameters for which there were statistically significant differences between sucromalt and sucrose are denoted with an asterisk in the first column; the respective P values are presented in the second column.

**Table 26. Effects of sucromalt compared to sucrose on various metabolic parameters compared to sucrose (Cerestar, 2007)**

| Parameter | Observation |
|---|---|
| Glycemic response* | Lowered glucose peak compared to sucrose in both AM and PM. (AM: P=0.00; PM: P=0.034)<br>Lowered AUC compared to sucrose in PM but not in AM (PM: P=0.01) |
| Insulinemic response* | Lowered insulinemic peak compared to sucrose in AM but not in PM. (PM: P=0.00) |
| Free fatty acid response | No differences compared to sucrose in AM or PM |
| Triacylglycerol response | No differences compared to sucrose in AM or PM |
| Energy expenditure* | Higher peak energy expenditure compared to sucrose during PM (PM: P=0.049) |
| Respiratory quotient | No differences compared to sucrose in AM or PM |
| Total carbohydrate oxidation | No differences compared to sucrose in AM or PM |
| Exogenous carbohydrate oxidation* | Greater response compared to sucrose in AM and PM (PM: P=0.023) |
| Fat oxidation* | Greater response compared to sucrose in AM and PM (AM: P=0.00; PM: P=0.009) |
| Ghrelin* | Lower response compared to sucrose in AM (PM: P=0.018) |
| GLP-1 | No differences compared to sucrose in AM or PM |

AM = morning, following the 8:00 exposure; AUC = area under the dose-response curve; GLP-1 = glucagon-like peptide-1; PM = afternoon, following the 1:00 exposure.

These findings support previous observations of lowered glycemic and insulinemic responses following sucromalt intake compared to sucrose intake. There were no reported adverse events by study participants' in response to any of the evaluated test substances (Cerestar, 2007).

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[65] A gastrointestinal hormone produced by stomach fundal epithelial cells. They appear to stimulate appetite and feeding as well as growth hormone secretion from the anterior pituitary. (Dictionary. com. http://dictionary.reference.com/search?q=ghrelin&r=66. Site visited 02 January 2008.)

## 8.0 EVALUATION

Cargill has developed sucromalt, a product derived from sucrose and maltose that is intended for use as a low glycemic carbohydrate in various food products. Sucromalt is prepared using alternansucrase, which transfers the glucose moiety from sucrose, making it available to form oligosaccharides with maltose to which it is joined by α-(1→3) and α-(1→6) glycosidic linkages. The raw materials used in the production of sucromalt are of food-grade purity and are safe. Sucromalt contains 35-45% fructose, 7-15% leucrose, ≤5% other mono- and disaccharides, and >40% higher oligosaccharides, all determined on a dry weight basis. Sucromalt has a glycemic index that is approximately 65% that of high fructose corn syrup, which is thought attributable to the slower digestibility of α-(1,3)- and α-(1,6)-linked glucooligosaccharides.

The safety of sucromalt is supported by (1) the long-time safe presence of its constituents (glucose, fructose, maltose, leucrose, and oligosaccharides) in the human diet, (2) the digestion of these saccharides by normal metabolic pathways, and (3) the corroborative findings of *in vitro*, animal and human studies. Fructose and maltose, for example, are naturally present in the human diet in varieties of corn syrup, substances considered GRAS by the FDA with no limitations other than cGMP; and leucrose is a component of honey. The digestive and absorptive characteristics of sucromalt's components and breakdown products are well understood and are described in the published scientific literature as well as in standard biochemistry textbooks.

The results of *in vitro* and *in vivo* studies suggest that sucromalt is well digested, and the oligosaccharides present in sucromalt, which are slowly digested and absorbed, are not expected to result in adverse effects following ingestion; an expectation supported by human studies. Any undigested sucromalt oligosaccharides are not expected to be of toxicological concern because they are composed of naturally occurring monosaccharides and disaccharides - also fermented naturally by endogenous bacteria - which, if not digested/metabolized to short-chain fatty acids, will be excreted in the feces. Although nondigestible oligosaccharides may contribute to gastrointestinal discomfort (*e.g.*, bloating, stool softening) in some individuals due to their high fermentation rate and osmotic properties, these effects are common to all nondigested or fermented dietary substrates and should not be considered unique to sucromalt

Sucromalt was not mutagenic in the Ames bacterial mutagenicity assay. There were no adverse macroscopic, microscopic or clinical chemistry findings suggestive of toxicity following administration of dietary sucromalt to male and female rats for 28 days at levels up to 19,800 g/kg/day in females and 18,560 mg/kg/day in males; these levels are the NOAELs and were the highest doses tested.[66] No signs of malabsorption (measured *via* breath hydrogen) were observed in a clinical investigation in which ten healthy subjects were administered single doses of either 50 g or 80 g sucromalt. In addition, sucromalt was well tolerated by triathletes during running and cycling, with no differences is gastrointestinal effects compared to sucrose, isomaltulose and an isomaltulose/trehalose mixture; sucromalt also had no effect on human postprandial glycemia and/or insulinemia. The estimated mean *per capita* and eaters-only daily intakes of sucromalt are 890 and 1090 mg/kg/day, respectively, and the 90$^{th}$ percentile eaters-only intake is 2540 mg/kg/day. These estimates are approximately 20.9-fold, 17-fold and 7.3-fold less, respectively, than the male rat NOAEL of 18,560 mg/kg/day, the highest dose tested in a 28-day feeding study. Because sucromalt (1) is intended to replace traditional carbohydrates in food, (2) is composed of the same saccharides found in those traditional carbohydrates, and (3) does not confer different absorption, distribution, metabolism and elimination (ADME) characteristics to those saccharides, it is reasonable to compare daily consumption estimates of sucromalt and traditional carbohydrates to gain a better understanding of their relative expected contribution to daily human consumption. In this case, the common metric of mean *per capita* consumption exists for each. Comparing these data shows that the mean *per capita* consumption estimate for sucromalt (*i.e.*, 890 mg/kg/day) is approximately 30% of the USDA-derived mean *per capita* consumption estimate of cane and beet sugar, high fructose corn syrup, glucose syrups and dextrose (*i.e.*, 2933 mg/kg/day). Because (1) sucromalt's predominant constituent carbohydrates are the same as currently consumed carbohydrates, and (2) the ADME characteristics of both are the same, one can conclude that the replacement of sucromalt for those traditional dietary carbohydrates, which are consumed in much greater quantities than comparable estimates of sucromalt consumption, will not result in harm.

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[66] Because these NOAEL's represent the highest concentration tested in the 28-day feeding study, considering the lack of any observed toxic effects, they may very well have been higher if a higher experimental sucromalt concentration been fed in that study.

## 9.0 EXPERT PANEL CONCLUSION

The undersigned, an independent panel of recognized experts (the "Expert Panel") qualified by scientific training and relevant national/international experience to evaluate the safety of food ingredients, was commissioned by Cargill, Incorporated, to determine whether the proposed use of sucromalt in various foods with no limitations other than current Good Manufacturing Practices (cGMP) would entail a reasonable certainty of no harm and could be considered Generally Recognized as Safe (GRAS) through scientific procedures Sucromalt contains 35-45% fructose, 7-15% leucrose, ≤5% other mono- and disaccharides and 40-60% higher oligosaccharides. It is derived from the reaction of nine parts sucrose and one part maltose catalyzed by alternansucrase, a food-processing enzyme derived from *Leuconostoc citreum*, or a recombinant strain of *Bacillus licheniformis*, both organisms which have well established safe strain lineages. The Expert Panel, individually and collectively, critically evaluated a compilation of published and unpublished sucromalt documentation supportive of a GRAS determination, as well as other documentation deemed applicable The members of the Expert Panel conferred by telephone on several occasions and unanimously concluded that the intended uses of sucromalt, meeting appropriate food grade specification and manufactured and used consistent with cGMP are safe and suitable and Generally Recognized As Safe (GRAS) based on scientific procedures. The basis for these conclusions includes the safe strain lineage of organisms involved in enzyme production, negative Ames assay, 28-day rat study with NOAELs of 19,800 mg/kg/day (females) and 18,560 mg/kg/day (males), uncomplicated human digestion *via* well-established metabolic pathways without adverse effects, current safe consumption of constituents, and intake estimates much lower that current carbohydrate consumption.

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## 10.0 SAFE LINEAGE EVALUATION OF *LEUCONOSTOC CITREUM* STRAINS

After critical evaluation of the discussion herein regarding *Leuconostoc* species production strains, the taxonomy, fermentation profile and morphology of the mutant strains of *L. citreum*, I, Michael W. Pariza, Ph.D., conclude that safe strain lineages for *L citreum* strains NRRL B-1355, B-21297, B-30821 and B-30894 are fully demonstrated.

Michael W. Pariza, Ph.D.
Michael W. Pariza Consulting LLC

1) May 08
Date

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## APPENDIX 1 FRUITS THAT CONTAIN FRUCTOSE

| Species | Part | Quantity | Reference |
|---|---|---|---|
| *Phoenix dactylifera* L. -- Date Palm | Fruit | 308,000 ppm | Duke, 1992 |
| *Cichorium intybus* L. -- Chicory, Succory, Witloof | Root | 220,000 ppm | Duke, 1992 |
| *Allium cepa* L. -- Onion, Shallot | Bulb | 162,600 ppm | Duke, 1992 |
| *Curcuma longa* L. -- Indian Saffron, Turmeric | Rhizome | 120,000 ppm | Duke, 1992 |
| *Tamarindus indica* L. -- Indian Tamarind, Kilytree, Tamarind | Fruit | 120,000 ppm | Duke, 1992 |
| *Asimina triloba* (L.) DUNAL -- Pawpaw | Fruit | 119,658 ppm | Duke, 1992 |
| *Ceratonia siliqua* L. -- Carob, Locust Bean, St.John's-Bread | Fruit | 118,300 ppm | Duke, 1992 |
| *Solanum nigrum* L. -- Black Nightshade | Fruit | 100,000 ppm | Duke, 1992 |
| *Prunus cerasus* L. -- Sour Cherry | Fruit | 72,400 ppm | Duke, 1992 |
| *Prunus domestica* L. -- Plum | Fruit | 61,000 ppm | Duke, 1992 |
| *Malus domestica* BORKH. -- Apple | Fruit | 60,800 ppm | Duke, 1992 |
| *Vaccinium vitis-idaea var. minus* LODD -- Cowberry, Lingen, Lingonberry | Fruit | 50,000 ppm | Duke, 1992 |
| *Mangifera indica* L. -- Mango | Fruit | 48,300 ppm | Duke, 1992 |
| *Opuntia ficus-indica* (L.) MILL. -- Indian Fig, Nopal, Nopalito, Prickly Pear | Fruit | 44,100 ppm | Duke, 1992 |
| *Prunus armeniaca* L. -- Apricot | Fruit | 42,000 ppm | Duke, 1992 |
| *Ribes uva-crispa* L. -- Gooseberry | Fruit | 41,000 ppm | Duke, 1992 |
| *Ribes nigrum* L. -- Black Currant | Fruit | 36,700 ppm | Duke, 1992 |
| *Musa x paradisiaca* L. -- Banana, Plantain | Fruit | 35,000 ppm | Duke, 1992 |
| *Arnica montana* L. -- Leopard's-Bane, Mountain Tobacco | Rhizome | 25,000 ppm | Duke, 1992 |
| *Citrus sinensis* (L.) OSBECK -- Orange | Fruit | 23,800 ppm | Duke, 1992 |
| *Ananas comosus* (L.) MERR. -- Pineapple | Fruit | 23,000 ppm | Duke, 1992 |
| *Ficus carica* L. -- Echte Feige (Ger.), Feigenbaum (Ger.), Fico (Ital.), Fig, Figueira (Port.), Figuier Commun (Fr.), Higo (Sp.), Higuera Comun (Sp.) | Fruit | 22,950 ppm | Duke, 1992 |
| *Lycoris radiata* HERB. -- Spider Lily | Bulb | 21,400 ppm | Duke, 1992 |
| *Polypodium vulgare* L. -- Common Polypody, Sweet Fern | Rhizome | 21,000 ppm | Duke, 1992 |
| *Humulus lupulus* L. -- Hops | Fruit | 20,000 ppm | Duke, 1992 |

| Species | Part | Quantity | Reference |
|---|---|---|---|
| *Ribes rubrum* L. -- Red Currant, White Currant | Fruit | 19,300 ppm | Duke, 1992 |
| *Physalis ixocarpa* BROT. -- Tomatillo | Fruit | 18,980 ppm | Duke, 1992 |
| *Spondias pinnata* L. -- Yellow Mombin, Yellow Plum | Fruit | 18,000 ppm | Duke, 1992 |
| *Phoenix dactylifera* L. -- Date Palm | Seed | 15,300 ppm | Duke, 1992 |
| *Phytolacca americana* L. -- Pokeweed | Fruit | 14,000 ppm | Duke, 1992 |
| *Citrus paradisi* MacFAD -- Grapefruit | Plant | 12,400 ppm | Duke, 1992 |
| *Lycopersicon esculentum* MILLER -- Tomato | Fruit | 11,700 ppm | Duke, 1992 |
| *Terminalia chebula* RETZ. -- Black Myrobalan, Chebulic Myrobalan, Ink Nut, Myrobalan | Fruit | 10,000 ppm | Duke, 1992 |
| *Valeriana officinalis* L. -- Common Valerian, Garden-Heliotrope, Valerian | Root | 9,000 ppm | Duke, 1992 |
| *Vanilla planifolia* JACKS. -- Bourbon Vanilla, Vanilla | Fruit | 7,500 ppm | Duke, 1992 |
| *Zea mays* L. -- Corn | Fruit | 4,000 ppm | Duke, 1992 |
| *Cocos nucifera* L. -- Coconut, Coconut Palm, Cocotero (Sp.), Copra, Kokospalme (Ger.), Nariyal | Resin, Exudate, Sap | 2,100 ppm | Duke, 1992 |
| *Triticum aestivum* L. -- Wheat | Seed | 600 ppm | Duke, 1992 |

## APPENDIX 2. FOODS INCLUDED IN CONSUMPTION ESTIMATE[67]

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Milk, cow's, fluid, filled w/ veg oil, ns as to fat | 3 |
| Milk, cow's, fluid, filled w/ veg oil, whole | 3 |
| Milk, cow's, fluid, filled w/ veg oil, lowfat | 3 |
| Milk, condensed, sweetened, undiluted | 15 |
| Milk, condensed, sweetened, diluted | 15 |
| Milk, imitation, fluid, soy based | 5 |
| Milk, soy, ready-to-drink, not baby | 2 |
| Milk, soy, ready-to-drink, not baby's, chocolate | 5 |
| Milk, imitation, fluid, nonsoy, sweetened, not chocolate | 5 |
| Yogurt, ns as to type of milk/flavor | 8 |
| Yogurt, vanilla, lemon, coffee, ns as to milk type | 8 |
| Yogurt, vanilla, lemon, coffee, whole milk | 8 |
| Yogurt, vanilla, lemon, coffee, lowfat milk | 8 |
| Yogurt, vanilla, lemon, coffee, nonfat milk | 8 |
| Yogurt, vanilla, lemon, coffee, nonfat milk, low cal sweet | 8 |
| Yogurt, chocolate, ns as to type of milk | 8 |
| Yogurt, chocolate, whole milk | 8 |
| Yogurt, chocolate, nonfat milk | 8 |
| Yogurt, fruit variety, ns as to milk type | 8 |
| Yogurt, fruit variety, whole milk | 8 |
| Yogurt, fruit variety, lowfat milk | 8 |
| Yogurt, fruit variety, nonfat milk | 8 |
| Yogurt, fruited, nonfat milk, low cal sweetener | 8 |
| Yogurt, fruit & nuts, ns as to type of milk | 8 |
| Yogurt, fruit & nuts, lowfat milk | 8 |
| Yogurt, frozen, ns as to flavor, ns to type of milk | 15 |
| Yogurt, frozen, not chocolate, type of milk ns | 15 |
| Yogurt, frozen, chocolate, type of milk ns | 15 |
| Yogurt, frozen, ns as to flavor, lowfat milk | 15 |
| Yogurt, frozen, chocolate, lowfat milk | 15 |
| Yogurt, frozen, not chocolate, lowfat milk | 15 |
| Yogurt, frozen, ns as to flavor, nonfat milk | 15 |
| Yogurt, frozen, chocolate, nonfat milk | 15 |
| Yogurt, frozen,not chocolate,w/ sorbet/sorbet-coated | 15 |
| Yogurt, frozen, not chocolate, nonfat milk | 15 |
| Yogurt, frozen, ns as to flavor, whole milk | 15 |

[67] U.S. Department of Agriculture, Agriculture Research Service (2006) What We Eat In America, NHANES 2003-2004: Documentation and Data Files. Available: http://www.ars.usda.gov/ba/bhnrc/fsrg and http://www.cdc.gov/nchs/about/major/nhanes/nhanes2003-2004/nhanes03-04.htm.

