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December 31, 2020



Susan J. Carlson, PhD
Director, Division of Food Ingredients
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

RE: GRAS Notice for Maltosyltrehalose Syrup (TG4 Syrup)

Dear Dr. Carlson:

The attached GRAS Notice for Maltosyltrehalose Syrup {TG4 Syrup) is submitted on behalf of the Notifier, Hayashibara Co., Ltd. of Okayama, Japan (Hayashibara), in accordance with 21 CFR Part 170, subpart E. Hayashibara has concluded that TG4 Syrup is generally recognized as safe (GRAS) for its intended use based on scientific procedures, and can therefore can be excluded from premarket approval requirement of the Food, Drug, and Cosmetic Act.

The intended use of TG4 syrup is as a food ingredient that can be used to substitute for other substantially equivalent commercial carbohydrates such as glucose syrups, dextrin and maltodextrin at concentrations not to exceed the amounts required to accomplish the intended effect. TG4 is a substance composed of only glucose molecules in relatively short linear chains (DE about 19 on dry basis) including only a-1,4, and to a lesser extent a-1, 1 glycosidic bonds.

TG4 has been consumed in Japan and Taiwan since 2003, and classified as a starch syrup and food ingredient, respectively. No known untoward reports have been received at any time since its introduction.

If the Agency has questions or requires additional information please contact me at the above address, phone number or email.

Sincerely,

Alan B. Richards, PhD
President, VRSI

Enclosures

# GRAS Notice for Maltosyltrehalose Syrup (TG4 Syrup)

## Manufactured by Hayashibara Co., Ltd.

December 29, 2020

**Prepared by**

**Vanguard Regulatory Services, Inc.**
**1311 Iris Circle**
**Broomfield, CO 80020**

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# Part 1. Signed Statements and Certification

## 1.1 Compliance statement

Vanguard Regulatory Services, Inc., on behalf of Hayashibara Co., Ltd. (Hayashibara), of Okayama, Japan, submits this GRAS notice of Maltosyltrehalose Syrup in accordance with 21 CFR §170 subpart E.

## 1.2 Name and address of Notifier

Hayashibara Co., Ltd.
675-1 Fujisaki, Naka-ku
Okayama 702-8006, JAPAN

**All communications regarding this document should be addressed to:**

Alan B. Richards, PhD
Vanguard Regulatory Services, Inc.
1311 Iris Circle
Broomfield, CO 80020
Office: (303) 464-8636
Mobile: (720) 989-4590
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## 1.3 Manufacturer

Hayashibara Co., Ltd.
675-1 Fujisaki, Naka-ku
Okayama 702-8006, JAPAN

## 1.4 Name of notified substance

Maltosyltrehalose Syrup, which will be abbreviated in the text as TG4 Syrup.

## 1.5 Intended conditions of use of the notified substance

TG4 Syrup will be used as a carbohydrate source that can be substituted for standard starch-based syrups. TG4 Syrup can also provide the following technical effects, as listed in 21 CFR §170.3(o): antioxidant, flavor enhancer, flour treating agent, formulation aid, nutritive sweetener, processing aid, solvent and vehicle, stabilizer and thickener, surface-active agent, surface-finishing agent, and texturizer.

It is expected that TG4 Syrup will be used in foods and beverages for consumption by adults and children 2 years old and older.

Table 1-1 provides the food categories (21 CFR §170.3(n) and percentages of the estimated maximum and average TG4 Syrup that could be used. However, please note that the amounts listed are the estimated amounts of conventional starch syrups that are believed to be used in these product categories. TG4 Syrup could theoretically, but not practically, be used at these percentages.