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Yogurt, frozen, chocolate, whole milk | 15 |
| Yogurt, frozen, not chocolate, whole milk | 15 |
| Yogurt, frozen, chocolate-coated | 15 |
| Yogurt, frozen, carob-coated | 15 |
| Yogurt, frozen, sandwich | 15 |
| Yogurt, frozen, cone, chocolate | 15 |
| Yogurt, frozen, cone, not chocolate | 15 |
| Yogurt, frozen, cone, not chocolate, lowfat milk | 15 |
| Yogurt, froz, cone, chocolate, lowfat milk | 15 |
| Milk, chocolate, nfs | 10 |
| Milk, chocolate, whole milk based | 10 |
| Milk, chocolate, red fat, 2% | 10 |
| Milk, chocolate, skim milk based | 10 |
| Milk, chocolate, lowfat milk-based | 10 |
| Spanish-style hot chocolate drink, puerto rican style, made | 5 |
| Cocoa & sugar mixture, skim milk added | 10 |
| Chocolate syrup milk added, ns as to type of milk | 25 |
| Chocolate syrup, whole milk added | 25 |
| Chocolate syrup, red fat milk added | 25 |
| Chocolate syrup, lowfat milk added | 25 |
| Chocolate syrup, skim milk added | 25 |
| Milk beverage, not chocolate, w/ whole milk | 5 |
| Milk, not chocolate, whole milk based | 5 |
| Milk, malted, unfortified, flavor ns | 10 |
| Milk, malted, unfortified, chocolate flavor | 10 |
| Milk, malted, unfortified, choc, made w/ skim milk | 10 |
| Milk, malted, unfortified, natural flavor | 10 |
| Milk,malted,fortified,natural flavor (incl ovaltine | 10 |
| Milk, malted, fortified, chocolate (incl ovaltine) | 10 |
| Milk, malted, fortified, (incl ovaltine) | 10 |
| Eggnog, made w/ whole milk (include egg nog, nfs) | 8 |
| Eggnog, made w/ 2% reduced fat milk | 8 |
| Milk shake, ns as to flavor or type | 11 |
| Milk shake,homemade/ fountain-type, ns as to flavor | 11 |
| Milk shake, homemade or fountain-type, chocolate | 11 |
| Milk shake, homemade/fountain-type, not chocolate | 11 |
| Milk shake with malt (incl malted milk w/ice cream) | 11 |
| Milk shake, made w/ skim milk, chocolate | 11 |
| Milk shake,made w/ skim milk, not chocolate | 11 |
| Carry-out milk shake, ns as to flavor | 11 |
| Carry-out milk shake, chocolate | 11 |
| Carry-out milk shake, not chocolate | 11 |
| Milk fruit drink (incl licuado) | 11 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Orange julius | 11 |
| Fruit smoothie drink, w/ fruit and dairy products | 5 |
| Fruit smoothie drink, nfs | 5 |
| Choc-flavored drink, whey-&milk-based(incl yoo-hoo) | 7.5 |
| Milk drink, whey&milk-base, not choc (incl yoo-hoo) | 7.5 |
| Cafe con leche prepared w/ sugar | 5 |
| Cream, heavy, whipped, sweetened | 8 |
| Cream, whipped, pressurized container | 8 |
| Whipped topping, dairy based, fat free, pressurized containe | 8 |
| Cream substitute, ns as to frozen,liquid or powder | 11 |
| Cream substitute, frozen | 11 |
| Cream substitute, liquid (include coffee whitner) | 11 |
| Cream substitute, light, liquid | 11 |
| Whipped topping, nondairy, ns as to cnd/frz/powder | 8 |
| Whipped topping, nondairy, pressurized can | 8 |
| Whipped topping, nondairy, frozen (incl cool whip) | 8 |
| Whipped topping, nondairy, fzn, lowfat (incl cool) | 8 |
| Dip, sour cream base (include buttermilk-type dip) | 3 |
| Dip, sour cream base, reduced calorie | 3 |
| Ice cream, nfs | 20 |
| Ice cream, regular, not chocolate | 20 |
| Ice cream, regular, chocolate | 20 |
| Ice cream, rich, flavors other than chocolate | 20 |
| Ice cream, rich, chocolate | 20 |
| Ice cream, rich, ns as to flavor | 20 |
| Ice cream, soft serve, not chocolate | 20 |
| Ice cream, soft serve, chocolate | 20 |
| Ice cream, soft serve, ns as to flavor | 20 |
| Ice cream bar or stick, not choc- or cake-covered | 9 |
| Ice cream bar/stick, chocolate covered | 9 |
| Ice cream bar, chocolate/caramel covered, w/ nuts | 9 |
| Ice cream bar,rich choc ice cream,thick choc cover | 9 |
| Ice cream bar,rich ice cream,thick choc cover | 9 |
| Ice cream bar/stick,rich ice cream,choc cover,w/nut | 9 |
| Ice cream bar/stick, choc ice cream, choc cover | 9 |
| Ice cream bar, cake-covered | 9 |
| Ice cream bar/stick w/ fruit | 9 |
| Ice cream sandwich | 9 |
| Ice cream cookie sandwich (include chipwich) | 9 |
| Ice cream cone, w/ nuts, not chocolate | 9 |
| Ice cream cone, choc-covered, w/ nuts, not choc | 9 |
| Ice cream cone, choc-covered or dipped, not choc | 9 |
| Ice cream cone, no topping, not chocolate | 9 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Ice cream cone, no topping, ns as to flavor | 9 |
| Ice cream cone, w/nuts, chocolate ice cream | 9 |
| Ice cream cone, choc-covered, choc ice cream | 9 |
| Ice cream cone, no topping, chocolate ice cream | 9 |
| Ice cream cone, choc-covered, w/nut, choc ice cream | 9 |
| Ice cream sundae cone (incl drumstick, all flavors) | 9 |
| Ice cream soda, not chocolate | 9 |
| Ice cream soda, chocolate | 9 |
| Ice cream sundae, topping ns, w/ whipped cream | 9 |
| Ice cream sundae, fruit topping, w/ whipped cream | 9 |
| Ice cream sundae, prepackaged, not chocolate | 9 |
| Ice cream sundae,chocolate topping,w/ whipped cream | 9 |
| Ice cream sundae, not fruit/ choc top,w/ whip cream | 9 |
| Ice cream sundae, fudge topping, w/ cake | 9 |
| Ice cream pie, no crust | 9 |
| Ice cream pie,cookie crust,fudge topping,whip cream | 9 |
| Ice cream, fried | 9 |
| Lt ice cream, ns flav ( ice milk) | 9 |
| Light ice cream,not chocolate (formerly ice milk) | 9 |
| Light ice cream,chocolate (formerly ice milk) | 9 |
| Light ice cream,soft serve, ns flavor (formerly ice milk) | 11 |
| Light ice cream,soft serve, not choc (formerly ice milk) | 11 |
| Light ice cream,soft serve choc (tastee frz, dairy queen) | 11 |
| Light ice cream,soft serve cone,not choc (dairy queen) | 11 |
| Light ice cream,soft serve cone, choc (formerly ice milk) | 11 |
| Light ice cream,soft serve cone, ns flav(formerly ice milk) | 11 |
| Light ice cream,sandwich (dairy queen) (formerly ice milk) | 11 |
| Light ice cream,bar/stick, choc-coated (formerly ice milk) | 11 |
| Light ice cream,bar, choc covered,w/nuts (formerly ice milk) | 11 |
| Light ice cream,cone, nfs (formerly ice milk) | 11 |
| Light ice cream,cone, not chocolate (formerly ice milk) | 11 |
| Light ice cream,cone, chocolate (formerly ice milk) | 11 |
| Light ice cream,sundae,soft serve,choc/fudge top (ice milk) | 11 |
| Light ice cream,sundae,soft serve,fruit topping (ice milk) | 11 |
| Light ice cream,sundae,soft serve,not fruit/choc topping | 11 |
| Light ice cream,sundae,choc / fudge top (w/o whip cream) | 11 |
| Light ice cream,sundae,fruit top (w/o whip cream)(ice milk) | 11 |
| Light ice cream,sundae,no fruit/choc top (w/o whip cream) | 11 |
| Light ice cream,creamsicle or dreamsicle (formerly ice milk) | 11 |
| Light ice cream,fudgesicle (formerly ice milk) | 11 |
| Milk dessert bar/stick, frozen, w/ coconut | 11 |
| Sherbet, all flavors | 11 |
| Fat free ice cream, flavors other than choc | 11 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Fat free ice cream, choc | 11 |
| Fat free ice cream, ns as to flavor | 11 |
| Milk dessert bar, frozen, made from lowfat milk | 11 |
| Milk dessert sandwich bar, frozen, dietary | 11 |
| Milk dessert sandwich bar,frz,w/low-cal sweet,lofat | 11 |
| Milk des bar, frozen, lofat milk&lo cal sweetener | 11 |
| Light ice cream,bar/stick, w/ low-cal sweetener, choc coat | 11 |
| Pudding, nfs | 18 |
| Pudding, bread (include w/ raisins) | 10 |
| Puerto rican bread pudding made w/ evap milk & rum | 10 |
| Diplomat pudding, p.r. (budin diplomatico) | 10 |
| Pudding, mexican bread (capirotada) | 10 |
| Pudding, mexican bread (capirotada), lower fat | 10 |
| Pudding, chocolate, rte, ns from dry mix or canned | 10 |
| Rice flour cream, p.r. (majarete, manjar blanco) | 10 |
| Spanish custard, p.r. (natilla espanol) | 10 |
| Pudding, not choc, rte, ns from dry mix or canned | 10 |
| Custard | 15 |
| Custard, puerto rican style (flan) | 15 |
| Pudding, rice | 10 |
| Pudding, tapioca,chocolate,made w/ milk | 15 |
| Pudding, coconut | 15 |
| Pudding, indian (milk, molasses, cornmeal-based) | 15 |
| Pudding, pumpkin | 15 |
| P.r. pumpkin pudding (flan de calabaza) | 10 |
| Pudding, can, choc, reduced fat (incl jell-o light) | 10 |
| Pudding, can, choc, fat free | 10 |
| Pudding, canned, not choc, reduced fat | 10 |
| Pudding, canned, not choc, fat free | 10 |
| Pudding, canned, not chocolate | 10 |
| Pudding, canned, chocolate | 10 |
| Pudding, canned, choc & non-choc flavors combined | 10 |
| Pudding, canned, tapioca | 10 |
| Pudding, canned, tapioca, fat free | 10 |
| Pudding, w/ fruit & vanilla wafers | 10 |
| Mousse, chocolate | 10 |
| Mousse, not chocolate | 10 |
| Coconut custard, p.r. (flan de coco) | 10 |
| Milk dessert or milk candy, p.r. (dulce de leche) | 15 |
| Barfi/burfi,indian dessert,from milk/cream/ricotta | 15 |
| Tiramisu | 10 |
| Hoisin sauce | 12.5 |
| Tofu yogurt | 8 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Teriyaki sauce (include oriental barbecue sauce) | 10 |
| Teriyaki sauce, reduced sodium | 10 |
| Worcestershire sauce | 5 |
| Tofu frozen dessert, not chocolate (incl tofutti) | 12.5 |
| Tofu frozen dessert, chocolate (include tofutti) | 12.5 |
| Energy bars (like a power bar) | 50 |
| Meal replacement bars (like slim-fast) | 50 |
| Bread, ns as to major flour | 3 |
| Bread, ns as to major flour, toasted | 3 |
| Bread, homemade/purch at a bakery, ns as to flour | 3 |
| Bread, homemade/purch at a bakery, toastd,ns flour | 3 |
| Roll, ns as to major flour | 3 |
| Roll, ns as to major flour, toasted | 3 |
| Roll, homemade/purch at a bakery, ns as to flour | 3 |
| Roll, homemade/purch at a bakery, toastd,ns flour | 3 |
| Roll, hard, ns as to major flour | 3 |
| Roll, bran, ns as to type of bran | 3 |
| Roll, bran, ns as to type of bran, toasted | 3 |
| Bread, cinnamon | 5 |
| Bread, cinnamon, toasted | 5 |
| Bread, cornmeal and molasses | 5 |
| Bread, cornmeal and molasses, toasted | 5 |
| Bread, egg, challah | 5 |
| Bread, egg, challah, toasted | 5 |
| Bread, lowfat, 98% fat free | 2 |
| Bread, lowfat, 98% fat free, toasted | 2 |
| Bread, reduced calorie/ high fiber, w/ fruit/nuts | 2 |
| Bread, reduced calorie/hi fiber, w/fruit/nuts,toast | 2 |
| Bread, milk & honey (include arnold's) | 3 |
| Bread, milk & honey, toasted (include arnold's) | 3 |
| Bread, raisin | 3 |
| Bread, raisin, toasted | 3 |
| Bread, sweetpotato | 3 |
| Bread dough, fried | 3 |
| Roll, egg bread | 3 |
| Roll, egg bread, toasted | 3 |
| Roll, hoagie, submarine, | 2 |
| Roll, hoagie, submarine, toasted | 2 |
| Roll, mexican, bolillo | 2 |
| Roll, sweet | 5 |
| Roll, sweet, toasted | 5 |
| Roll, sweet, cinnamon bun, no frosting | 5 |
| Roll, sweet, cinnamon bun, frosted | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Roll, sweet, w/ fruit, no frosting | 5 |
| Roll, sweet, w/ fruit, frosted | 10 |
| Roll, sweet, w/ fruit, frosted, diet | 5 |
| Roll, sweet, w/ nuts, frosted | 10 |
| Roll, sweet, w/ fruit, frosted, fat free | 10 |
| Roll, sweet, w/ fruit & nuts, no frosting | 5 |
| Roll, sweet, w/ fruit & nuts, frosted | 10 |
| Roll, sweet, w/ nuts, no frosting | 5 |
| Roll, sweet, no topping, mexican (pan dulce) | 5 |
| Roll sweet, crumb topping, mexican (pan dulce) | 10 |
| Roll, sweet, sugar topping, mexican (pan dulce) | 10 |
| Roll,sweet,w/ raisins & icing,mexican (pan dulce) | 5 |
| Coffee cake, yeast type | 5 |
| Coffee cake, yeast, homemade/purch at a bakery | 5 |
| Coffee cake, yeast type, fat free, chol free, w/ fruit | 5 |
| Croissant, chocolate | 5 |
| Croissant, fruit | 5 |
| Brioche | 2 |
| Coffee bread, spanish | 5 |
| Bagel | 5 |
| Bagel, toasted | 5 |
| Bagel, w/ raisins | 5 |
| Bagel, w/ raisins, toasted | 5 |
| Bagel w/ fruit other than raisins | 5 |
| Bagel w/ fruit other than raisins, toasted | 5 |
| Croutons | 2 |
| Muffin, english (include sour dough) | 3 |
| Muffin, english, toasted | 3 |
| Muffin, english, w/ raisins | 4 |
| Muffin, english, w/ raisins, toasted | 4 |
| Muffin, english, cheese | 4 |
| Muffin, english, cheese, toasted | 4 |
| Muffin, english, w/ fruit other than raisins | 4 |
| Muffin, english, w/ fruit other than raisins, tstd | 4 |
| Pannetone (italian-style sweet bread) | 5 |
| Bread, 100% whole wheat | 2 |
| Bread, 100% whole wheat, toasted | 2 |
| Bread, 100% whole wheat, home-made | 2 |
| Bread, 100% whole wheat, home-made, toasted | 2 |
| Bread, 100% whole wheat, w/ raisins | 2 |
| Bread, 100% whole wheat, w/ raisins, toasted | 2 |
| Bread, pita, 100% whole wheat | 2 |
| Bread, pita, 100% whole wheat, toasted | 2 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Muffin, english, 100% whole wheat | 2 |
| Muffin, english, 100% whole wheat, toasted | 2 |
| Muffin, english, 100% whole wheat, w/ raisins | 2 |
| Muffin, english, whole wheat, w/ raisins, toasted | 2 |
| Bread, wheat germ | 2 |
| Bread, wheat germ, toasted | 2 |
| Bread, sprouted wheat | 2 |
| Bread, sprouted wheat, toasted | 2 |
| Bagel, 100% whole wheat | 2 |
| Bagel, 100% whole wheat, toasted | 2 |
| Bagel, 100% whole wheat, w/ raisins | 2 |
| Bagel, 100% whole wheat, w/ raisins, toasted | 2 |
| Roll, 100% whole wheat | 2 |
| Roll, 100% whole wheat, toasted | 2 |
| Roll, 100% whole wheat, home recipe/bakery | 2 |
| Roll, 100% whole wheat, home recipe/bakery, toasted | 2 |
| Bread, whole wheat, other than 100%/ns as to 100% | 2 |
| Bread, whole wheat, other than 100%/ns 100%, toast | 2 |
| Bread, "whole" wheat, home recipe/bakery | 2 |
| Bread, "whole" wheat, home recipe/bakery, toasted | 2 |
| Bread,puri/poori(indian puff bread)ww,ns 100%,fried | 2 |
| Bread, whole wheat, ns as to 100%, w/ raisins | 2 |
| Bread, whole wheat,ns as to 100%,w/ raisins,toasted | 2 |
| Bread, wheat or cracked wheat | 2 |
| Bread, wheat or cracked wheat, toasted | 2 |
| Bread, cracked wheat, home recipe/bakery | 2 |
| Bread, cracked wheat, home recipe/bakery, toasted | 2 |
| Bread, wheat or cracked wheat, w/ raisins | 2 |
| Bread, wheat or cracked wheat, w/ raisins, toasted | 2 |
| Bread, cracked wheat, reduced calorie/ high fiber | 2 |
| Bread, cracked wheat, red calorie/ hi fiber, toast | 2 |
| Bread, french, "whole" wheat, homemade | 2 |
| Bread, french, "whole" wheat, homemade, toasted | 2 |
| Bread, pita, "whole" wheat | 2 |
| Bread, pita, "whole" wheat, toasted | 2 |
| Bread, pita, cracked wheat | 2 |
| Bread, pita, cracked wheat, toasted | 2 |
| Bagel, wheat | 2 |
| Bagel, wheat, toasted | 2 |
| Bagel, "whole" wheat | 2 |
| Bagel, "whole" wheat, toasted | 2 |
| Bagel, wheat, w/ raisins | 2 |
| Bagel, wheat, w/ raisins, toasted | 2 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Bagel, wheat, w/ fruits & nuts | 2 |
| Bagel, wheat, w/ fruits & nuts, toasted | 2 |
| Bagel, wheat bran | 2 |
| Bagel, wheat bran, toasted | 2 |
| Bread, wheat bran | 2 |
| Bread, wheat bran, toasted | 2 |
| Bread, wheat bran, w/ raisins | 2 |
| Bread, wheat bran, w/ raisins, toasted | 2.5 |
| Muffin, english, wheat bran | 2.5 |
| Muffin, english, wheat bran, toasted | 2.5 |
| Muffin, english, wheat bran, w/ raisins | 2.5 |
| Muffin, english, wheat bran, w/ raisins, toasted | 2.5 |
| Muffin, english, wheat or cracked wheat | 2 5 |
| Muffin, english, wheat or cracked wheat, toasted | 2.5 |
| Muffin, english, "whole" wheat | 2.5 |
| Muffin, english, "whole" wheat, toasted | 2.5 |
| Muffin, english, wheat or cracked wheat w/ raisins | 2.5 |
| Muffin, english, wheat w/ raisins, toasted | 2.5 |
| Muffin, english, "whole" wheat, w/ raisins | 2.5 |
| Muffin, english, "whole" wheat, w/ raisins, toasted | 2.5 |
| Roll, whole wheat, ns as to 100% | 2 |
| Roll, whole wheat, ns as to 100%, toasted | 2 |
| Roll, "whole" wheat, home recipe/bakery | 2 |
| Roll, "whole" wheat, home recipe/bakery, toasted | 2 |
| Bread, rye | 2 |
| Bread, rye, toasted | 2 |
| Bread, marble rye & pumpernickel | 2 |
| Bread, marble rye & pumpernickel, toasted | 2 |
| Bread, rye, reduced calorie/ high fiber (incl less) | 2 |
| Bread, rye, reduced calorie/ high fiber, toasted | 2 |
| Muffin, english, rye | 2 |
| Muffin, english, rye, toasted | 2 |
| Bread, pumpernickel | 2 |
| Bread, pumpernickel, toasted | 2 |
| Bagel, pumpernickel | 2 |
| Bagel, pumpernickel, toasted | 2 |
| Muffin, english, pumpernickel | 2 |
| Muffin, english, pumpernickel, toasted | 2 |
| Bread, black | 2 |
| Bread, black, toasted | 2 |
| Roll, rye | 2 |
| Roll, pumpernickel | 2 |
| Roll, pumpernickel, toasted | 2 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Bread, oatmeal | 5 |
| Bread, oatmeal, toasted | 5 |
| Bread, oat bran | 5 |
| Bread, oat bran, toasted | 5 |
| Bread, oat bran, reduced calorie/ high fiber | 5 |
| Bread, oat bran reduced calorie/hi fiber, toasted | 5 |
| Bagel, oat bran | 5 |
| Bagel, oat bran, toasted | 5 |
| Roll, oatmeal | 2 |
| Roll, oatmeal, toasted | 2 |
| Roll, oat bran | 2 |
| Roll, oat bran, toasted | 2 |
| Muffin, english, oat bran | 3 |
| Muffin, english, oat bran, toasted | 3 |
| Muffin, english, oat bran, with raisins | 3 |
| Muffin, english, oat bran, w/ raisins, toasted | 3 |
| Bread, multigrain, toasted | 3 |
| Bread, multigrain | 3 |
| Bread, multigrain, w/ raisins | 3 |
| Bread, multigrain, w/ raisins, toasted | 3 |
| Bread, multigrain, reduced calorie/ high fiber | 3 |
| Bread, multigrain, reduced calorie/ hi fiber, toast | 3 |
| Roll, multigrain | 2 |
| Roll, multigrain, toasted | 2 |
| Bagel, multigrain | 3 |
| Bagel, multigrain, toasted | 3 |
| Bagel, multigrain, w/ raisins | 3 |
| Bagel, multigrain, w/ raisins, toasted | 3 |
| Muffin, english, multigrain | 2.5 |
| Muffin, english, multigrain, toasted | 2.5 |
| Bread, barley | 2.5 |
| Bread, triticale | 2.5 |
| Bread, triticale, toasted | 2.5 |
| Bread, buckwheat | 2.5 |
| Bread, buckwheat, toasted | 2.5 |
| Bread, soy | 2.5 |
| Bread, soy, toasted | 2.5 |
| Bread, sunflower meal | 2.5 |
| Bread, sunflower meal, toasted | 2.5 |
| Bread, rice | 2.5 |
| Bread, rice, toasted | 2.5 |
| Bread, low gluten | 2.5 |
| Bread, low gluten, toasted | 2.5 |

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| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Biscuit, baking powder or buttermilk type, nfs | 2.5 |
| Biscuit dough, raw | 2.5 |
| Biscuit dough, fried | 2.5 |
| Crumpet | 2.5 |
| Crumpet, toasted | 2.5 |
| Biscuit, baking powder or buttermilk, from mix | 2.5 |
| Biscuit,baking pwr/butter milk,refrig dough,lowfat | 2.5 |
| Biscuit, bak powder or buttermilk, from refrg dough | 2.5 |
| Biscuit, baking powder/buttermilk type, commercially baked | 2.5 |
| Biscuit, baking powder or buttermilk, homemade | 2.5 |
| Biscuit, whole wheat | 2.5 |
| Biscuit, cheese | 2.5 |
| Biscuit, cinnamon-raisin | 2.5 |
| Scones | 5 |
| Scones, whole wheat | 5 |
| Scone, with fruit | 5 |
| Cornbread, prepared from mix | 5 |
| Cornbread, homemade | 5 |
| Cornbread, made with egg substitute, homemade | 5 |
| Cornbread stuffing | 5 |
| Cornbread muffin, stick, round | 5 |
| Cornbread muffin, stick, round, toasted | 5 |
| Cornbread muffin, stick, round, homemade | 5 |
| Johnnycake | 10 |
| Tortilla, flour (wheat) | 3 |
| Tortilla, whole wheat | 3 |
| Cornmeal bread, dominican | 5 |
| Muffin, nfs | 10 |
| Muffin, fruit & or nuts | 10 |
| Muffin, fruit, fat free, chol free | 10 |
| Muffin, chocolate chip | 10 |
| Muffin, chocolate | 10 |
| Muffin, whole wheat | 10 |
| Muffin, wheat | 10 |
| Muffin, buckwheat | 10 |
| Muffin, wheat bran (include w/ raisins & nuts) | 10 |
| Muffin,bran,w/ fruit, lowfat | 10 |
| Muffin, bran w/ fruit, no fat, no cholesterol | 10 |
| Muffin, oatmeal | 10 |
| Muffin, oat bran | 10 |
| Muffin, oat bran with fruit and/or nuts | 10 |
| Muffin, plain | 10 |
| Muffin, cheese | 5 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Muffin, pumpkin, w/ raisins | 10 |
| Muffin, zucchini | 10 |
| Muffin, carrot (incl w/ raisins/nuts) | 10 |
| Muffin, multigrain, w/ nuts | 10 |
| Muffin, multigrain, with fruit | 10 |
| Toaster muffin, fruit, untoasted | 5 |
| Toaster muffin, fruit, toasted | 5 |
| Bread, boston brown | 3 |
| Bread, nut | 10 |
| Bread, pumpkin (include w/ raisins) | 10 |
| Bread, fruit, w/o nuts | 10 |
| Bread, fruit & nut | 10 |
| Bread, whole wheat, w/ nuts | 3 |
| Bread, zucchini (incl squash bread; w/ nuts) | 10 |
| Bread, irish soda | 5 |
| Cake, batter, chocolate, raw | 15 |
| Cake, batter, raw, not chocolate | 15 |
| Cake, ns as to type, w/ or w/o icing | 15 |
| Cake, angel food, ns as to icing | 15 |
| Cake, angel food, w/o icing | 15 |
| Cake, angel food, w/ icing | 15 |
| Cake, angel food, w/ fruit & icing/filling | 15 |
| Cake, angel food, choc, without icing | 15 |
| Cake, applesauce, ns as to icing | 15 |
| Cake, applesauce w/o icing | 15 |
| Cake, applesauce w/ icing | 15 |
| Cake, applesauce, diet, without icing | 15 |
| Cake, banana, ns as to icing | 15 |
| Cake, banana, w/o icing | 15 |
| Cake, banana, w/ icing | 15 |
| Cake, black forest (choc-cherry) | 15 |
| Cake, boston cream pie | 15 |
| Cake, butter, ns as to icing | 15 |
| Cake, butter, w/o icing | 15 |
| Cake, butter, w/ icing | 15 |
| Cake, carrot, ns as to icing | 15 |
| Cake, carrot, no icing | 15 |
| Cake, carrot, w/ icing | 15 |
| Carrot cake, diet | 15 |
| Cake, coconut, w/ icing | 15 |
| Cheesecake | 15 |
| Cheesecake, diet | 15 |
| Cheesecake, w/ fruit | 15 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Cheesecake, diet, with fruit | 15 |
| Cheesecake-type dessert, made w/ yogurt, w/ fruit | 15 |
| Cheesecake, chocolate | 15 |
| Cheesecake, chocolate, reduced fat | 15 |
| Cake, choc, made w/ mayonnaise, ns as to icing | 15 |
| Cake, choc, made w/ mayonnaise, w/o icing | 15 |
| Cake, choc, made w/ mayonnaise, w/ icing or filling | 15 |
| Cake,choc,devil's food/fudge,stndrd mix,ns icing | 15 |
| Cake, choc, devil's food/fudge, homemade, ns icing | 15 |
| Cake,choc,devils food/fudge,stndrd mix, w/o icing | 15 |
| Cake,choc,devil's food/fudge, w/o icing, homemade | 15 |
| Cake,choc,devil's food/fudge,stndrd mix,w/icing | 15 |
| Cake,choc,devil's food/fudge,w/icing, homemade | 15 |
| Cake, german choc, w/ icing and filling | 15 |
| Cake, choc, w/ icing, diet | 15 |
| Cake,choc/dvl fd, pudd mix, lite recipe, w/ icing | 15 |
| Cake,choc,devil food/fudge,pud type.no chol,ns icin | 15 |
| Cake,choc,devil food/fudge,pud type,no chol,no icin | 15 |
| Cake,choc,devil food/fudge,pud type,no chol, | 15 |
| Cake, choc,devil's food/fudge,pudding mix, ns icing | 15 |
| Cake,choc,devil's food/fudge,pudding mix, w/o icing | 15 |
| Cake, choc,devil's food/fudge, pudding mix, w/icing | 15 |
| Cake, poor man's (spice-type), w/o icing | 15 |
| Cake, cream, w/o icing or topping | 15 |
| Cake, cupcake, ns as to type and icing | 15 |
| Cake, cupcake, ns as to type, w/o icing | 15 |
| Cake, cupcake, ns as to type, w/icing | 15 |
| Cake, cupcake, chocolate, ns as to icing | 15 |
| Cake, cupcake, chocolate, w/o icing | 15 |
| Cake, cupcake, chocolate, w/ icing or filling | 15 |
| Cake, cupcake, not chocolate, ns as to icing | 15 |
| Cake, cupcake, not chocolate, w/o icing or filling | 15 |
| Cake, cupcake, not chocolate, w/ icing or filling | 15 |
| Cake, cupcake, not choc, w/ icing, lowfat, no chol | 15 |
| Cupcake, not chocolate, w/ fruit & cream filling | 15 |
| Cupcake,choc,w/orw/o icing,fruit/cream fill,lowfat | 15 |
| Cake,dobos torte(non-choc cake w/choc fill & icing) | 15 |
| Cake, fruitcake, light/dark, holiday type cake | 15 |
| Cake, plum pudding | 15 |
| Cake, gingerbread, w/o icing | 15 |
| Cake, graham cracker, w/o icing | 15 |
| Cake, ice cream & cake roll, chocolate | 15 |
| Cake, ice cream & cake roll, not chocolate | 15 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Cake,frozen yogurt & cake layer,not choc,w/ icing | 15 |
| Cake,frozen yogurt & cake layer,chocolate,w/ icing | 15 |
| Cake, ice box, w/ fruit & whipped cream | 15 |
| Cake, jelly roll | 15 |
| Cake, lemon, ns as to icing | 15 |
| Cake, lemon, w/o icing | 15 |
| Cake, lemon, w/ icing | 15 |
| Cake, lemon, low fat, ns as to icing | 15 |
| Cake, lemon, low fat, w/o icing | 15 |
| Cake, lemon, low fat, w/ icing | 15 |
| Cake, marble, ns as to icing | 15 |
| Cake, marble, w/o icing | 15 |
| Cake, marble, w/ icing | 15 |
| Cake, nut, ns as to icing | 15 |
| Cake, nut, w/o icing | 15 |
| Cake, nut, w/ icing | 15 |
| Cake, oatmeal, w/o icing | 15 |
| Cake, oatmeal, w/icing | 15 |
| Cake, peanut butter, with icing | 15 |
| Cake,pineapple,fat free,w/ icing | 15 |
| Cake, poppyseed, w/o icing | 15 |
| Cake, pound, w/o icing | 15 |
| Cake, pound, w/ icing | 15 |
| Cake, pound, chocolate | 15 |
| Cake, pound, choc, fat free, no chol | 15 |
| Cake, pound, p.r. (ponque) | 15 |
| Cake, pound, fat free, no chol | 15 |
| Cake, pound, reduced fat, no cholesterol | 15 |
| Cake,pumpkin,ns as to icing | 15 |
| Cake, pumpkin, w/o icing | 15 |
| Cake,pumpkin,w/ icing | 15 |
| Cake, raisin-nut, w/o icing | 15 |
| Cake, raisin-nut, w/ icing | 15 |
| Cake, ravani (made w/ farina) | 15 |
| Cake, rice flour, w/o icing | 15 |
| Cake, quezadilla, el salvadorian style | 15 |
| Cake, soy flour, w/o icing | 15 |
| Cake, spice, ns as to icing | 15 |
| Cake, spice, w/o icing | 15 |
| Cake, spice, w/ icing | 15 |
| Cake, sponge, ns as to icing | 15 |
| Cake, sponge, w/o icing | 15 |
| Cake, sponge, w/ icing | 15 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Cake, sponge, chocolate, w/o icing | 15 |
| Cake, sponge, chocolate, w/ icing | 15 |
| Cake, sweetpotato, w/ icing (incl with glaze) | 15 |
| Cake, rum flavored w/o icing | 15 |
| Cake, torte | 15 |
| Cake, chiffon, ns as to icing | 15 |
| Cake, chiffon, w/o icing | 15 |
| Cake, chiffon, w/ icing | 15 |
| Cake, chiffon, chocolate, w/o icing | 15 |
| Cake, chiffon, chocolate, w/ icing | 15 |
| Cake, upside down (all fruits) | 15 |
| Cake, white, standard mix, ns as to icing | 15 |
| Cake, white, homemade, ns as to icing | 15 |
| Cake, white, standard mix, w/o icing | 15 |
| Cake, white, w/o icing, homemade | 15 |
| Cake, white, standard mix, w/ icing | 15 |
| Cake, white, w/ icing, homemade | 15 |
| Cake, white, pudding-type mix, ns as to icing | 15 |
| Cake, white, pudding-type mix, w/o icing | 15 |
| Cake, white, pudding-type mix, w/ icing | 15 |
| Cake, white, eggless, lowfat | 15 |
| Cake, whole wheat, w/ fruit & nuts, w/o icing | 15 |
| Cake, yellow, standard mix, ns as to icing | 15 |
| Cake, yellow, homemade, ns as to icing | 15 |
| Cake, yellow, standard mix, w/o icing | 15 |
| Cake, yellow, w/o icing, homemade | 15 |
| Cake, yellow, standard mix, w/ icing | 15 |
| Cake, yellow, w/ icing, homemade | 15 |
| Cake, yellow, pudding mix, ns as to icing | 15 |
| Cake, yellow, pudding mix, w/o icing | 15 |
| Cake, yellow, pudding mix, w/ icing | 15 |
| Cake, shortcake, biscuit, w/ whipped cream & fruit | 15 |
| Cake, shortcake, biscuit, w/ fruit | 15 |
| Cake, shortcake, sponge, w/ whipped cream & fruit | 15 |
| Cake, shortcake, sponge, w/ fruit | 15 |
| Cake, shortcake, w/ whip topping & fruit, diet | 15 |
| Cake, zucchini, ns as to icing | 15 |
| Cake, zucchini, w/o icing | 15 |
| Cake, zucchini, w/ icing | 15 |
| Cookie, batter / dough, raw, not chocolate | 10 |
| Cookie, ns as to type | 10 |
| Cookie, almond | 10 |
| Cookie, applesauce | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Cookie, fruit, baby | 10 |
| Cookie, baby (incl gerber animal shaped cookies) | 10 |
| Cookie, biscotti | 10 |
| Cookie, brownie, ns as to icing | 10 |
| Cookie, brownie, w/o icing | 10 |
| Cookie, brownie, w/ icing | 10 |
| Cookie, brownie, w/ cream cheese filling, w/o icing | 10 |
| Cookie, brownie, w/ peanut butter fudge icing | 10 |
| Brownie, diet, ns as to icing | 10 |
| Cookie, brownie, lowfat, w/ icing | 10 |
| Cookie, brownie, lowfat, w/o icing | 10 |
| Cookie, brownie, fat free, no chol, w/ icing | 10 |
| Cookie, brownie, fat free, w/o icing | 10 |
| Cookie, butterscotch, brownie | 10 |
| Cookie, butterscotch chip | 10 |
| Cookie, caramel coated, w/ nuts | 10 |
| Cookie, carob | 10 |
| Cookie, carob & honey brownie | 10 |
| Cookie, chocolate chip | 10 |
| Cookie, chocolate chip w/ raisins | 10 |
| Cookie, choc chip, homemade or purchased at bakery | 10 |
| Cookie, choc chip, reduced fat | 10 |
| Cookie,rich,chocolate chip,w/ chocolate filling | 10 |
| Cookie, chocolate chip sandwich | 10 |
| Cookie, chocolate, made with rice cereal | 10 |
| Cookie, chocolate, made w/ oatmeal & coconut | 10 |
| Cookie, chocolate fudge | 10 |
| Cookie, chocolate, w/ choc filling/coating, fat free | 10 |
| Cookie, chocolate-covered, marshmallow | 10 |
| Cookie, chocolate-covered, marshmallow pie | 10 |
| Cookie, choc,choc sandwich/chocolate-coated/striped | 10 |
| Cookie,choc-cover,sugar waffer,creme/caramel filled | 10 |
| Cookie, chocolate sandwich, reduced fat | 10 |
| Cookie, chocolate sandwich, chocolate covered | 10 |
| Cookie, chocolate, sandwich, w/ extra filling | 10 |
| Cookie, chocolate & vanilla sandwich | 10 |
| Cookie, chocolate wafer | 10 |
| Cookie, graham cracker sandwich w/choc&marshmellow | 10 |
| Cookie, graham crax w/ marshmallow | 10 |
| Cookie, bar, w/ chocolate, nuts, & graham crackers | 10 |
| Cookie, coconut | 10 |
| Cookie, coconut & nut | 10 |
| Cookie, fruit-filled | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Cookie, fruit-filled bar, fat free | 10 |
| Cookie, date bar | 10 |
| Cookie, fig bar | 10 |
| Cookie, fig bar, fat free | 10 |
| Cookie, fortune | 10 |
| Cookie, cone shell, ice cream type,wafer / cake | 10 |
| Cookie, cone shell, ice cream type, brown sugar | 10 |
| Cookie, gingersnaps | 10 |
| Cookie, granola | 10 |
| Cookie, lady finger | 10 |
| Cookie, lemon bar | 10 |
| Cookie, macaroon | 10 |
| Cookie, marshmallow, w/ coconut | 10 |
| Cookie, marshmallow, w/ rice cereal (no-bake) | 10 |
| Cookie, marshmallow, w/ rice cereal & choc chips | 10 |
| Cookie, marshmallow & peanut butter, w/ oat cereal (no-bake) | 10 |
| Cookie, marshmallow pie, nonchocolate coating | 10 |
| Cookie, meringue | 10 |
| Cookie, molasses | 10 |
| Cookie, lebkuchen | 10 |
| Cookie, multigrain, high fiber | 10 |
| Cookie, oatmeal | 10 |
| Cookie, oatmeal, w/ raisins or dates | 10 |
| Cookie, oatmeal, w/ fruit filling | 10 |
| Cookie,oatmeal,fat free,w/ raisins(incl w/ dates) | 10 |
| Cookie, oatmeal, red fat, w/ raisins | 10 |
| Cookie, oatmeal sandwich, w/ creme filling | 10 |
| Cookie, oatmeal, w/ chocolate chips | 10 |
| Cookie, oatmeal sandwich, w/ peanut butter & jelly filling | 10 |
| Cookie,oatmeal,w/ choc & peanut butter (no-bake) | 10 |
| Cookie, oat bran | 10 |
| Cookie, peanut butter (include pb wafer) | 10 |
| Cookie, peanut butter w/ oatmeal | 10 |
| Cookie, peanut butter, w/ chocolate (incl nassau) | 10 |
| COOKIE, PEANUT BUTTER W/ RICE cereal (NO-BAKE) | 10 |
| Cookie, peanut | 10 |
| Cookie, w/ peanut butter filling, chocolate-coated | 10 |
| Cookie, pfeffernusse | 10 |
| Cookie, pizzelle (italian style wafer) | 10 |
| Cookie, pumpkin | 10 |
| Cookie, raisin | 10 |
| Cookie, raisin sandwich, cream-filled | 10 |
| Cookie, rum ball (no-bake) | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Cookie, sandwich type, not chocolate or vanilla | 10 |
| Cookie, shortbread | 10 |
| Cookie, shortbread, reduced fat | 10 |
| Cookie, shortbread, with chocolate filling | 10 |
| Cookie, butter or sugar | 10 |
| Cookie, butter/sugar cookie, w/ fruit and/ or nuts | 10 |
| Cookie, sugar wafer | 10 |
| Cookie, teething, baby food | 10 |
| Cookie, toffee bar | 10 |
| Cookie, vanilla sandwich | 10 |
| Cookie, vanilla sandwich, reduced fat | 10 |
| Cookie, rich, all chocolate, w/ choc filling or choc chips | 10 |
| Cookie, butter/sugar, w/ chocolate icing / filling | 10 |
| Cookie, butter/sugar, w/ icing/filling other than choc | 10 |
| Cookie, vanilla waffle creme | 10 |
| Cookie, tea, japanese | 10 |
| Cookie, vanilla wafer, ns as to type | 10 |
| Cookie, vanilla wafer, reduced fat | 10 |
| Cookie, vanilla w/ caramel, coconut, choc coating | 10 |
| Cookie, whole wheat, dried fruit & nuts | 10 |
| Cookie, rugelach | 10 |
| Cookie, dietetic, chocolate chip | 10 |
| Cookie, lemon wafer, lowfat | 10 |
| Cookie, dietetic, oatmeal w/ raisins | 10 |
| Cookie, dietetic, sandwich type | 10 |
| Cookie, dietetic, sugar or plain | 10 |
| Cookie, p.r. (mantecaditos polvorones) | 10 |
| Pie, nfs | 15 |
| Pie, individual size or tart, nfs | 15 |
| Pie, fried, nfs | 15 |
| Pie, apple, two crust | 15 |
| Pie, apple, individual size or tart | 15 |
| Pie, apple, fried (include mcdonald's) | 15 |
| Pie, apple, one crust (incl w/ crumb topping) | 15 |
| Pie, apple, diet | 15 |
| Pie, apricot, two crust | 15 |
| Pie, apricot, individual size or tart | 15 |
| Pie, apricot, fried | 15 |
| Pie, blackberry, two crust | 15 |
| Pie, blackberry, individual size or tart | 15 |
| Pie, berry not black;blue,boysen,rasp....,two crust | 15 |
| Pie, berry, one crust | 15 |
| Pie, berry, individual size or tart | 15 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Pie, blueberry, two crust | 15 |
| Pie, blueberry, one crust | 15 |
| Pie, blueberry, individual size or tart | 15 |
| Pie, cherry, two crust | 15 |
| Pie, cherry, one crust | 15 |
| Pie, cherry, individual size or tart | 15 |
| Pie, cherry, fried (include mcdonald's) | 15 |
| Pie, lemon (not cream or meringue) | 15 |
| Pie, lemon (not cream or meringue), individual size | 15 |
| Pie, lemon, fried | 15 |
| Pie, mince, two crust | 15 |
| Pie, mince, individual size or tart | 15 |
| Pie, peach, two crust | 15 |
| Pie, peach, one-crust | 15 |
| Pie, peach, individual size or tart | 15 |
| Pie, peach, fried | 15 |
| Pie, pear, two crust | 15 |
| Pie, pear, individual size or tart | 15 |
| Pie, pineapple, two crust | 15 |
| Pie, pineapple, individual size or tart | 15 |
| Pie, plum, two crust | 15 |
| Pie, prune, one crust | 15 |
| Pie, raisin, two crust | 15 |
| Pie, raisin, individual size or tart | 15 |
| Pie, raspberry, one crust | 15 |
| Pie, raspberry, two crust | 15 |
| Pie, rhubarb, two crust | 15 |
| Pie, rhubarb, one crust | 15 |
| Pie, rhubarb, individual size or tart | 15 |
| Pie, strawberry, one crust | 15 |
| Pie, strawberry-rhubarb, two crust | 15 |
| Pie, strawberry, individual size or tart | 15 |
| Pie, apple-sour cream | 15 |
| Pie, cherry, w/ cream cheese & sour cream | 15 |
| Pie, banana cream | 15 |
| Pie, banana cream, individual size or tart | 15 |
| Pie, buttermilk | 15 |
| Pie, chess (incl lemon chess pie) | 15 |
| Pie, chocolate cream | 15 |
| Pie, chocolate cream, individual size or tart | 15 |
| Pie, coconut cream | 15 |
| Pie, coconut cream, individual size or tart | 15 |
| Pie, custard | 15 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Pie, custard, individual size or tart | 15 |
| Pie, lemon cream | 15 |
| Pie, lemon cream, individual size or tart | 15 |
| Pie, peanut butter cream | 15 |
| Pie, pineapple cream | 15 |
| Pie, pumpkin | 15 |
| Pie, pumpkin, individual size or tart | 15 |
| Pie, raspberry cream | 15 |
| Pie, sour cream, raisin | 15 |
| Pie, squash | 15 |
| Pie, strawberry cream | 15 |
| Pie, strawberry cream, individual size or tart | 15 |
| Pie, sweetpotato | 15 |
| Pie, vanilla cream | 15 |
| Pie, yogurt, frozen | 15 |
| Pie, chiffon, not chocolate | 15 |
| Pie, chiffon, chocolate | 15 |
| Pie, chiffon, w/ liqueur | 15 |
| Pie, black bottom | 15 |
| Pie, lemon meringue | 15 |
| Pie, lemon meringue, individual size or tart | 15 |
| Pie, chocolate-marshmallow | 15 |
| Pie, pecan | 15 |
| Pie, pecan, individual size | 15 |
| Pie, oatmeal | 15 |
| Pie, pudding, not chocolate | 15 |
| Pie, pudding, not choc, individual size | 15 |
| Pie, pudding, choc, w/ choc coating, individ size | 15 |
| Pie, pudding, not choc, choc-coated, individ size | 15 |
| Pie, toll house chocolate chip | 15 |
| Pie, shoo-fly | 15 |
| Pie, tofu w/ fruit | 15 |
| Pie shell, graham cracker | 10 |
| Pie shell, chocolate wafer | 10 |
| Vanilla wafer dessert base | 10 |
| Blintz, cheese-filled | 5 |
| Blintz, fruit-filled | 5 |
| Cobbler, apple (include fruit cobbler) | 10 |
| Cobbler, apricot | 10 |
| Cobbler, berry | 10 |
| Cobbler, cherry | 10 |
| Cobbler, peach | 10 |
| Cobbler, pear | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Cobbler, pineapple | 10 |
| Cobbler, plum | 10 |
| Cobbler, rhubarb | 10 |
| Crisp, apple, apple dessert | 10 |
| Fritter, apple | 10 |
| Fritter, banana | 10 |
| Fritter, berry | 10 |
| Crisp, blueberry | 10 |
| Crisp, cherry | 10 |
| Crisp, peach | 10 |
| Crisp, rhubarb | 10 |
| Cream puff/eclair, custard/cream-filled, ns icing | 10 |
| Cream puff/eclair, custard/cream-filled, not iced | 10 |
| Cream puff/eclair, custard/cream-filled, iced | 10 |
| Cream puff/eclair, custard/cream-filled, iced, reduced fat | 10 |
| Sopaipilla w/ syrup or honey | 5 |
| Crepe, dessert type, ns as to filling | 5 |
| Crepe, dessert type, chocolate-filled | 5 |
| Crepe, dessert type, fruit-filled | 5 |
| Crepe suzette | 5 |
| Crepe, dessert type, ice cream-filled | 20 |
| Tamale, sweet | 10 |
| Tamale, sweet, w/ fruit | 10 |
| Strudel, apple (include strudel, nfs) | 10 |
| Strudel, berry | 10 |
| Strudel, cherry | 10 |
| Strudel, cheese | 10 |
| Strudel, peach | 10 |
| Strudel, pineapple | 10 |
| Strudel, cheese & fruit | 10 |
| Baklava (include kadayif) | 10 |
| Basbousa (semolina dessert dish) | 10 |
| Turnover or dumpling, apple | 10 |
| Turnover or dumpling, berry | 10 |
| Turnover or dumpling, cherry | 10 |
| Turnover or dumpling, lemon | 10 |
| Turnover or dumpling, peach | 10 |
| Turnover, guava | 10 |
| Turnover, pumpkin | 10 |
| Pastry, fruit-filled | 10 |
| Pastry,oriental,made w/ bean or lotus seed paste | 10 |
| Pastry,oriental,w/bean paste&salted egg yolk(baked) | 10 |
| Pastry, chinese (include 9-layer pudding) | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Pastry, cookie type, fried(incl polish paczki) | 10 |
| Pastry, italian, w/ cheese (include cannoli) | 10 |
| Pastry, puff, custard/cream filled, iced/not iced | 10 |
| Empanada, mexican turnover, fruit-filled | 10 |
| Empanada, mexican turnover, pumpkin | 10 |
| Breakfast pastry, nfs | 20 |
| Toaster bagel, w/ fruit and cream cheese | 5 |
| Danish pastry, plain/spice (incl w/ icing) | 20 |
| Danish pastry, w/ fruit | 20 |
| Danish pastry, w/ nuts | 20 |
| Danish pastry, w/ cheese | 10 |
| Danish pastry, w/ cheese, fat free, no chol | 10 |
| Doughnut, ns as to cake or yeast | 10 |
| Doughnut, cake type | 10 |
| Doughnut, chocolate, cake type | 10 |
| Doughnut, cake type, chocolate covered | 10 |
| Doughnut, cake type, chocolate covered, w/ peanuts | 10 |
| Doughnut, chocolate, cake type, with chocolate icing | 10 |
| Churros (incl mexican cruellers) | 10 |
| Doughnut, oriental | 10 |
| Cruller, nfs | 10 |
| French cruller | 10 |
| Doughnut, chocolate, raised or yeast, with chocolate icing | 10 |
| Doughnut, raised / yeast | 10 |
| Doughnut, chocolate, raised or yeast | 10 |
| Doughnut, raised or yeast, chocolate covered | 10 |
| Doughnut, jelly | 10 |
| Doughnut, custard-filled | 10 |
| Doughnut, chocolate cream-filled | 10 |
| Doughnut, custard-filled, with icing | 10 |
| Doughnut, wheat | 10 |
| Doughnut, wheat, chocolate covered | 10 |
| Coffee cake, nfs | 10 |
| Coffee cake, crumb or quick-bread type | 10 |
| Coffee cake,crumb/quick bread type,red fat,no chol | 10 |
| Coffee cake, crumb or quick-bread type, w/ fruit | 10 |
| Coffeecake, crumb or quick-bread type, cheese filld | 10 |
| Coffee cake, quick-bread type, custard filled | 10 |
| Popcorn, sugar syrup or caramel coated | 10 |
| Popcorn, sugar syrup or caramel coated, w/ nuts | 10 |
| Popcorn, sugar syrup/caramel coated, fat free | 10 |
| Pretzel, hard, chocolate coated | 10 |
| Pretzel, yogurt covered | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Pretzels, cheese-filled (incl combos) | 5 |
| Pancakes, plain (include pancakes, nfs) | 10 |
| Pancakes, reduced calorie, high fiber | 10 |
| Pancakes, w/ fruit (include blueberry pancakes) | 10 |
| Pancakes w/ choc chips | 10 |
| Pancakes, buckwheat | 10 |
| Pancakes, cornmeal | 10 |
| Pancakes, whole wheat | 10 |