**Table 1-1 Food Categories, Estimated Maximum and Average Use Percentages of TG4 Syrup**

| FDA 21 CFR §170.3(n) Food Category | Estimated Maximum Use Percentage | Estimated Average Use Percentage |
|---|---|---|
| (1) Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours, and mixes requiring preparation before serving. | 10 | 5 |
| (2) Beverages, alcoholic, including malt beverages, wines, distilled liquors, and cocktail mix. | 10 | 5 |
| (3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks. | 10 | 5 |
| (4) Breakfast cereals, including ready-to-eat and instant and regular hot cereals. | 10 | 5 |
| (5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses. | 10 | 5 |
| (6) Chewing gum, including all forms. | 10 | 5 |
| (7) Coffee and tea, including regular, decaffeinated, and instant types. | 10 | 5 |

**Table 1-1 Food Categories, Estimated Maximum and Average Use Percentages of TG4 Syrup (continued)**

| FDA 21 CFR §170.3(n) Food Category | Estimated Maximum Use Percentage | Estimated Average Use Percentage |
|---|---|---|
| (8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs. | 10 | 5 |
| (9) Confections and frostings, including candy and flavored frostings, marshmallows, baking chocolate, and brown, lump, rock, maple, powdered, and raw sugars. | 40 | 10 |
| (10) Dairy product analogs, including nondairy milk, frozen or liquid creamers, coffee whiteners, toppings, and other nondairy products. | 10 | 5 |
| (11) Egg products, including liquid, frozen, or dried eggs, and egg dishes made therefrom, i.e., egg roll, egg for young, egg salad, and frozen multicourse egg meals, but not fresh eggs. | 15 | 5 |
| (12) Fats and oils, including margarine, dressings for salads, butter, salad oils, shortenings and cooking oils. | 10 | 5 |
| (13) Fish products, including all prepared main dishes, salads, appetizers, frozen multicourse meals, and spreads containing fish, shellfish, and other aquatic animals, but not fresh fish. | 10 | 5 |
| (14) Fresh eggs, including cooked eggs and egg dishes made only from fresh shell eggs. | 15 | 5 |
| (15) Fresh fish, including only fresh and frozen fish, shellfish, and other aquatic animals. | 10 | 5 |
| (16) Fresh fruits and fruit juices, including only raw fruits, citrus, melons, and berries, and home-prepared "ades" and punches made therefrom. | 30 | 5 – 10 |
| (17) Fresh meats, including only fresh or home-frozen beef or veal, pork, lamb or mutton and home-prepared fresh meat-containing dishes, salads, appetizers, or sandwich spreads made therefrom. | 10 | 5 |
| (18) Fresh poultry, including only fresh or home-frozen poultry and game birds and home-prepared fresh poultry-containing dishes, salads, appetizers, or sandwich spreads made there from. spreads made there from. | 10 | 5 |
| (20) Frozen dairy desserts and mixes, including ice cream, ice milks, sherbets, and other frozen dairy desserts and specialties. | 10 | 5 |

**Table 1-1 Food Categories, Estimated Maximum and Average Use Percentages of TG4 Syrup (continued)**

| FDA 21 CFR §170.3(n) Food Category | Estimated Maximum Use Percentage | Estimated Average Use Percentage |
|---|---|---|
| (21) Fruit and water ices, including all frozen fruit and water ices. | 10 | 5 |
| (22) Gelatins, puddings, and fillings, including flavored gelatin desserts, puddings, custards, parfaits, pie fillings, and gelatin base salads. | 10 | 5 |
| (23) Grain products and pastas, including macaroni and noodle products, rice dishes, and frozen multicourse meals, without meat or vegetables. | 10 | 5 |
| (24) Gravies and sauces, including all meat sauces and gravies, and tomato, milk, buttery, and specialty sauces. | 10 | 5 |
| (25) Hard candy and cough drops, including all hard type candies. | 40 | 10 – 20 |
| (26) Herbs, seeds, spices, seasonings, blends, extracts, and flavorings, including all natural and artificial spices, blends, and flavors. | 10 | 5 |
| (27) Jams and jellies, home-prepared, including only home-prepared jams, jellies, fruit butters, preserves, and sweet spreads. | 20 | 5 |
| (28) Jams and jellies, commercial, including only commercially processed jams, jellies, fruit butters, preserves, and sweet spreads. | 20 | 10 |
| (26) Herbs, seeds, spices, seasonings, blends, extracts, and flavorings, including all natural and artificial spices, blends, and flavors. | 10 | 5 |
| (27) Jams and jellies, home-prepared, including only home-prepared jams, jellies, fruit butters, preserves, and sweet spreads. | 20 | 5 |
| (28) Jams and jellies, commercial, including only commercially processed jams, jellies, fruit butters, preserves, and sweet spreads. | 20 | 10 |
| (29) Meat products, including all meats and meat containing dishes, salads, appetizers, frozen multicourse meat meals, and sandwich ingredients prepared by commercial processing or using commercially processed meats with home preparation. | 10 | 5 |