| Pancakes, sourdough | 10 |
| Pancakes, rye | 10 |
| Waffle, plain | 4 |
| Waffle, wheat, bran, or multigrain | 4 |
| Waffle, fruit | 4 |
| Waffle, nut & honey (incl eggo) | 4 |
| Waffle, cornmeal | 4 |
| Waffle, 100% whole wheat or 100% whole grain | 4 |
| Waffle, oat bran | 4 |
| Waffle, multi-bran (include eggo nutrigrain) | 4 |
| Waffle, plain, fat free | 4 |
| Waffle, plain, lowfat | 4 |
| French toast, plain (include roman meal) | 4 |
| French toast sticks, plain | 4 |
| Bread fritters, p.r. (torrejas, galician fritters) | 4 |
| Cake made w/ glutinous rice | 10 |
| Cake or pancake made w/ rice flour &/or dried beans | 10 |
| Cake made w/ glutinous rice & dried beans | 10 |
| Funnel cake | 10 |
| Cereal, nfs | 15 |
| Kashi cereal, ns as to ready-to-eat or cooked | 15 |
| Oat cereal, nfs | 10 |
| Cereal, ready-to-eat, nfs | 10 |
| All-bran cereal | 10 |
| All bran cereal w/ extra fiber | 10 |
| Alpen cereal | 10 |
| Alpha-bits cereal | 30 |
| Alpha-bits w/ marshmallows cereal | 30 |
| Apple cinnamon cheerios | 30 |
| Apple cinnamon squares mini-wheats cereal, kellogg's | 20 |
| Apple jacks cereal | 30 |
| Banana nut crunch cereal (post) | 20 |
| Basic 4 (rte cereal) | 15 |
| Berry berry kix | 15 |
| Blueberry morning, post | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Booberry cereal | 30 |
| All-bran bran buds cereal, kellogg's (formerly bran buds) | 12.5 |
| Bran chex cereal | 10 |
| Cap'n crunch cereal | 30 |
| Cap'n crunch's christmas crunch cereal | 30 |
| Cap'n crunch's crunch berries cereal | 30 |
| Cap'n crunch's peanut butter crunch cereal | 30 |
| Chex cereal, nfs | 10 |
| Chocolate flavored frosted puffed corn cereal | 20 |
| Cinnamon grahams cereal, generalmills | 15 |
| Cinnamon toast crunch cereal | 15 |
| Honey nut clusters cereal | 15 |
| Cocoa krispies cereal | 15 |
| Cocoa blasts cereal, quaker | 25 |
| Cocoa pebbles cereal | 20 |
| Cocoa puffs cereal | 20 |
| Common sense oat bran cereal, plain | 10 |
| Cookie-crisp cereal (include all flavors) | 20 |
| Crunchy corn bran cereal, quaker | 10 |
| Corn chex cereal | 5 |
| Corn flakes, nfs (include store brands) | 5 |
| Corn flakes, low sodium | 5 |
| Corn flakes, kellogg | 5 |
| Corn puffs cereal | 5 |
| Total corn flakes | 5 |
| Count chocula cereal | 10 |
| Cracklin' oat bran cereal | 5 |
| Crisp crunch cereal | 5 |
| Crispix cereal | 5 |
| Crispy brown rice cereal | 5 |
| Harmony cereal, gen mills | 15 |
| Crispy rice cereal | 7 |
| Crispy wheats'n raisins cereal | 5 |
| Disney cereals, kellogg's | 20 |
| Familia cereal | 15 |
| Bran flakes cereal, nfs (formerly 40% bran flakes, nfs) | 10 |
| Complete wheat bran flakes, kellogg's (form.40% bran flakes) | 10 |
| Natural bran flakes cereal, post | 10 |
| Frankenberry cereal | 30 |
| French toast crunch cereal, general mills | 30 |
| Froot loops cereal | 30 |
| Fruit & fibre cereal, nfs | 10 |
| Fruit & fibre cereal, w/ dates, raisins, & walnuts | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Fruit rings, nfs (include store brands) | 20 |
| Fruit whirls cereal | 20 |
| Fruity pebbles cereal | 20 |
| Golden grahams cereal | 15 |
| Granola, nfs | 12.5 |
| Granola, homemade | 12.5 |
| Granola, lowfat, kellogg's | 15 |
| Granola w/ raisins, lowfat, kellogg's | 15 |
| Grape-nuts cereal | 5 |
| Grape-nut flakes | 5 |
| Great grains, raisin, date, & pecan,whole grain cereal, post | 5 |
| Great grains double pecan whole grain cereal, post | 5 |
| Healthy choice almond crunch cereal w/ raisins | 5 |
| Honey bunches of oats cereal | 10 |
| Honey bunches of oats w/ almonds, post | 10 |
| Honeycomb cereal, plain | 10 |
| Honeycomb cereal, strawberry | 10 |
| Honey crunch corn flakes cereal, kellogg's | 10 |
| Honey nut chex cereal | 10 |
| Honey nut cheerios | 10 |
| Honey nut shredded wheat cereal, post | 10 |
| Smacks, Kellogg's (formerly Honey Smacks) | 20 |
| Jenny o's | 10 |
| Just right cereal | 10 |
| Just right fruit & nut cereal (w/ raisins, dates, nuts) | 10 |
| Pokemon, kellogg's | 20 |
| Kaboom cereal | 10 |
| King vitaman cereal | 20 |
| Kix cereal | 5 |
| Life cereal (plain & cinnamon) | 10 |
| Lucky charms cereal | 20 |
| Malt-o-meal coco-roos cereal | 20 |
| Malt-o-meal corn bursts cereal | 20 |
| Malt-o-meal crispy rice cereal | 5 |
| Malt-o-meal honey & nut toasty o's cereal | 20 |
| Malt-o-meal marshmallow mateys cereal | 20 |
| Malt-o-meal golden puffs cereal (formerly sugar puffs) | 20 |
| Malt-o-meal tootie fruities (rte cereal) | 20 |
| Marshmallow safari cereal, quaker | 20 |
| Mueslix cereal, nfs | 15 |
| Muesli with raisins, dates, and almonds | 15 |
| Multi bran chex | 10 |
| Multi grain cheerios | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Natural muesli, jenny's cuisine | 15 |
| Nature valley granola, w/ fruit & nuts | 12.5 |
| Nu system cuisine toasted grain circles cereal | 10 |
| Nutty nuggets (ralston) | 10 |
| Oat bran flakes, health valley | 5 |
| Apple cinnamon oatmeal crisp cereal (oatmeal crisp w/ apples | 15 |
| Oatmeal crisp w/ almonds cereal | 15 |
| Oatmeal raisin crisp cereal | 15 |
| Oh's, honey graham cereal | 20 |
| Oh's, fruitangy cereal | 20 |
| Sun country 100% natural granola, with almonds | 10 |
| 100 % natural cereal, w/ oats,honey & raisins,quaker | 10 |
| 100% natural wholegrain cereal w/ raisins, lowfat, quaker | 5 |
| Oreo o's cereal, post | 20 |
| Sweet crunch cereal, quaker (formerly popeye) | 10 |
| Sweet puffs cereal, quaker | 20 |
| Product 19 cereal | 5 |
| Quaker oat bran cereal | 5 |
| Quaker oatmeal squares cereal (formerly quaker oat squares) | 5 |
| Quisp cereal | 20 |
| Raisin bran cereal, nfs | 5 |
| Raisin bran cereal, kellogg | 5 |
| Raisin bran cereal, post | 5 |
| Raisin bran, total | 5 |
| Raisin nut bran cereal | 5 |
| Raisin squares mini-wheats cereal (formerly raisin squares) | 5 |
| Reese's peanut butter puffs cereal | 20 |
| Rice chex cereal | 5 |
| Rice flakes, nfs | 5 |
| Rice krispies cereal | 5 |
| Rice krispies treats cereal (kellogg's) | 5 |
| Smart start, kellogg's | 15 |
| Special k cereal | 5 |
| Toasted oatmeal, honey nut (quaker) | 15 |
| Corn pops cereal | 20 |
| Strawberry squares mini-wheats cereal(strawberry squares) | 10 |
| Golden crisp cereal | 20 |
| Toasties, post | 5 |
| Malt-o-meal apple & cinnamon toasty o's | 20 |
| Total cereal | 8 |
| Trix cereal | 20 |
| Uncle sam's hi fiber cereal | 5 |
| Waffle crisp cereal, post | 20 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Wheat chex cereal | 5 |
| Wheat germ cereal, w/ sugar & honey | 15 |
| Wheat, puffed, presweetened w/ sugar | 20 |
| Lemon pie filling | 12 |
| Grapefruit juice, canned, bottled, carton, w/ sugar | 5 |
| Orange juice, canned/bottled/carton, w/ sugar | 5 |
| Tangerine juice, canned, w/ sugar | 5 |
| Grapefruit & orange juice, canned, w/ sugar | 5 |
| Pineapple-grapefruit juice, canned, w/ sugar | 5 |
| Pineapple-orange juice, canned, ns as to sweetener | 5 |
| Pineapple-orange juice, canned, w/ sugar | 5 |
| Apple, dried, cooked, w/ sugar | 9 |
| Apricot, dried, cooked, w/ sugar | 12 |
| Currants, dried | 20 |
| Cranberries, dried | 20 |
| Fig, dried, cooked, w/ sugar | 11 |
| Mango, dried | 6 |
| Peach, dried, cooked, w/ sugar | 11 |
| Pear, dried, cooked, w/ sugar | 15 |
| Prune, dried, cooked, w/ sugar | 15 |
| Applesauce, stewed apples, ns as to added sweetener | 9 |
| Applesauce, stewed apples, w/ sugar | 9 |
| Applesauce / other fruits(include mott's fruit pak) | 9 |
| Apple, baked, w/ sugar | 15 |
| Carambola (starfruit), cooked, w/ sugar | 11 |
| Cherry pie filling | 14 |
| Peach, frozen, w/ sugar | 11 |
| Rhubarb, frozen, w/ sugar | 11 |
| Blueberry pie filling | 18 |
| Cranberries, cooked or canned (incl cranberry sce) | 15 |
| Raspberries, frozen, w/ sugar | 11 |
| Strawberries, frozen, ns as to added sweetner | 12 |
| Strawberries, frozen, w/ sugar | 12 |
| Cranberry-raspberry sauce | 12 |
| Fruit salad, p.r. style (ensalada de fruta) | 8 |
| Apple, candied (include caramel apples) | 8 |
| Banana whip | 8 |
| Prune whip | 8 |
| Fruit salad (no citrus) w/ salad dressing | 5 |
| Fruit salad (no citrus) w/ cream | 5 |
| Fruit salad (no citrus) w/ cream substitute | 5 |
| Fruit salad (no citrus) w/ marshmallows | 5 |
| Fruit salad (w/ citrus) w/ pudding | 5 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Fruit salad (no citrus fruits) w/ pudding | 5 |
| Fruit salad (incl citrus fruits) w/ salad dressing | 5 |
| Fruit salad (incl citrus fruits) w/ cream | 5 |
| Fruit salad w/ cream substitute | 5 |
| Fruit salad w/ marshmallows | 5 |
| Fruit dessert, nfs | 5 |
| Fruit dessert w/ cream & or pudding & nuts | 5 |
| Pineapple salad w/ dressing | 8 |
| Pineapple salad w/ cream cheese | 8 |
| Fruit juice bar, frozen, orange flavor | 12.5 |
| Fruit juice bar, frozen, flavor other than orange | 12.5 |
| Sorbet, fruit, noncitrus flavor | 35 |
| Sorbet, fruit, citrus flavor | 35 |
| Tomato juice cocktail | 2 |
| Tomato catsup | 25 |
| Tomato catsup, low sodium | 25 |
| Tomato chili sauce (catsup type) | 25 |
| Salsa, nfs | 5 |
| Salsa, red, ckd, not hom (incl taco, creole, picante sauces) | 5 |
| Barbecue sauce (include arby's sauce) | 12 |
| Barbecue sauce, low sodium | 12 |
| Steak sauce, tomato-base (include a-1) | 7 |
| Cocktail sauce | 6 |
| Cucumber pickles, sweet | 8 |
| Cucumber pickles, fresh (include bread & butter) | 8 |
| Cucumber pickle, sweet, reduced salt | 8 |
| Orange sauce for duck | 15 |
| Sandwich spread | 7 |
| Tartar sauce | 5 |
| Honey butter | 10 |
| Salad dressing, nfs | 8 |
| Bacon & tomato dressing | 8 |
| Coleslaw dresssing | 8 |
| French dresssing | 20 |
| Fruit dressing w/ fruit juice & cream | 8 |
| Fruit dressing, made w/ honey, oil, water | 8 |
| Honey mustard dressing | 15 |
| Russian dressing | 20 |
| Mayonnaise-type salad dressing | 8 |
| Mayonnaise-type salad dressing, cholesterol-free | 8 |
| Boiled, cooked-type dressing | 8 |
| Green goddess dressing | 8 |
| Creamy dressing, w/sour cream/buttermilk & oil | 8 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Cream cheese dressing | 8 |
| Milk, vinegar & sugar dressing | 8 |
| Poppy seed dressing | 8 |
| Peppercorn dressing | 8 |
| Celery seed dressing | 8 |
| Sesame dressing | 8 |
| Sweet & sour dressing | 8 |
| Thousand island dressing | 15 |
| Yogurt dressing | 8 |
| Coleslaw dressing, reduced calorie | 8 |
| Mayonnaise-type salad dressing, fat-free | 8 |
| Vinegar, sugar & water dressing | 8 |
| Korean dressing or marinade | 8 |
| Syrup, nfs | 25 |
| Pancake syrup, nfs | 85 |
| Cane & corn pancake syrup | 85 |
| Corn syrup, light or dark | 85 |
| Buttered blends syrup (incl mrs butterworth) | 85 |
| Fruit syrup | 30 |
| Chocolate syrup, thin type | 50 |
| Chocolate syrup, thin type, light | 8 |
| Fruit flavored syrup used for milk beverages | 20 |
| Maple & corn &/or cane pancake syrup blends | 85 |
| Syrup, pancake, reduced calorie | 10 |
| Topping, butterscotch or caramel | 40 |
| Topping, chocolate, thick, fudge type | 40 |
| Topping, fruit | 40 |
| Topping, marshmallow | 40 |
| Topping, nut (wet) | 40 |
| Topping, peanut butter, thick fudge type | 40 |
| Topping, choc flavor hazelnut spread (incl nutella) | 40 |
| Topping, milk chocolate w/ cereal | 20 |
| Topping, chocolate, hard coating | 30 |
| Icing, chocolate | 30 |
| Icing, white | 30 |
| Sweet & sour sauce (include vietnamese sauce) | 10 |
| Fruit sauce (include all fruits) | 10 |
| Raisin sauce | 10 |
| Plain dessert sauce (include vanilla, rum sauce) | 10 |
| Duck sauce (include chaisni sauce) | 10 |
| Plum sauce, oriental style | 10 |
| Jelly, all flavors | 60 |
| Jam, preserves, all flavors | 35 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Marmalade, all flavors | 25 |
| Jelly, reduced sugar, all flavors | 12 |
| Jam preserves,marmalades,sweet w/ fruit juice conc | 25 |
| Gelatin dessert | 8 |
| Gelatin snacks | 8 |
| Gelatin dessert w/ fruit | 8 |
| Gelatin dessert w/ whipped cream | 8 |
| Gelatin dessert w/ fruit & whipped cream | 8 |
| Gelatin dessert w/ cream cheese | 8 |
| Gelatin dessert w/ sour cream | 8 |
| Gelatin dessert w/ fruit & sour cream | 8 |
| Gelatin dessert w/ fruit & cream cheese | 8 |
| Gelatin dessert w/ fruit, vegetables, & nuts | 8 |
| Gelatin dessert w/ fruit & whipped topping | 8 |
| Danish dessert pudding | 8 |
| Tastes good to me, p.r. (bien me sabe) (dessert) | 8 |
| Pineapple custard, p.r. (flan de pina) | 8 |
| Haupia (coconut pudding) | 8 |
| Gelatin,froz,whipped,on stick(incl jello gltn pops) | 8 |
| Ice, fruit | 20 |
| Ice pop | 20 |
| Ice pop filled w/ ice cream, all flavor varieties | 20 |
| Snow cone, slurps | 20 |
| Candy, nfs | 30 |
| Butterscotch morsels | 30 |
| Caramel candy, choc-flavor roll (incl tootsie roll) | 30 |
| Caramel candy, not chocolate | 30 |
| Caramel candy, w/ nuts | 30 |
| Caramel candy, chocolate covered | 30 |
| Caramel candy, w/ nuts & cereal, chocolate-covered | 30 |
| Caramel candy, w/ nuts, chocolate-covered | 30 |
| Rolos candy | 30 |
| Toblerone,milk chocolate w/ honey & almond nougat | 20 |
| Twix cookie bars | 20 |
| Twix chocolate fudge cookie bars | 20 |
| Twix peanut butter cookie bars | 20 |
| Bar none candy bar | 20 |
| Whatchamacallit candy | 20 |
| Milk chocolate candy, plain | 20 |
| Milk chocolate candy, with cereal | 20 |
| Kit kat candy bar | 20 |
| Chocolate, milk, w/ nuts, not almonds or peanuts | 10 |
| Milk chocolate candy, with fruit and nuts | 20 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Milk chocolate candy, with almonds | 20 |
| Chocolate, milk, w/ peanuts (include mr goodbar) | 20 |
| Coconut candy, chocolate-covered | 20 |
| Coconut candy, no chocolate covering | 20 |
| Coconut candy, p.r. style | 20 |
| Fondant candy | 20 |
| Fondant candy, chocolate covered | 20 |
| Fruit peel, candied | 20 |
| Fruit candy bar | 20 |
| Soft fruit confection | 20 |
| Fruit leather (include fruit roll-up) | 20 |
| Fun fruits creme supremes candy | 20 |
| Tamarind candy | 20 |
| Fruit snack candy, w/ vit c | 20 |
| Fudge, chocolate, chocolate-coated | 5 |
| Fudge, chocolate, chocolate-coated, w/ nuts | 5 |
| Fudge, chocolate | 5 |
| Fudge, chocolate, w/ nuts | 5 |
| Fudge, peanut butter | 5 |
| Fudge, peanut butter, w/ nuts | 5 |
| Fudge, vanilla | 5 |
| Fudge, vanilla, w/ nuts | 5 |
| Fudge, divinity | 5 |
| Fudge, brown sugar (panuchi) | 5 |
| Fudge, caramel and nut, chocolate-coated candy | 5 |
| Snickers candy bar | 20 |
| Baby ruth candy bar | 20 |
| 100 grand bar (incl $100,000 bar) | 20 |
| Halvah, plain | 20 |
| Halvah, chocolate-covered | 20 |
| Honey-combed hard candy, peanut butter | 20 |
| Honey-combed candy, peanut butter, choc-covered | 20 |
| Butterfinger candy bar | 20 |
| Jimmies (include chocolate-flavored sprinkles) | 20 |
| Ladoo, round ball, asian-indian dessert | 20 |
| Licorice candy | 20 |
| Marshmallow | 30 |
| Marshmallow, chocolate-covered | 30 |
| Marshmallow, candy-coated | 30 |
| Marshmallow, coconut-coated | 30 |
| Nougat candy, plain | 30 |
| Nougat candy, w/ caramel, chocolate-covered | 30 |
| Milky way bar | 20 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Milky way dark bar | 20 |
| Mars bar | 20 |
| Nougat candy, chocolate-covered | 30 |
| 3 musketeers candy bar | 20 |
| Nuts, chocolate-covered, not almonds or peanuts | 20 |
| Nut roll, fudge or nougat, caramel & nuts | 20 |
| M&m's peanut butter chocolate candies | 20 |
| Reese's peanut butter cups | 20 |
| Reese's pieces candy | 20 |
| Reese sticks | 20 |
| Peanut butter morsels candy | 20 |
| Pineapple candy, p.r. style | 30 |
| Raisins, chocolate-covered | 10 |
| Raisins, carob-covered | 10 |
| Raisins, yogurt-covered | 10 |
| Sesame crunch candy (sahadi) | 10 |
| Skittles candy | 20 |
| Sugar-coated chocolate discs candy | 20 |
| M&m's plain chocolate candies | 20 |
| Easter egg, candy-coated chocolate | 20 |
| Taffy | 40 |
| Toffee, plain | 20 |
| Toffee, chocolate covered (incl heath bar, skor) | 20 |
| Toffee, chocolate-coated, w/ nuts | 20 |
| Truffles | 20 |
| Wax candy, liquid filled | 20 |
| Mints, dietetic or low calorie | 20 |
| Chewing gum, nfs | 15 |
| Chewing gum, sugared | 15 |
| Rice beverage (incl rice tea) | 5 |
| Soft drink, nfs | 10 |
| Carbonated water,sweetend(incl tonic,quinine water) | 5 |
| Soft drink, cola-type | 10 |
| Soft drink, cola-type, w/ higher caffeine (incl jolt) | 10 |
| Soft drink, cola-type, decaffeinated | 10 |
| Soft drink, pepper-type (incl dr. Pepper, mr. Pibb) | 10 |
| Soft drink, pepper-type, decaffeinated | 10 |
| Cream soda | 12 |
| Soft drink, fruit-flavored, caffeine free | 12 |
| Soft drink, fruit-flavored, w/ caffeine | 12 |
| Ginger ale | 10 |
| Root beer | 10 |
| Chocolate-flavored soda | 10 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Cola w/ fruit or vanilla flavor | 10 |
| Cola w/ chocolate flavor | 10 |
| Mavi drink | 5 |
| Soft drink, ale type (include ale-8) | 10 |
| Carbonated juice drink, ns as to type of juice | 10 |
| Carbonated citrus juice drink | 10 |
| Carbonated noncitrus juice drink | 10 |
| Apple-cherry drink | 10 |
| Apple juice drink | 10 |
| Apple-cranberry-grape juice drink | 10 |
| Apple-orange-pineapple juice drink | 10 |
| Apricot-pineapple juice drink | 10 |
| Banana-orange drink | 10 |
| Black cherry drink | 10 |
| Fruit drink (include fruit punch & fruit ade) | 10 |
| Fruit juice drink, nfs | 10 |
| Tamarind drink, p.r. (refresco de tamarindo) | 10 |
| Fruit punch, made w/ fruit juice & soda | 10 |
| Fruit punch, made w/ soda, fruit juice & sherbet | 10 |
| Grapeade & grape drink | 10 |
| Grape juice drink | 10 |
| Grapefruit juice drink | 10 |
| Guava juice drink | 10 |
| Lemonade, frozen concentrate, not reconstituted | 45 |
| Lemonade | 10 |
| Lemon-limeade | 10 |
| Limeade | 10 |
| Orange-mango juice drink | 10 |
| Orange drink (include orange ade, yaba daba dew) | 10 |
| Orange-apricot juice drink | 10 |
| Orange-lemon drink | 10 |
| Citrus fruit juice drink (incl 5-alive) | 10 |
| Orange-cranberry juice drink | 10 |
| Orange-peach juice drink | 10 |
| Orange-grape-banana juice drink | 10 |
| Papaya juice drink | 10 |
| Pineapple-grapefruit juice drink | 10 |
| Pineapple-orange juice drink | 10 |
| Orange-raspberry juice drink | 10 |
| Raspberry-flavored drink | 10 |
| Strawberry-flavored drink | 10 |
| Frozen daiquiri mix, concentrate, not reconstituted | 45 |
| Frozen daiquiri mix, from froz conc, reconstituted | 6 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Pina colada, nonalcoholic | 15 |
| Whiskey sour, nonalcoholic (incl lemix) | 15 |
| Black cherry drink w/ vitamin c added | 10 |
| Cherry drink w/ vitamin c added | 10 |
| Citrus drink w/ vitamin c added | 10 |
| Cranberry juice drink w/vit c added(incl cocktail) | 10 |
| Cranberry-apple juice drink w/ vitamin c added | 10 |
| Fruit punch/drink/ade w/ vit c added (incl hi-c) | 10 |
| Grape drink w/ vitamin c added | 10 |
| Grapefruit juice drink w/ vitamin c added | 10 |
| Guava juice drink w/ vit c added | 10 |
| Lemonade w/ vitamin c added | 10 |
| Vegetable & fruit juice drink, w/ vit c | 10 |
| Orange drink & orangeade w/ vitamin c added | 10 |
| Orange breakfast drink, from frozen concentrate | 10 |
| Orange breakfast drink | 10 |
| Pineapple-grapefruit juice drink w/ vit c added | 10 |
| Pineapple-orange juice drink w/ vitamin c added | 10 |
| Pineapple-orange-grapefruit juice drink w/vitamin c | 10 |
| Srawberry-flavored drink w/ vitamin c added | 10 |
| Orange-cranberry juice drink,low cal,w/ vit c added | 10 |
| Fruit-flavored thirst quencher beverage | 6 |
| Fluid replacemnt,electrolyte solutn(incl pedialyte) | 2.5 |
| Fluid replacement, 5% glucose in water | 5 |
| Citrus juice drink, calcuim fortified | 10 |
| Orange breakfast drink, calcium fortified | 10 |
| Horchata, p.r. (beverage) | 5 |
| Coconut beverage, p.r | 5 |
| Oatmeal beverage, p.r. | 5 |
| Oatmeal beverage w/ milk | 5 |
| Rice beverage, mexican (horchata) | 5 |
| Sugar cane beverage, p.r. | 5 |
| Atole (cornmeal beverage) | 5 |
| Corn bev w/ choc & milk(champurrado,atole de choc) | 5 |
| Red bull energy drink | 10 |
| Nonalcoholic malt beverage | 8 |
| Shirley temple | 5 |
| Cordial or liqueur | 10 |
| Cocktails, nfs | 10 |
| Bacardi cocktail | 5 |
| Daiquiri | 5 |
| Grasshopper | 5 |
| Mint julep | 20 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Screwdriver (include harvey wallbanger, slo-screw) | 5 |
| Whiskey sour(incl scotch,vodka,apricot,brandy sour) | 5 |
| Mixed drinks (for recipe modifications) | 5 |
| Rum & cola | 5 |
| Pina colada | 15 |
| Coquito, p.r. (coconut, rum) | 5 |
| Sloe gin fizz | 5 |
| Fruit punch, alcoholic | 10 |
| Singapore sling | 5 |
| Mai tai | 5 |
| Tequila sunrise | 5 |
| Long island iced tea | 5 |
| Fuzzy navel cocktail | 5 |
| Irish coffee (incl coffee royale) | 5 |
| Liqueur w/ cream | 10 |
| Frozen daiquiri | 10 |
| Frozen margarita | 10 |
| Eggnog, alcoholic | 5 |
| Wine cooler | 5 |
| Sangria | 5 |
| Sangria, puerto rican style | 5 |
| Rum cooler | 5 |
| Milk, soy, ready-to-drink, not baby | 5 |
| Milk, soy, ready-to-drink, not baby's, chocolate | 5 |
| Milk fruit drink (incl licuado) | 2 |
| Fruit smoothie drink, w/ fruit and dairy products | 11 |
| Fruit smoothie drink, nfs | 11 |
| Choc-flavored drink, whey-&milk-based(incl yoo-hoo) | 11 |
| Milk drink, whey&milk-base, not choc (incl yoo-hoo) | 11 |
| Cafe con leche prepared w/ sugar | 11 |
| Honey-combed hard candy, peanut butter | 30 |
| Honey-combed candy, peanut butter, choc-covered | 30 |
| Nougat candy, chocolate-covered | 30 |
| Mint julep | 5 |
| Milk, cow's, fluid, filled w/ veg oil, ns as to fat | 3 |
| Milk, cow's, fluid, filled w/ veg oil, whole | 3 |
| Milk, cow's, fluid, filled w/ veg oil, lowfat | 3 |
| Milk, condensed, sweetened, undiluted | 15 |
| Milk, condensed, sweetened, diluted | 15 |
| Milk, imitation, fluid, soy based | 5 |
| Milk, soy, ready-to-drink, not baby | 2 |
| Milk, soy, ready-to-drink, not baby's, chocolate | 5 |
| Milk,imitation,fluid,nonsoy,sweetened,not chocolate | 5 |

| SPECIFIC FOOD DESCRIPTION | CONCENTRATION (%) |
|---|---|
| Yogurt, ns as to type of milk/flavor | 8 |
| Yogurt, vanilla, lemon, coffee, ns as to milk type | 8 |
| Yogurt, vanilla, lemon, coffee, whole milk | 8 |
| Yogurt, vanilla, lemon, coffee, lowfat milk | 8 |
| Yogurt, vanilla, lemon, coffee, nonfat milk | 8 |
| Yogurt, vanilla, lemon, coffee, nonfat milk, low cal sweet | 8 |
| Yogurt, chocolate, ns as to type of milk | 8 |
| Yogurt, chocolate, whole milk | 8 |
| Yogurt, chocolate, nonfat milk | 8 |
| Yogurt, fruit variety, ns as to milk type | 8 |
| Yogurt, fruit variety, whole milk | 8 |
| Yogurt, fruit variety, lowfat milk | 8 |
| Yogurt, fruit variety, nonfat milk | 8 |
| Yogurt, fruited, nonfat milk, low cal sweetener | 8 |
| Yogurt, fruit & nuts, ns as to type of milk | 8 |

NFS = Not food specified; NS = non-specific

## APPENDIX 3. ESTIMATED CONSUMPTION OF SUCROMALT FROM FOOD CATEGORIES SPECIFIED IN APPENDIX 2; EXACT NUMBERS FROM BURDOCK GROUP (2008)

| | mg/day | mg/kg/day |
|---|---|---|
| Mean *per capita* | 52,923 | 890.41* |
| Mean, weighted, eaters-only*** | 64,851 | 1091.09** |
| 90th Percentile, weighted, eaters-only*** | 147,600 | 2538.27** |

* = body weight in this calculation is based on the general assumption of the average person in the U.S. population weighing 60 kg; ** = the body weights used in these calculations were based on respondent ages, derived from Portier *et al.* (2007); *** = estimates are based on individuals in the U.S. that consume the foods identified in Table 10, *i.e.*, eaters-only In the 2003-2004 survey, 466,778,866 individuals consumed the targeted foods

## APPENDIX 4. MEAN, WEIGHTED EATERS-ONLY CONSUMPTION OF SUCROMALT BROKEN DOWN BY AGE GROUPS; EXACT NUMBERS FROM BURDOCK GROUP (2008)

| Age groups | | | Consumption | |
|---|---|---|---|---|
| Age range (yrs) | Average weight (kg)* | # of eaters | mg/day | mg/kg/day |
| 1-2 | 11.4 | 1022 | 31,304.62 | 2746.46 |
| 3-5 | 18.2 | 947 | 48,145.45 | 2655.50 |
| 6-11 | 33.5 | 1708 | 71,425.41 | 2133.36 |
| 12-19 | 64.7 | 4035 | 88,990.13 | 1372.20 |
| 20-29 | 77.6 | 1456 | 93,578.93 | 1203.40 |
| 30-39 | 81.4 | 1326 | 74,843.16 | 918.25 |
| 40-49 | 82.5 | 1317 | 63,336.95 | 769.31 |
| 50-59 | 82.9 | 981 | 49,721.63 | 595.29 |
| 60-69 | 81.5 | 1267 | 36,953.00 | 455.03 |
| 70+ | 73.7 | 1766 | 33,088 41 | 449.92 |
| | | $\Sigma$=15,825 | | |

* = body weights in this column are derived from Portier *et al.* (2007)

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November 12, 2008

**VIA OVERNIGHT MAIL**
Robert Martin, Ph.D.
Deputy Director, Division of Biotechnology & GRAS Notice Review
Office of Food Additive Safety, CFSAN
U.S. Food and Drug Administration
4300 River Road, HFS-255
College Park, MD 20740-3835

**Re: Amendment to Cargill's Sucromalt GRAS Notification – GRN 258 -- Novozymes' GRAS Documentation Package for Sucrozyme®**

Dear Dr. Martin:

In support of Cargill's GRAS Notification for Sucromalt (GRN 258), Novozymes is providing a copy of our GRAS notification package for Sucrozyme®, an Alternansucrase preparation by *Bacillus licheniformis* expressing a gene encoding a modified Alternansucrase from *Leuconostoc mesenteroides*. Sucrozyme® is used as a processing aid in the starch industry for production of low glycemic sweeteners for use in food and beverages as a replacement of sucrose and other traditional sweeteners. Novozymes' self-affirmation GRAS of Sucrozyme® was reviewed and confirmed by an external expert panel consisting of Drs. Joseph F. Borzelleca and Michael W. Pariza.

Should you have any questions or comments regarding Sucrozyme®, please contact me directly at (919) 494-3152.

Sincerely yours,

(b)(6)

Denise Bernstein
Staff Specialist
DCBe@novozymes.com

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2007-98542-01

**Statement of the GRAS Expert Panel on Sucrozyme®**

We, Professors Borzelleca and Pariza, members of the Expert Panel, have independently and critically evaluated the information on **Sucrozyme®** (Alternansucrase, PPN 26587) assembled by Novozymes including Decision Tree Analysis of Safe Strain Lineage of Enzyme Products derived from Bacillus licheniformis, test reports of three toxicity studies, and Summary of Toxicity Data and other information deemed appropriate or necessary and conclude that the proposed uses of **Sucrozyme®** produced consistent with current Good Manufacturing Practice and meeting appropriate food grade standards, are safe. We further conclude that these proposed uses are Generally Recognized As Safe (GRAS) based on scientific procedures.
It is our opinion that other qualified experts would concur with our conclusions.