**Table 1-1 Food Categories, Estimated Maximum and Average Use Percentages of TG4 Syrup (continued)**

| FDA 21 CFR §170.3(n) Food Category | Estimated Maximum Use Percentage | Estimated Average Use Percentage |
|---|---|---|
| (30) Milk, whole and skim, including only whole, low fat, and skim fluid milks. | 10 | 5 |
| (31) Milk products, including flavored milks and milk drinks, dry milks, toppings, snack dips, spreads, weight control milk beverages, and other milk origin products. | 20 | 10 |
| (32) Nuts and nut products, including whole or shelled tree nuts, peanuts, coconut, and nut and peanut spreads. | 20 | 10 |
| (33) Plant protein products, including the National Academy of Sciences / National Research Council "reconstituted vegetable protein" category, and meat, poultry, and fish substitutes, analogs, and extender products made from plant proteins. | 10 | 5 |
| (34) Poultry products, including all poultry and poultry-containing dishes, salads, appetizers, frozen multicourse poultry meals, and sandwich ingredients prepared by commercial processing or using commercially processed poultry with home preparation. | 10 | 5 |
| (35) Processed fruits and fruit juices, including all commercially processed fruits, citrus, berries, and mixtures; salads, juices and juice punches, concentrates, dilutions, "ades", and drink substitutes made therefrom. | 30 | 5 – 10 |
| (36) Processed vegetables and vegetable juices, including all commercially processed vegetables, vegetable dishes, frozen multicourse vegetable meals, and vegetable juices and blends. | 10 | 5 |
| (37) Snack foods, including chips, pretzels, and other novelty snacks | 10 | 5 |
| (38) Soft candy, including candy bars, chocolates, fudge, mints, and other chewy or nougat candies. | 40 | 10 – 20 |
| (39) Soups, home-prepared, including meat, fish, poultry, vegetable, and combination home-prepared soups. | 10 | 5 |
| (40) Soups and soup mixes, including commercially prepared meat, fish, poultry, vegetable, and combination soups and soup mixes. | 10 | 5 |

**Table 1-1 Food Categories, Estimated Maximum and Average Use Percentages of TG4 Syrup (continued)**

| FDA 21 CFR §170.3(n) Food Category | Estimated Maximum Use Percentage | Estimated Average Use Percentage |
|---|---|---|
| (43) Sweet sauces, toppings, and syrups, including chocolate, berry, fruit, corn syrup, and maple sweet sauces and toppings. | 60 | 30 – 40 |

## 1.6 Statutory Basis for GRAS Status

The Notifier, Hayashibara Co., Ltd., has concluded, and is therefore notifying the Agency, that the intended use of Maltosyltrehalose Syrup (TG4 Syrup) as an ingredient in human food and beverage products is generally recognized as safe (GRAS) based on scientific procedures, in accordance with 21 CFR §170.30(a) and (b).

## 1.7 Exemption from premarket approval

The Notifier has concluded that the notified substance, Maltosyltrehalose Syrup (TG4 Syrup), is not subject to the premarketing approval requirements of the Federal Food, Drug, and Cosmetic Act based on the company's conclusion that the notified substance is GRAS under the conditions of its intended use.

## 1.8 Availability of information for FDA review

### 1.8.1 Availability and Copying

All information and data, both favorable and unfavorable, from which this GRAS Notice was derived is available to the FDA for review and copying during normal business hours at:

Vanguard Regulatory Services, Inc.
1311 Iris Circle
Broomfield, CO 80020
Tel: 303-464-8636

The data can also be supplied to the Agency either in electronic format or in paper copy.