(b)(6)

05 October 2007
Date

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Medical College of Virginia
VA. Commonwealth University
8718 September Drive
Richmond, VA 23229-7319

(b)(6)

20 Oct 2007
Date

Michael W. Pariza, Ph.D.
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Safe Strain Lineage of Enzyme Products Derived from Bacillus Licheniformis

After evaluation of the Decision Tree Analysis of Safe Strain Lineage of Enzyme Products derived from *Bacillus licheniformis* - Sucrozyme® an alternansucrase preparation by *Bacillus licheniformis* expressing a gene encoding a modified alternansucrase from *Leuconostoc mesenteroides* supplied by Novozymes, I, Michael W. Pariza, conclude that the enzyme preparation products derived from the *Bacillus licheniformis* SJ1707 including the alternansucrase, which is the subject of this review, are derived from a safe strain lineage.

(b)(6)

Michael W. Pariza, Ph.D.
7102 Valhalla Trail
Madison, WI 53719

5 September 2007
Date

Member, Michael W. Pariza Consulting LLC

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September 5, 2007

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# Decision Tree Analysis of Safe Strain Lineage of Enzyme Products Derived from *Bacillus licheniformis*

## Sucrozyme®

**an alternansucrase preparation by *Bacillus licheniformis***

**expressing a gene encoding a modified alternansucrase**

**from *Leuconostoc mesenteroides***

# 1. A Decision Tree Analysis of *Bacillus licheniformis* Production Organisms Derived from a Safe Strain Lineage

## 1.1 Overview

Sucrozyme® is the Novozymes trade name used for an alternansucrase preparation produced by submerged fermentation of *Bacillus licheniformis* carrying a modified gene coding (C-truncated version) for alternansucrase from *Leuconostoc mesenteroides*. Alternansucrase catalyzes alternating α(1→6) and α(1→3) glucosidic linkages from sucrose to generate a D-glucan called alternan.[31,48 & 49] The alternansucrase production strain is constructed using rDNA techniques from a *Bacillus licheniformis* host strain TH049 derived from the *Bacillus licheniformis* strain SJ1707 that is documented as a safe strain in the literature[11,14,15,19,20,26,27&30] and is responsible for several products produced by Novozymes for over 30 years. The alternansucrase enzyme preparation is used as a processing aid in the starch industry for production of low glycemic sweeteners for use in food and beverages as a replacement of sucrose and other traditional sweeteners including high fructose corn syrup.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983; Pariza and Johnson, 2001). The *B. licheniformis* strain SJ1707 has been used as a host strain for several years in the construction of production strains for several other Novozymes enzyme products. For each of the products summarized in Table 1 (section 4.2.2), *B. licheniformis* SJ1707 was used as a common parent strain for the construction of the final production strain by standard transformation procedures using well-known plasmid vectors and well-characterized DNA sequences. In each case, the recombinant plasmid DNA was integrated into the *B. licheniformis* host strain chromosome by homologous recombination. All of these production strains meet the criteria for a safe production strain as described by Pariza and Foster[1], Pariza and Johnson[2], and other expert groups.[3, 13, 18, 26-28, & 34-37] In addition, the production strains comply with current GMP and OECD's criteria for GILSP.[4] The following evaluation uses the Decision Tree set forth by Pariza and Johnson (2001)[2] to establish safe strain lineage of the *B. licheniformis* host production organism SJ1707 and its derivatives.

Further, the safety of the enzyme preparations derived from these *B. licheniformis* production strains was confirmed by toxicological testing. No toxicological effects were observed for any of the test substances produced by these strains derived from the *B. licheniformis* strain SJ1707. See a summary of toxicological testing in section 4.2.2, Table 1.

The Food and Drug Administration has affirmed that mixed carbohydrase and protease enzyme products derived from *B. licheniformis* are generally recognized as safe (GRAS) in the production of certain foods including nutritive sweeteners (21CFR§184.1027), which includes low glycemic sweeteners. Further, FDA has summarized some of the published literature outlining the safe production and use of food enzymes from recombinant microorganisms such as *B. licheniformi* (see Appendices B-D).[26,27,28] Merker and Olempska-Beer outlined FDA's perspective on a case study of enzymes including

GRAS Notice 022, which is an alpha-amylase derived from *B. licheniformis* host strain SJ1707 (Appendix C).[27]

Based upon the work of Pariza and Johnson[2], a decision tree analysis of the safe strain lineage of *Bacillus licheniformis* SJ1707 and products derived from SJ1707 is conducted below. Sections 2-6 provide support for the analysis.

**Safe Strain Lineage of *Bacillus licheniformis***

1. Is the production strain genetically modified? If yes, go to 2. If no, go to 6.

   *Answer: The production strain is genetically modified.*

2. Is the production strain modified using rDNA techniques? If yes, go to 3. If no, go to 5.

   *Answer: The production strain was modified using standard recombinant DNA techniques.*

3. Issues relating to the introduced DNA are addressed in 3a-3e.
   a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food? If yes, go to 3c. If no, go to 3b.

   *Answer: The enzyme product produced is a truncated wild-type Alternansucrase from Leuconostoc mesenteroides, which is substantially equivalent to a wild-type alternansucrase from Leuconostoc mesenteroides currently in commercial use. Note: both 3b & c have been addressed below.*

   b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety? If yes, go to 3c. If no, go to 12.

   *Answer: Although it is concluded that the truncated wild-type alternansucrase is substantially equivalent to the wild-type enzyme used to produce low glycemic sweeteners used in commercially available products, a NOAEL for the truncated wild-type alternansucrase for a two-week oral toxicity study in rats (NZ 20076014) is 792.7 mg TOS/kg/day (TOS is the Total Organic Solids determined as 100 – water – ash – diluents). It is concluded to be sufficiently high to ensure safety. See safety margins established for human consumption scenarios in Section 4.9.*

   c. Is the test article free of transferable antibiotic resistance gene DNA? If yes, go to 3e. If no, go to 3d.

   *Answer: No, none of the DNA inserts that have been inserted into the host strain contains any antibiotic resistance genes. Thus, no antibiotic resistance markers have been added to the strain.*

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   d. Does the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animal? If yes, go to 12. If

no, go to 3e.

e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products? If yes, go to 4. If no, go to 12.

*Answer: The introduced DNA is well characterized and free of attributes that would render it unsafe for constructing microorganisms used to produced food-grade products.*

4. Is the introduced DNA randomly integrated into the chromosome? If yes, go to 5. If no, go to 6.

*Answer: No, the DNA has been integrated through specific integration into the pre-selected amyL locus by double homologous recombination. Specifically, the amyL coding region was replaced by a Leuconostoc mesenteroides truncated asr gene, by double homologous recombination.*

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed? If yes, go to 6. If no, go to 7.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? If yes, the test article is ACCEPTED. If no, go to 7.

*Answer: DN2717, the parent strain is a sporulation-deficient derivative of a natural isolate and SJ1707, the host strain is derived from the parent, which is host for several Novozymes production strains. The parent and host strains were produced over forty years ago. Thus, it is concluded that the Bacillus licheniformis strain is a safe lineage used to produce the truncated wild-type alternansucrase. Accordingly, it is concluded that the Bacillus licheniformis strain used to produce the truncated wild-type alternansucrase is ACCEPTED under the Decision Tree guidelines.*

***STOP**, strain is accepted; no need to address 7-12 below.*

7. Is the organism nonpathogenic? If yes, go to 8. If no, go to 12.

8. Is the test article free of antibiotics? If yes, go to 9. If no, go to 12.

9. Is the test article free of oral toxins known to be produced by other members of the same species? If yes, go to 11. If no, go to 10.

10. Are the amounts of such toxins in the test article below levels of concern? If yes, go to 11. If no, go to 12.

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety? If yes, the test article is ACCEPTED. If no, go to 12.

12. An undesirable trait or substance may be present and the test article is not acceptable for food use. If the genetic potential for producing the undesirable or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.

It is concluded that based upon the decision tree analysis, the host production organism Bacillus licheniformis SJ1707 and production strains derived from SJ1707 are considered to be derived from a safe strain lineage. This conclusion is further supported by the knowledge that 1) common recombinant techniques are used in the construction of the production strain; 2) genetic modifications are well-characterized; 3) the introduced genetic material does not encode and express any known harmful or toxic substances; and 4) current good manufacturing practices are used. Therefore, based on the foregoing Decision Tree analysis of *Bacillus licheniformis* as a type species, which is widely accepted as a non-pathogenic and non-toxigenic host organism, the genetically modified Bacillus licheniformis strains derived from the *Bacillus licheniformis* SJ1707 are considered to be safe production strains for food enzymes and their intended uses.

## 2. TAXONOMY OF ENZYME AND MICROORGANISM

### 2.1 Enzyme

| | |
|---|---|
| IUB nomenclature: | Alternansucrase |
| Systematic name: | Sucrose-1,6(3)-α-glucan 6(3)-α-glucosyltransferase |
| E.C. number: | 2.4.1.140 |
| CAS number: | 100630-46-4 |

Reaction:
Alternansucrase is an enzyme that transfers an α-D-glucosyl residue from sucrose to the 6-position and the 3-position of the non-reducing terminal residue of an α-D-glucan, thus producing a glucan having alternating α-(1,6)- and α-(1,3)-linkages.

The Sucrozyme Product Sheet is enclosed (Appendix A).

### 2.2 Microorganism – host strain

The classification of the host strain is based on the taxonomic characteristics (Priest, F.G. and Alexander, B. Journal of General Microbiology, 134:3011-3018, 1988.

| | |
|---|---|
| Name: | *Bacillus licheniformis* |
| Order: | *Bacillaceae* |
| Genus: | *Bacillus* |
| Species: | *licheniformis* |

(Reference: Bergey's Manual of Systematic Bacteriology).

## 3.0 CONSTRUCTION OF PRODUCTION ORGANISMS DERIVED FROM SJ1707 HOST STRAIN

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The construction of the *Bacillus licheniformis* production strain JA2444, from the parent strain DN2717, via the host strain SJ1707, uses cGMP, well-characterized and commonly used *Bacillus* regulatory sequences (Appendix G – plamisd map and chart), and equivalent manufacturing processes as all other production strains derived from SJ1707. The host strain belongs to the species *Bacillus licheniformis* (Ref.: Gordon, R.E.: The Genus *Bacillus*, Agricultural Handbook No. 427, United States Department of Agriculture, 1973). The strain designations are the Novozymes Identifications Numbers.

Parent Strain: DN2717 is a sporulation-deficient derivate of a natural isolate of *B. licheniformis*, ATCC 9789. This strain naturally produces a subtilisin type protease and an α-amylase. This DN2717 strain is the ancestor of *Bacillus licheniformis* strains that have been used safely for industrial production of enzymes marketed and sold since 1972.[11, 14, 15, 19, 27, 32, 33]

Host Strains: SJ1707 was derived from the Novozymes *Bacillus licheniformis* DN2717 cell line by replacement of the α-amylase (*amyL*) gene with the Thermoanaerobacter cyclomaltodextrin glucanotransferase (CGTase) gene and the introduction of approximately 135 base pair internal deletion in the subtilisin type alkaline protease (*aprE*) gene. MDT 227 is a marker-free strain derived from SJ1707. MDT 227 is deleted for the *aprE* homologue, which encodes a subtilisin type alkaline protease and the *mpr* homologoue, which encodes another protease termed the C-component. TH049 is a derivative of MDT227 and is deleted additionally of five serine protease genes termed *vpr*, *bprA*, *bprB*, *epr*, and *wpr*.

Production Strain: JA2444 was constructed by transformation of TH049 with the truncated wild-type gene of the alternansucrase under control of commonly used *Bacillus* regulatory sequences that have a long history of safe use by Novozymes.

## 4. SAFETY EVALUATION

### 4.1 Overview of the Safety of the Production Strain

The safety of *Bacillus licheniformis* production strain JA2444 was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (Pariza & Johnson[2] IFBC, 1990[3], Olempska-Beer, 2006[26]). Based upon the published literature, the host strain *B. licheniformis* SJ1707 has been thoroughly characterized. In addition, the introduced DNA used in the construction of production strains derived from SJ1707 are well-known and characterized. In addition, the procedures used to modify the host organism are well-defined and commonly used in the industry. The safety assessment of an enzyme preparation to be used in food or food processing should include an evaluation of the safety of the enzyme source (production organism), the enzyme component, and the manufacturing process, as well as a consideration of dietary exposure.

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Novozymes has used the decision tree in Pariza & Johnson, 2001, as a basis for our safety assessment. The production strains including the host strain SJ1707 and all derivatives including the current production strain JA2444, are genetically modified by rDNA techniques commonly known and used in the industry. The expressed enzyme product is an alternansucrase.3[2,48&49] More specifically, the enzyme product is a truncated wild-type alternansucrase from *Leuconostoc mesenteroides*. The introduced DNA is well-characterized and safe for the construction of microorganisms to be used in the production of food-grade products. The DNA has been integrated through specific integration into pre-selected loci. Production strains are well-characterized by qualified scientists and technicians. Novozymes has extensive experience working with *B. licheniformis* production strains and has developed expertise in identifying and characterizing these strains in order to prevent contamination and ensure continuing acceptable, economic yields of a functional enzyme product. Research scientists, fermentation engineers, chemical operators, and quality control technicians follow standard aseptic microbiological produces as well as specific Novozymes protocols for monitoring the biological activity, growth, and physiological characteristics of the production organism during strain improvement programs and during large scale industrial fermentations. In addition, the final commercial enzyme product must perform reproducibly, meet Novozymes' technical service department requirements, and consistently meet the needs of customers in the food industry. All of these periodic and continuous monitoring activities serve not only to guarantee customer satisfaction with Novozymes' enzyme products, but also indicate that there are no unexpected secondary effects of the genetic modifications.

As outlined in the decision tree analysis above, the production strain is derived from a safe strain lineage. Novozymes has used *B. licheniformis* production strains for over 30 years. Table 1 below in Section 4.2.2 outlines some of the Novozymes' products produced by *B. licheniformis* production strains and a summary of safety studies conducted on products derived from Bacillus licheniformis production strains derived from the SJ1707 strain. Thus, the strain SJ1707 and derived production strains meet the elements set forth in Pariza & Johnson[2] to establish safe strain lineage for the food industry.

## 4.2 Safety of Production Organism and Production Strain

### 4.2.1 Safety of *B. licheniformis*

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*B. licheniformis* is widely used as a production organism for the production of enzymes. The history of safe fermentations using B. licheniformis has been documented for over a half century.[28,30] FDA has published a review on the safety of microorganisms used as hosts for the enzyme-encoding genes, the construction of recombinant strains including bacterial host strains, and a list of enzymes from recombinant microorganisms based on FDA regulations, GRAS affirmation petitions, and GRAS notices.[26] An FDA overview on the safety of microbial enzymes used in food: FDA perspective is a synopsis of the regulations and examples of safety assessments of case studies for a lipase enzyme preparation from *Penicillium camembertii*, a chymosin derived from *E. coli K-12*, and an alpha-amylase enzyme preparation from *B. licheniformis*[27] (Appendix C: Merker & Olempska-Beer, Safety of Microbial Enzymes Used in Food: FDA Perspective, presentation).

#### 4.2.2.1 Overview of GRAS Enzymes Derived from *B. licheniformis*

A review of GRAS affirmation petitions and GRAS notices of enzymes from recombinant microorganisms including B. licheniformis is summaried by Olempksa-Beer[26] (Table 1, p. 146; (www.cfsan.fda.gov/~rdb/opa_gras.htm)). In addition, both

carbohydrase and protease enzyme mixtures from *B. licheniformis* have been affirmed by the FDA as generally recognized as safe (GRAS)[11,20] based partly on published literature establishing that *B. licheniformis* is widely recognized as a harmless contaminant of food and unpublished studies which corroborate that food uses of enzyme products from *B. licheniformis* are safe.[11]

### 4.2.2 Safety of *B. licheniformis* strain SJ1707 and its derivatives

The recipient host *B. licheniformis* strain, TH049, used in the construction of the Sucrozyme alternansucarase production strain JA2444, is derived from *B. licheniformis* strain, SJ1707, a sporulation deficient and alkaline protease negative derivative of a natural isolate of *B. licheniformis*, ATCC 9789. The *B. licheniformis* strain SJ1707 is the ancestor of *B. licheniformis* strains that have been used safely for industrial production of enzymes since 1972. SJ1707 and derivatives thereof has been used as host strain in the construction of several production strains for other Novozymes enzyme products. For these production strains, *B. licheniformis* strain SJ1707 or derivatives was used as host strain for the construction of these final production strains by standard transformation procedures using well-known plasmid vectors and well-characterized DNA sequences. In each case, the recombinant plasmid DNA was integrated into the *B. licheniformis* host strain chromosome by homologous recombination. All of these production strains comply with the OECD criteria for GILSP microorganisms[4] and meet the criteria for a safe production microorganism as described by Pariza and Foster[1] and Pariza and Johnson[2] and several expert groups.[3, 13, 18, 34-37] No toxicological effects were observed for any of the test substances produced by strains derived from *B. licheniformis* strain SJ1707.

The outcome of the appropriate safety evaluations outlined by these expert groups supports the determination that the Sucrozyme production strain, JA2444, would not present a pathogenic or toxigenic risk greater than that of its "parent" strain, SJ1707, or any of its derivatives described above and therefore can be regarded as a safe production microorganism for the modified Sucrozyme alternansucrase enzyme.

Novozymes has used Bacillus licheniformis production strains for over thirty years. Table 1 below outlines some of Novozymes' products produced by B. licheniformis and the safety studies conducted on these products. SJ1707 was reviewed by FDA, which summarizes that these studies are published and unpublished.[26] Toxicological testing, confirming the safety of enzyme preparations derived from these *B. licheniformis* production strains, was done as indicated in the table below. An "X" indicates the study was done, a "-" indicates the study was not done.

Table 1.

| Test substance | Termamyl | Duramyl | Toruzyme | Termamyl LC | Termamyl SC | Liquozyme X | Sucrozyme |
|---|---|---|---|---|---|---|---|
| Batch no. | PPY 5499 | PPY 5067 | PPA 4357 | PPY 5977 | PPY 6347 | PPY 7075 | PPN 26587 |
| **Geno tox:** | | | | | | | |
| Ames test | x | x | x | x | x | x | x |
| Chromosome aberration test | x | x | x | x | x | x | x |
| Mouse lymphoma test | - | - | x | - | - | - | - |
| **In vivo tox:** | | | | | | | |
| 4-week oral in rats | x | x | - | - | - | - | x* |
| 13 week oral in rats | - | - | x | x | x | x | - |
| Comments | No treatment related effects | No treatment related effects | No treatment related effects | No treatment related effects | No treatment related effects | No treatment related effects | No treatment related effects |
| NOAEL/NOEL | 1.32 g TOS/ kg bw /day | 1.8 g TOS/ kg bw /day | 2.6 g TOS/ kg bw/day | 0.58 g TOS/ kg bw/day | 0.87 g TOS/ kg bw/day | 1.02 g TOS/ kg bw/day | 0.79 g TOS/kgbw/d |

*2 wk oral test in rats

The chromosome aberration test is an *in vitro* cytogenetic test using cultured human lymphocytes in the presence or absence of S-9 mix. The mouse lymphoma test is a gene mutation assay testing for the ability to induce mutations at the tk locus in mouse lymphoma cells in the presence or absence of S-9 mix. No toxicological effects were observed for any of the test substances produced by strains derived from *B. licheniformis* strain SJ1707. The cyclodextrin glycosyl transferase enzyme preparation from the Toruzyme *B. licheniformis* production strain is generally recognized as safe.[19]

The test article for this study was a test batch of the Sucrozyme alternansucrase enzyme preparation produced according to our standard operating procedures. The test article was administered daily by oral (gavage) to rats for two consecutive weeks. There were no unscheduled deaths, no treatment related clinical signs, no treatment related effects in body weight gains or food consumption, no treatment related changes in hematological or serum clinical chemistry parameters, and no organ weight changes or macroscopic changes that were considered related to treatment. Daily administration of the test article for two consecutive weeks by the oral route (gavage) to rats at low, intermediate, and high doses did not induce any signs of systemic toxicity. No treatment related effects were observed at up to 0.79 g TOS/kg body weight/day. This toxicological test confirmed the safety of the Sucrozyme production strain, JA2444, and the C-truncated wild-type alternansucrase enzyme.

All of the *B. licheniformis* strain SJ1707 derived production strains meet the criteria for a safe production microorganism as described previously by several expert groups. The outcome of the appropriate safety evaluations outlined by these expert groups supports the determination that the Sucrozyme production strain, JA2444, would not present a pathogenic or toxigenic risk greater than that of its "parent" strain, SJ1707, or any of its

derivatives described above and therefore can be regarded as a safe production microorganism for the modified Sucrozyme alternansucrase enzyme.

### 4.3 Safety of the Donor

The donor for the alternansucrase gene is *Leuconostoc mesenteroides* strain BRL 1355. The *Leuconostoc mesenteroides* microorganism is GRAS[21] and has been authorized for food production in Europe.[22,23] *Leuconostoc*[25] *mesenteroides* is used in the production of traditional fermented foods like yogurt, sauerkraut, pickles, and green olives,[3] (see table 18 on page S115).

### 4.4 Safety of the Alternansucrase Enzyme

Alternansucrase is found in a variety of foods including yogurt, pickles, and sauerkraut, for example. Enzyme proteins themselves do not generally raise safety concerns.[1, 16, 17]

### 4.5 Antibiotic Resistance Genes

None of the DNA inserts that been inserted into the host strain contains any antibiotic resistance genes. As a result, no antibiotic resistance markers have been added to the strain.

### 4.6 Absence of Production Organism in Product

The absence of the production organism is an established specification and recombinant DNA was not detected in the Sucrozyme product. As a result, the production organism does not end up in food. Therefore, the first step in the safety assessment as described by IFBC[3] is satisfactorily addressed.

### 4.7 Safety of the Manufacturing Process

Sucrozyome alternansucrase meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. The alternansucrase preparation is produced in accordance with cGMP, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.[6-9]

### 4.8 Safety Studies

#### 4.8.1 Sucromalt

**Sucromalt is a marketed low glycemic sweetener that is produced from sucrose and maltose by an enzymatic reaction with glycosyltransferase from L. mesenteroides.[42]**

**Two safety studies have recently become available.[24,42] A 28-day oral toxicity study of sucromalt in rats concluded that "consumption of high concentrations of sucromalt for 28 days is not associated with obvious signs of toxicity" (Appendix F).[42]**
**A second study is a random, double-blind clinical study concluding that "the reduced glucose and insulin responses elicited by sucromalt are not explained malabsorption and are more likely related to differences in either rate of digestion and absorption or post absorption handling by the body" (Appendix E). [24]**

### 4.8.2 Sucrozyme

**As indicated in section 4.2.1, toxicity studies on Sucrozyme and other products from *B. licheniformis* production strains exhibited no signs of toxicity.**

## 4.9 Potential Dietary Exposure

### 4.9.1 Estimates of Human Consumption and Safety Margin

The enzyme is used as a processing aid and is subjected to temperatures that inactivate the enzyme during the manufacture of the sweetener. In order to illustrate a 'worse-case' situation, however, the following calculations are made assuming that all enzyme activity is retained in the final product.

Sucrozyme has an activity of 150 ASRU/g and an approximate content of 12% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

#### 4.9.1.1 Estimates of Human Consumption - calculation of 'worse-case' enzyme residue in low glycemic sugar/sweetener

In order to demonstrate the 'worse-case' situation, an exaggerated human intake is estimated using the following information:

It is assumed that all enzyme activity is retained in the sugar and sweeteners.
It is assumed that all low glycemic sweetners produced by Sucrozyme will replace 25 percent of traditional sucrose & sweeteners.

Figures used in the calculation for determing the 'worse-case' enzyme residue in the low glycemic sweetener:

| | | |
|---|---|---|
| Maximum Sucrozyme dosage (1ASR (unit)/g) | = | 0.001g/g |
| Activity Sucrozyme | = | 150 ASRU/g |
| "Enzyme" Total Organic Solids | = | 12% |
| Weight of average person | = | 60 kg |
| Consumption of sugar/sweeteners* | = | 143 g/day |
| 25% Market share replacement of sucrose | = | 37.75 g/day |

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*The average human intake of sugar and sweeteners is estimated by using well-established statistics. In the United States, statistics from the U.S. Department of Agriculture (USDA) are used to calculate the potential dietary exposure of the enzyme.

U.S. per capital consumption of sugar per year: "Sugar and sweeteners-situation and outlook report," USDA, Economic Research Service, June 1991: Consumption of refined sugar and high-fructose corn syrup per person per day: 143 g

A twenty-five percent market share replacement of the low glycemic sweetener for sucrose is conservatively estimated to be 35.75 g per person per day. Thus, 35.75 grams per person per day is used in the calculation below. Isomaltulose, a nutritive sweetener, was estimated to assume a 5-10 percent market share replacement of sucrose (GRN 184).

"Worse-case" daily intake per person of Sucrozyme Enzyme TOS from sugar and sweeteners:

35.75 g/day x 0.001 g/g x 0.12 g/g TOS = $4.3 \times 10^{-3}$ g TOS per day

For an average person weighing 60 kg this number corresponds to
$7.2 \times 10^{-5}$ g TOS per kg body weight per day.

#### 4.9.1.2 Safety Margin

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 2 weeks oral toxicity study in rats is 792.7 mg TOS/kg/day, corresponding to 1063 ASR/kg body weight/day.

The safety margin is calculated to be $0.792 / 7.2 \times 10^{-5}$ or approximately 11,014

### 4.10 Results and Conclusions

On the basis of the evaluation contained in this analysis, a review of the published literature, the history of safe use of Bacillus licheniformis, confirmed toxicological studies, and the limited and well-defined nature of the genetic modifications that are not known to encode or express any harmful or toxic substances, it is concluded that alternansucrase as well as other enzymes can be safely produced by Bacillus licheniformis host strain SJ1707 and production strains derived from host strain SJ1707 for use as processing aids in the starch industry for the production of low glycemic sweeteners.

## 5.0 APPENDICES

Appendix A Sucrozyme Product Sheet
Appendix B Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D., and DiNovi, M.J. Food-processing enzymes from recombinant microorganims- a review. Regulatory Tox. Pharmacology, 45, 144-158, 2006.
Appendix C Merker, R.I. and Olempksa-Beer, Z.S. Safety of Microbial Enzymes Used In Food: FDA Perspective, presentation, www.anbio.org.br/palestras/palestra_fda.ppt, accessed 30-July-2007.
Appendix D Merker, R.I. Enzymes from recombinant microorganisms, presentation to the Enzyme Technical Association, 2Nov2006.

Appendix E Grysman, A., Carlson, T. & Wolever, T.M.S. (published online 22Aug2007).Effects of sucromalt on postprandial responses in human subjects. Eur. J.Clinical Nutrition.

Appendix F Eapen, A.K., Chengelis, C.P., Jordan, N.P., Baumgartner, R.E., Zheng, T. & Carlson, T. A 28-day oral (dietary) toxicity study of sucromalt in Sprague-Dawley rats. Food and Chemical Toxicology 1016, 2007.06.008 (in press).

Appendix G Plasmid map and chart.

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Appendix A

# Product Data sheet

novozymes

# Alternan Sucrase

## Valid from: Preliminary Draft

**Product Characteristics:**

| | |
|---|---|
| Enzyme Class | Alternan sucrase |
| Declared activity | 150 ASR/g |
| Colour | Brown<br>Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity. Product may be hazy and contain slight precipitate; this does not affect enzyme activity or performance |
| Physical form | Liquid |
| Approximate Density (g/ml) | 1.25 |
| Viscosity (cPs) | 20-50 |
| Stabiliser | Glycerol<br>Sorbitol |
| Preservatives | Potassium sorbate |
| Production organism | Bacillus licheniformis<br><br>Produced by submerged fermentation of a genetically modified micro organism. The enzyme protein, which is protein engineered, is separated and purified from the production organism. |

## Product Specification:

| | Lower Limit | Upper Limit | Unit |
|---|---|---|---|
| Alternan sucrase Units ASR | 150 | | /g |
| Total Viable Count | - | 10000 | /g |
| Coliform Bacteria | - | 30 | /g |
| Enteropathogenic E.Coli | | None Detected /25g. | |
| Salmonella | | None Detected /25g. | |

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

## Packaging:

See the standard packaging list for more information.

## Recommended Storage:

| | |
|---|---|
| Best before | When stored as recommended, the product is best used within 3 months from date of delivery. |
| Storage temperature | 0-10°C (32°F-50°F) |
| Storage Conditions | In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement. |

## Safety and handling precautions:

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. The product may create easily inhaled aerosols if splashed or vigorously stirred. Spilled product may dry out and create dust. Spilled material should be flushed away with water. Avoid splashing.
Left over material may dry out and create dust. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes. A Material Safety Data Sheet is supplied with all products. See the Safety Manual for further information regarding how to handle the product safely.

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Appendix C

## Safety of Microbial Enzymes Used in Food: FDA Perspective

Robert I. Merker and Zofia S. Olempska-Beer
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety

## Overview

- REGULATORY OPTIONS
  - Food additive (petition)
    - subject to premarket review
  - GRAS determination – optional notice
    - not subject to premarket review
- SAFETY ASSESSMENT
  - Information provided in GRAS notices
  - Case studies
    - Lipase from a "conventional" microorganism
    - Chymosin from bioengineered microorganisms
    - α-amylase from a bioengineered microorganism

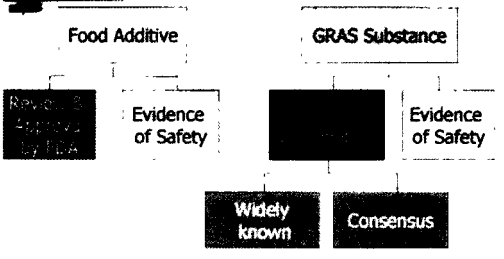
## Regulatory Basis

- 1958: Food Additive Amendments to Federal Food Drug and Cosmetic Act:
- In the United States, a food additive is defined as:
  - . any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food . if such substance is not generally recognized... to be safe under the conditions of its intended use. .
    - FFDCA: 201 (s)

## U.S. Regulatory Processes for Food Ingredients

- Food Additive Petition
  - Regulation in 21 CFR 172 or 173
- GRAS Affirmation Petition
  - Regulation in 21 CFR 184
- GRAS Notice
  - Primary route for submitting information on microbial enzymes to FDA
  - Information available on the Internet

## GRAS Criteria: Comparing a GRAS Substance to a Food Additive




## The GRAS Proposal: 62 FR 18938 April 17, 1997

- Proposed notification program for GRAS substances
  - Notifier prepares summary of basis for its GRAS determination
  - Not an iterative process
  - Three categories of response letters
    - "No questions"
    - "No basis"
    - Withdrawn by notifier

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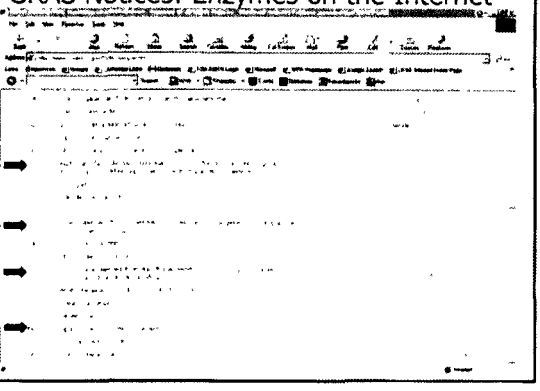
## The Summary of GRAS Notices

- At http://www.cfsan.fda.gov/~rdb/opa-gras.html
- Lists all Notices and information about each notice
  - Currently, 24 out of 101 notices are about enzymes
- Includes a link to the text of FDA's response letter

## GRAS Notices: Enzymes on the Internet



## GRAS Affirmation Regulations on Microbial Enzymes

In 21 CFR 184

| 21 CFR § | Enzyme | Source Organism |
|---|---|---|
| [illegible] | Mixed **carbohydrase** and **protease** | *Bacillus licheniformis* |
| [illegible] | Bacterially-derived **Carbohydrase** | *Bacillus subtilis / B. amyloliquefaciens* |
| [illegible] | Bacterially-derived **protease** | *Bacillus subtilis* or *amyloliquefaciens* |
| [illegible] | **Cellulase** | *Trichoderma longibrachiatum* |
| [illegible] | Insoluble **glucose isomerase** | Multiple sources |
| [illegible] | **Lactase** | Multiple sources |
| [illegible] | **Lipase** | *Rhizopus niveus* |
| [illegible] | **Urease** | *Lactobacillus fermentum* |

## Some Microbial Enzymes are Approved Food Additives

In 21 CFR 173 Subpart B- Enzyme Preparations and Microorganisms

| 21 CFR § | Enzyme | Organism |
|---|---|---|
| 173.110 | Aminoglucosidase | *Rhizopus niveus* |
| 173.120 | Carbohydrase, cellulase | *Aspergillus niger* |
| 173.130 | Carbohydrase | *Rhizopus oryzae* |
| 173.135 | Catalase | *Micrococcus lysodeikticus* |
| 173.140 | Esterase-lipase | *Mucor miehei* |
| 173.145 | Alpha-galactosidase | *Mortierella vinaceae* var. *raffinoseutilizer* |
| 173.150 | Milk Clotting enzymes | Various organisms |
| 173.170 | Aminoglycoside 3'-phosphotransferase II | Originally from *Tn5* (*E. coli*) |

## Enzyme Submissions in 2002

- GRAS Notices - most commonly used means for submitting information about microbial enzymes to FDA
  - Include published and non-published information
- Consistency in the categories of information provided in GRAS notices

## Information Provided About Microbial Enzyme Preparations in GRAS Notices

- Safety of the enzyme
- Safety of the production microorganism
- Safety of the manufacturing process
- Safety of the enzyme preparation

## The Enzyme

- Identity
  - (name, IUB classification, CAS Reg. No.)
- Enzymatic activity
- Physico-chemical characteristics
  - (e.g., MW, thermal stability, optimum pH)
- Genetic modifications at the DNA and amino acid levels (if applicable)

## The Production Microorganism

- Name and taxonomic classification
- Source
- Important characteristics
  - Nonpathogenic
  - Nontoxigenic
  - History of safe use
  - Modifications (classical mutagenesis; genetic engineering)

## For a Bioengineered Production Microorganism

- Characterization of the host strain
- Description of the expression plasmid (genetic material, its identity, organization, and origin)
- Description of the gene encoding the enzyme (source(s), modifications)
- Introduction of hereditary materials (DNA) into the host and characterization of the production strain

## The Manufacturing Process

- Fermentation (type of process, raw materials, controls)
- Removal of the microorganism
- Recovery (process, raw materials)
- Purification and concentration
- Formulation and standardization (e.g., water, sodium chloride, sucrose)
- Use of food grade materials

## The Enzyme Preparation

- Composition (including total organic solids – TOS)
- Specifications (Food Chemicals Codex, 4th edition, 1996; JECFA, 2001)
- Absence of transformable DNA (bioengineered microorganisms)

## The Enzyme Preparation, cont.

- Use in food
- Level in food (generally very low)
- Estimation of daily intake
- Toxicological studies (case-by case basis; may include e.g., gene mutation, chromosomal aberrations, toxicity in rats)
- Consideration of constituents derived from source, and manufacturing process

## Case studies

- Lipase enzyme preparation from *Penicillium camembertii*
- Chymosin enzyme preparation from bioengineered *Escherichia coli* K-12
- ✂-amylase enzyme preparation from bioengineered *Bacillus licheniformis*

## Case Studies: Lipase

- GRN 000068: Lipase enzyme preparation derived from *Penicillium camembertii*
- Intended Use: Production of fatty acids from fats and oils
- Similar to other lipases used in food processing
- Source Organism ( )
  - Nontoxigenic
  - Nonpathogenic
  - Long used in the production of Camembert cheese

## Lipase: The Manufacturing Process

- Major steps:
  - Fermentation using food grade ingredients
  - Enzyme secreted into medium
  - Filtration and heat – remove fungal cells
  - EtOH and acid precipitation
  - Centrifuged, dried, crushed, blended with diluent to desired activity

## Lipase: The Enzyme Preparation

- Specifications:
  - Complies with general and additional requirements of Food Chemicals Codex, 4th edition ( )
  - Does not contain detectable antibiotics or mycotoxins (unpublished)
- Estimated intake: 1 mg/person/day (unpublished)

## Lipase: Studies

- "Pathogenicity" study ( ) – organism (*P. camembertii)* injected into mice – no effects, not recovered
- Toxicological studies ( ) using concentrated lipase enzyme preparation
  - 90 day gavage study in rats up to 2000 mg/kg/d – no effect
  - Bacterial mutagenicity – no effects

## Lipase: FDA letter

"Based on the information provided by Amano Enzyme, Inc., as well as other information available to FDA, **the agency has no questions at this time regarding Amano's conclusion that the lipase enzyme preparation derived from *P. camembertii* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this lipase preparation.** As always, it is the continuing responsibility of Amano to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements."

## Case Studies: Chymosin from *Escherichia coli* K-12

- First FDA regulation issued on an enzyme preparation produced from a bioengineered organism (21 CFR 184.1685)
- Bovine enzyme expressed in *E. coli* K-12
- Subsequent regulations:
  - Bovine chymosin from *Kluyveromyces marxianus*
  - Bovine chymosin from *Aspergillus niger*

## Chymosin from *E. coli* K-12

- The host strain: *E. coli* K-12 JA198
  - Nonpathogenic
  - Nontoxigenic
- The production strain: *E. coli* K-12 GE81
  - Contains the expression plasmid pPFZ-87A based on the *E. coli* vector pBR322
  - The plasmid carries a gene encoding bovine prochymosin

## Chymosin from *E. coli* K-12

- The manufacturing process
  - Pure culture fermentation of *E. coli* K-12 GE81
  - Prochymosin accumulates within cells as "inclusion bodies"
  - Prochymosin is isolated, purified, and converted to chymosin
  - Chymosin is purified and shown to be identical to bovine chymosin present in rennet (published information)

## Case Studies: Bioengineered α-Amylase

- GRAS Notice 000022: α-Amylase enzyme preparation derived from a bioengineered strain of *Bacillus licheniformis*
- Bioengineered for enhanced stability at low pH, low calcium concentration and high temperature
- Intended use: starch hydrolysis in the production of syrups (e.g., high-fructose corn syrup) and alcohol

## Bioengineered α-Amylase: The Enzyme

- Gene encoding α-amylase derives from *B. licheniformis.*
  - Modifications for enhanced stability and a lower calcium requirement (as compared with other α-amylases):
    - 35 amino acids at amino-terminal region from *B. amyloliquefaciens* α- amylase
    - Five additional altered amino acids
  - DNA sequence: highly homologous to those encoding other α-amylases
  - Same enzymatic function as other α-amylases that have a history of safe use in food ( )

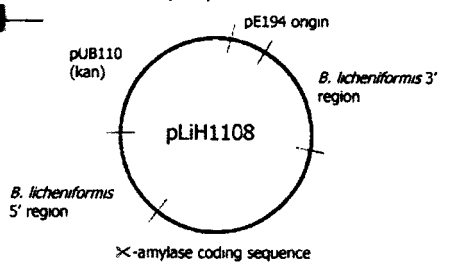
## Bioengineered α-Amylase: The Production Strain

- *B. licheniformis*
  - Nonpathogenic ( )
  - Nontoxigenic ( )
  - History of use as a source of food enzymes ( )
- Host strain: *B. licheniformis* strain SJ1707 (sporulation deficient and protease negative)
- Production strain: LiH 1159
  - contains plasmid pLiH1108 stably integrated into the host chromosome.

## Bioengineered α-Amylase: The Bioengineered Strain

- Plasmid pLiH1108 contains:
  - The bioengineered α-amylase gene
  - Selectable marker: the kanamycin resistance gene encoding aminoglycoside 3'-phosphotransferase II
  - DNA sequences necessary for plasmid integration
  - DNA sequences from the certified cloning vectors pE194 and pUB110

## Plasmid Map: pLiH1108




## Bioengineered α-Amylase: The Manufacturing Process

- Pure culture fermentation of the bioengineered production strain LiH 1159
- α-Amylase is recovered from the fermentation broth, concentrated, and formulated with sodium chloride, sucrose, and water
- Materials used in fermentation and recovery are food grade

## Bioengineered α-Amylase: The Enzyme Preparation

- Derived from the safe production strain.
- Contains the safe active component, bioengineered α-amylase.
- Complies with FCC and JECFA specifications ( ).
- Does not contain the production strain (unpublished).

## Bioengineered α-Amylase: The Enzyme Preparation, cont.

- Does not contain plasmid DNA (unpublished)
- Does not contain APH(3')II (unpublished)
- Is not carried over to food (syrups and alcohol)
- Estimated intake: negligible (unpublished)
- Toxicological studies: gene mutation, chromosomal aberrations, subchronic toxicity in rats, all negative (unpublished)

## Summary

- In the U.S.A., microbial enzymes intended for use in food are most commonly submitted to FDA as the subjects of GRAS notices, which do not require premarket review.
- GRAS Notices commonly contain and unpublished information about:
  - The Safety of the Enzyme
  - The Safety of the Production Microorganism
  - The Safety of the Manufacturing Process
  - The Safety of the Enzyme Preparation

Appendix D

# Enzymes from recombinant microorganisms

Robert I Merker Ph D
FDA/Center for Food Safety
and Applied Nutrition
Office of Food Additive Safety
November 2, 2006
Presentation to Enzyme Technical Association

## Highlights from the recently published review

Food-processing enzymes from recombinant microorganisms--a review.

by

Zofia Olempska-Beer, Robert Merker, Mary Ditto, and Michael DiNovi

Office of Food Additive Safety

(Regul. Toxicol. Pharmacol. 2006 Jul;45(2):144-158 )

## Abstract

Enzymes traditionally isolated from microorganisms, plants and mammalian tissue are often not well-adapted to the conditions used in modern food production methods. The use of recombinant DNA technology has made it possible to manufacture novel enzymes suitable for specific food-processing conditions

Such enzymes may be discovered by screening microorganisms sampled from diverse environments or developed by modification of known enzymes using modern methods of protein engineering or molecular evolution.

## Outline

- Introduction
- Development of recombinant production strains
- Characteristics of host strains
- Examples of host strains used for producing recombinant enzymes
- Safety of host organisms
- Constructing recombinant strains
- Sources of recombinant enzymes
- Production and assessment of enzyme preparations
- Conclusions

## Introduction

- Numerous enzymes currently used in food-processing are derived from recombinant microorganisms
- Enzymes derived from diverse sources (microorganisms, plants, animal tissue) can be efficiently produced by fermentation of well-characterized microorganisms

## Advantages of rDNA technology in enzyme production

- Enzyme properties can be modified to make them compatible with food-processing conditions (example: α-amylase for starch hydrolysis active at 90-105°C)
- Safety of enzyme production strains can be improved by reducing or eliminating their ability to produce toxic metabolites
- Multiple techniques to increase yield

## Development of recombinant production strains

- Development of the host (recipient) strain
- Construction of the expression vector
- Transformation of the host strain with rDNA (either the entire expression vector or a fragment of the vector)
- Identification of the best recombinant strain
- Additional improvements (e.g., by traditional mutagenesis)
- Characterization of the production strain

## Characteristics of host strains

- Nonpathogenic
  No history of conferring disease to humans
- Nontoxigenic
  Do not produce substances injurious to humans and animals

## Commonly Used Host Species

- Most derived from strains of *Bacillus subtilis*, *B licheniformis* *Aspergillus oryzae*, *A niger* as native sources of enzymes
  - Grow efficiently under industrial production conditions
  - Amenable to genetic manipulation
  - Secrete enzymes into fermentation media
  - Protease-deficient strains to increase enzyme yields

## Examples of host strains used for producing recombinant enzymes

| Source microorganism | Enzyme Preparation Submissions submitted to FDA |
|---|---|
| *Aspergillus niger* | Phytase, Chymosin, Lipase |
| *Aspergillus oryzae* | Esterase-lipase, Aspartic proteinase, Glucose oxidase, Laccase, Lipase, Pectin esterase, Phospholipase A1 |
| *Bacillus licheniformis* | Alpha-amylase, Pullulanase |
| *Bacillus subtilis* | Alpha-acetolactate decarboxylase, Alpha-amylase, Maltogenic amylase Pullulanase |

## New strains for production of recombinant enzyme preparations

- No prior history of use for food enzyme preparations
  - Bacteria: *Escherichia coli* K-12, *Pseudomonas fluorescens*
    Gram-negative bacteria accumulate (do not secrete) enzymes
    Adds steps to purification process
  - Fungus: *Fusarium venenatum*

## Enzymes Reviewed by FDA produced from uncommon strains

| Microorganism | Enzyme/submission or regulation |
|---|---|
| *Eschenchia coli K-12* | Chymosin 21 CFR 184.1685 |
| *Fusarium venenatum* | Xylanase GRN 54 |
| *Kluyveromyces marxianus* var. *lactis* | Chymosin 21 CFR 184.1685 |
| *Pseudomonas Fluorescens* Biovar I | alpha-amylase GRN 126 |
| *Trichoderma reesei* | Pectin lyase GRN 32 |

## Safety of host organisms- *Bacillus* spp.

- *B. subtilis*
  - Progenitor strain 168: Genome sequenced. Well-studied
    Safety of recombinant enzymes from *B subtilis* is documented in petitions, GRAS notices, publications
- *B. licheniformis*, (also *B. amyloliquefaciens, G stearothermophilus*)
  - Used successfully as hosts
  - Safety discussed in reviews
    Cytotoxicity tested, found nontoxic
  - No homology to genes encoding *B cereus* protein toxins

## *Bacillus* spp.: Traits used in hosts

Characteristics unrelated to safety

- Nonsporulating mutants
  Sporulation diverts energy from enzyme production
- Protease-deficient mutants - increase enzyme yield
- Secrete proteins into fermentation medium
- Auxotrophic mutants for selection purposes – avoids safety question of dissemination of antibiotic resistance markers

## Safety of Host Organisms: Gram-negative bacteria

- *Escherichia coli* K-12
  - Used for chymosin preparation production
    Safety based on long-term laboratory use (30+ years) and absence of genes for toxins
  - Produces enzyme intracellularly – inclusion bodies
- *Pseudomonas fluorescens Biovar I*
  - Used for production of thermostable α-amylase
    Not known to cause disease
  - Ubiquitous in environment
  - Used for large-scale production of *Bacillus thuringiensis* toxin (biopesticide)
  - Produces enzyme intracellularly – inclusion bodies

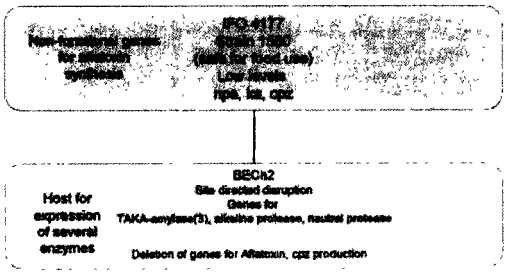
## Safety of Host Organisms: Fungal Hosts

- *Aspergillus oryzae* and *A. niger*
  - Long history of use in food production
    *A. oryzae*
    - Koji mold in production of soy sauce, soybean paste miso and rice wine sake
    - Genome sequenced 2005
    *A niger*
    - Used for production of citric acid (21 CFR 184 1033)
    Long history of use in production of enzymes used in baking, brewing, other food applications
    Historical use leads to consideration as nonpathogenic, nontoxigenic
    - Bioengineered strains used since 1980's, 1990's

## Safety of Host Organisms: *A. oryzae*

- Not pathogenic
- Related to *A. flavus* / lost ability to produce aflatoxins over time
  - Aflatoxin synthesis: in some strains structural and regulatory genes intact, but some products are nonfunctional; synthetic pathways are blocked
  - Other mycotoxins with low-to-moderate toxicity produced at low levels
    3-β-nitropropionic acid (npa), kojic acid (ka), cyclopiazonic acid (cpz)
    Maltoryzine, violacetin (not commonly found in enzyme producing strains)
    EPA assessment under TSCA – low risks, long history of commercial use, mycoxins produced, not highly toxic

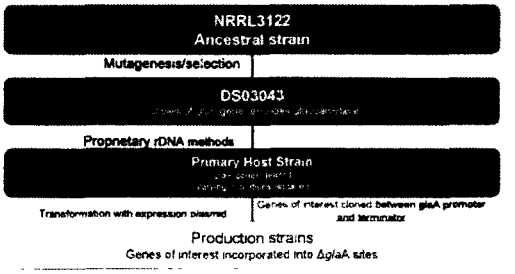
## *A. oryzae* Strain Development



## Safety of Host Organisms: *A. niger*

- Use as source of enzymes
  - (cellulase, carbohydrase) 21 CFR 173.120
  - recombinant chymosin (21 CFR 184 1685)
- Mycotoxin production
  - Ochratoxin A (nephrotoxic, carcinogenic) (suggest testing for presence)
  - Other potentially toxic secondary metabolites produced in some strains.

## *A. niger* Strain Development: One example




## Safe use of *A. oryzae and A. niger*

- Fungi: may produce low levels of toxic secondary metabolites
- Levels should not be
  - detectable
  - harmful under conditions of use
- Mycotoxins only produced under stress conditions
  - Can control stress by controlling fermentation conditions
- Precautionary measures
  - Production / host strains with history of safe use
  - Test for mycotoxin production under industrial fermentation conditions
  - Test new strains for mycotoxin production capability
  - Implement controls to ensure that mycotoxins will not be produced under fermentation conditions by mycotoxin-producing strains

## Safety of host organisms: *Fusarium venenatum*

- *F. venenatum*
  - Not a known pathogen
  - Mycotoxins
    - May produce trichothecenes, culmorrins fusarins, and cyclic peptide enniatin B (insecticidal, bactericidal activities),
    - Not produced under controlled fermentation conditions
  - Mycoprotein source – protein-rich product used in U K. since 1985
    - Mycoprotein routinely tested for trichothecenes and fusarins
  - Xylanase expressed from a gene from *Thermomyces lanuginosus* (GRN 54)
    - Strain with trichothecene synthase gene deletion - considered safe source

## Safety of Host Organisms: Other Fungal Hosts

- *Kluyveromyces marxianus* var *lactis*
  - Host for chymosin production
  - Source of lactase (affirmed GRAS 21 CFR 184 1388)
    - for hydrolysis of lactose to glucose and galactose (e.g., products of lactose intolerant individuals)
  - Nonpathogenic/nontoxigenic
- *Trichoderma reesei*
  - Cellulolytic enzymes
    - Cellulases used in baking, malting, grain alcohol production
  - Host for enzyme expression (pectin lyase from *A. niger* var *awamori*)
  - Single strain isolated from cotton canvas – QM6a parent of all existing strains
  - Taxonomic confusion:
    - previously considered identical to *T. longibrachiatum*,
    - now considered asexual form of tropical fungus *Hypocrea jecorina*
  - Production of antifungal metabolites
    - Validity of single report questioned

## Constructing Recombinant Strains

- Well-characterized host strains
- Introduce desired genes on expression vectors to increase expression
- Expression vectors
  - What they are
    - Plasmid DNA (extrachromosomal, self-replicating DNA)
    - Expression cassette
      - (minimally) a promoter, a gene encoding the enzyme a terminator
    - A selective marker (commonly antibiotic resistance but metabolic markers also used)
- May be maintained as extrachromosomal elements (usually in bacterial hosts) or integrated either at random or specific sites on the chromosome

000177

## Expression Vectors (2)

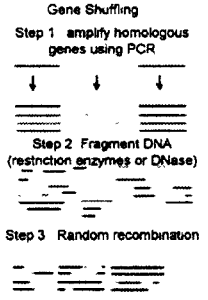
- On the plasmid backbone:
  - Replication origin compatible with replication in desired bacterial host
  - A selective marker that is active in bacterial hosts (*kan*$^r$, *amp*$^r$, *tet*) [often used in vector construction]
  - if designed for use in fungi, a selective marker for fungal hosts
    - (*amd*S, encodes acetaminidase growth on acetamide)
    - (URA3 – encodes Orotidine-5'-phosphate (OMP) decarboxylase – can complement mutations in prokaryotic and eukaryotic enzymes)

## Sources of Recombinant enzymes

- Sources: microbial, plant, animal sources
- Many identical to long-used food enzymes
  - Chymosin from *E coli, K marxianus* var *lactis, and A niger* var *awamori*
    - Identical to animal-derived rennet
- Commonly derived from well-characterized, culturable microorganisms
  - New screening techniques, new sources
- Direct DNA isolation from environment, create expression libraries in suitable hosts and screen for appropriate activities
  - Optimizing properties for industrial use use various techniques to adapt
    - Site-specific mutagenesis effective when structure-function relationships are well-elucidated

## Directed Evolution

- Stepwise process
- Often uses multiple parent genes from diversified sources encoding a specific enzyme activity
- Mutagenized in a random manner (alternative methods)
  - Error-prone PCR
  - Sequential random mutagenesis.
  - Gene shuffling
- High throughput screening
- Recycled through process until enzyme with appropriate activity generated




## FDA reviews of recombinant enzymes

- *B. licheniformis* alpha-amylase (GRN 22):
  - N- terminus: replace *B. licheniformis* sequences with *B. amyloliquefaciens* sequence and 5 additional aa substitutions
- Hybrid alpha-amylase (GRN 126)
  - Environmentally isolated DNA subjected to directed evolution through gene shuffling of environmental isolates from three Archaea, produced in *Pseudomonas fluorescens Biovar I.*

## Fine Tuning

- Genes: can incorporate into host genome
  - At different loci
  - In different copy numbers
    - On at multiple sites on chromosome
    - On high copy number plasmid
  - Assess expression levels
  - May use mutagenesis to increase expression

## Production: Fermentation

- Controlled fermentation of production strains
- Food grade medium components
  - Satisfy nutritional requirements of production strain
- Batch process: large-scale. aerated fermenters
  - Controlled parameters: pH, temperature, aeration
- Test for contaminants, discard if present
- Antifoams, alkali for pH adjustment

## Production: Processing

- Most enzymes:
  - secreted into medium
  - Concentrated by ultrafiltration and evaporation
  - Formulated with sucrose, maltose, maltodextrin, potassium sorbate, sodium benzoate, or other similar compounds
- Final product = enzyme preparation
  - Contains formulating ingredients;
  - May retain metabolites from production organism, compounds used in fermentation and processing

## Assessment

- Used in food processing at low levels
- Removed from some foods during processing
- Inactivated during processing, (i.e., cooking, baking)
- Exposure calculations:
  - Based on total organic solids (TOS)
    - [enzyme plus organic material, excluding formulating ingredients]
- Concentrated enzyme before formulation tested according to generally accepted procedures
- Specifications by Food Chemicals Codex, JECFA
- Test for transformable DNA encoding antibiotic resistance
  - Not detected in submissions to date

## Conclusions

- Recombinant methods
  - Allow for expression of enzymes in hosts that are well-adapted to large scale industrial fermentation
- Techniques to increase yield
  - Efficient promoters
  - Multiple copies
- Tailor properties to food processing conditions
  - Modify aa sequence for increased thermostability or altered pH stability

## Conclusions (2)

- Safety of Production Strains
  - Well-characterized, nonpathogenic, nontoxigenic, history of safe use in food manufacture
  - Minimize mycotoxins through controlled fermentation conditions
    - secondary metabolites of certain fungal production strains
- Targeting cloned genes into designated chromosomal loci to avoid potential unintended effects
- Improved host strains
  - Protease-deficient mutants
  - Sporulation-deficient strains

## Acknowledgements


- My co-authors:
  - Dr. Michael DiNovi, Dr. Mary Ditto;
  - Dr. Zofia Olempska Beer;
  - Thanks for their hard work on the review and assistance with this presentation
- Our Division Director Dr. Antonia Mattia and Deputy Division Director Dr. Robert L. Martin at FDA:
  - Thanks for support and helpful suggestions during the "birth" of the paper

Pages 000180-000208 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

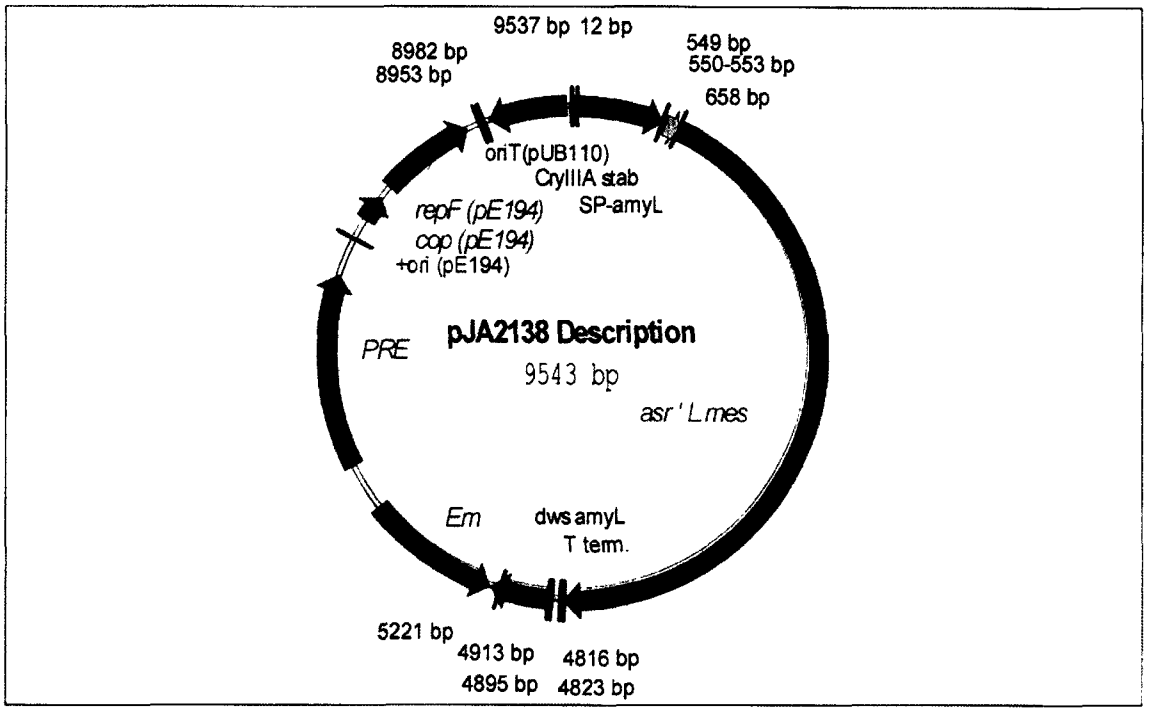
Pages 000209-000216 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

**Confidential Business Information**

## Appendix G

Appendix G

## Description of plasmid JA2138




| Position (bp) | Size (bp) | Element | Origin |
|---|---|---|---|
| 9538-12 | 18 | Linker region (*SacI, EcoRI, BglII*) | Synthetic |
| 13-549 | 537 | *cryIIIA* UTR | Bacillus thuringiensis cry3Aa gene |
| 550-553 | 4 | Linker (*MscI*) | Synthetic |
| 554-658 | 105 | *amyL* 5' end | B.licheniformis alpha-amylase (amyL) gene |
| 659-4816 | 4158 | C-terminal truncated mature *asr* | Leuconostoc mesenteroides NRRL B-1355 asr gene for alternansucrase |
| 4817-4822 | 6 | Linker (*SalI*) | Synthetic |
| 4823-4895 | 73 | *amyL* Terminator | B. licheniformis ATCC 14580, complete genome. |
| 4896-4912 | 17 | Linker (*BglII,ClaI*) | Synthetic |
| 4913-5221 | 309 | N-truncated *yvdE* (downstream region of *amyL* gene) | B. licheniformis ATCC 14580, complete genome. |
| 5222-5224 | 3 | Linker (AatI) | Synthetic |
| 5225-8953 | 3729 | pE194 | S.aureus plasmid pE194[40] ORF's A,B,C,D,E, & F |
| 8954-8982 | 29 | Linker (*XbaI, BamHI, XmaI, KpnI, SacI*) | Synthetic |
| 8983-9537 | 555 | pUB110 | Plasmid pUB110[39] (S.aureus) complete genome |

# *Reference List for Industry Submission, GRN 000258*

| *Pages* | *Author* | *Title* | *Publish Date* | *Source* | *BIB_Info* |
|---|---|---|---|---|---|
| 000153-000167 | Olempska-Beer, Zofia S.; Merker, Robert I.; Ditto, Mary D.; DiNovi, Michael J. | Food-processing enzymes from recombinant microorganisms - a review | 2006 | Regulatory Toxicology and Pharmacology | Volume 45, pgs 144-158 |
| 000180-000208 | Grysman, Atarah; Carlson, Ting; Woever, Thomas, MS | Effects of sucromalt on postprandial repsonses in human subjects | NA | NA | NA |
| 000209-000216 | Eapen, Alex K.; Chengelis, Christopher, P.; Jordan, Nancy P.; Baumgartner, Roxanne E.; Zheng, Ted; Carlson, Ting | A 28-day oral (dietary) toxicity study of a sucromalt in Sprague-Dawley rats | 2007 | Food and Chemical Toxicology | NA |

***NA- Not applicable***