1.8.2 Exemption from the Freedom of Information Act

The Notifier, Hayashibara Co., Ltd., requests that the commercial information regarding the amount of sales of TG4 Syrup in Japan provided in Parts 3.2 (p. 48), 3.5 (p. 54-56), 3.6 (p. 57), 4.0 (p. 61), and 6.6 (p. 93), be exempt from the Freedom of Information Act, 5 U.S.C. 552 as privileged. To make it easier to identify this information the specific numbers have been highlighted.

1.8.3 Trade Secrets to the United States Department of Agriculture Food Safety & Inspection Service

The Notifier authorizes the FDA to include any identified privileged information identified in this GRAS Notice to the USDA FSIS for their review.

## 1.9 Certification

The Notifier certifies that to the best our knowledge, this GRAS Notice is a complete, representative and balanced submission including both favorable and unfavorable information, if there is any, known to the Notifier, which is pertinent to the evaluation of the safety and GRAS status of the use of Maltosyltrehalose Syrup for general food consumption by humans.

[redacted]

December 29, 2020
Alan B. Richards, Ph.D.
Authorized Agent

# Part 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL AND TECHNICAL EFFECTS

## 2.1 Common or Usual Names and Identity, IUPAC Nomenclature, CAS Number and Name, and Synonyms, Other Common and Trade Names

The notified substance of this GRAS report is called Maltosyltrehalose Syrup (TG4 Syrup). It consists of two similar, but not identical, types of saccharides. The two main components are maltotetraose and maltosyltrehalose, as noted below. Maltotetraose is a simple tetraglucose (G4) molecule with the glucose molecules being bound by α-1,4 linkages (see Part 2.2.2). This linkage is the most common found in starch, starch syrups and maltodextrin, and are usually completely digested to individual glucose units in the small intestine. Maltotetraose comprises about 15% of the total composition of TG4 Syrup. TG4 Syrup also contains glucose (G1; 4.5%), maltose (G2; 7.1%) and maltotriose (G3; 9.8%), for a total of 36.3% with α-1,4 linkages, except G1.

The second and most common type of substance in TG4 Syrup is maltosyltrehalose (TG4), for which the product is named. Maltosyltrehalose constitutes approximately 52.5% of the TG4 Syrup. It is composed of four glucose units in which the terminal glycosidic bond at the reducing end of the glucose chain has been inverted. This results in the final bond between the 3$^{rd}$ and 4$^{th}$ glucose molecule being an α-1,1 linkage instead of the standard α-1,4 (see Part 2.2.2). The structure of the two end glucoses is the same as that of trehalose (Richards, et al., 2002; FDA, 2000). The change in linkage places the number 4 carbon on the terminal glucose exposed, which is much more stable and less susceptible to reaction with other substances in the final product. The chemical structure results in functional and technical properties which are less or more exaggerated than those of a conventional α-1,4 linked oligosaccharide found in starch (glucose) syrups. In addition to TG4, TG4 Syrup also contains glucosyltrehalose (TG3) molecules at about 3.5%, making a total of about 56% with terminal α-1,1 bonds. Maltosytrehalose and maltotetrose have the same empirical formula and molecular weight. As with maltotetraose, maltosytrehalose is also completely digested in the small intestine, as have been shown for the trehalose molecule (see Part 6.2.1; Richards, et al., 2002).

Finally there is approximately 7.7% of other saccharides that are in TG4 Syrup. These include larger molecules, like those that are produced by most all other starch syrup and maltodextrin production processes (White, 2014). Together the three types of saccharides constitute 99.9% of the dry weight of the TG4 Syrup, and only include glucose molecules.

### 2.1.1 Common or Usual Names and Identity

Maltosyltrehalose Syrup

### 2.1.2 IUPAC Nomenclature of the Main Constituents

Maltosyltrehalose: (2R,3R,4S,5S,6R)-2-{[(2R,3S,4R,5R,6R)-6-{[(2R,3S,4R,5R,6R)-4,5-dihydroxy-2-(hydroxymethyl)-6-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]oxy}tetrahydro-2H-pyran-3-yl]oxy}-4,5-dihydroxy-2-(hydroxymethyl)tetrahydro-2H-pyran-3-yl]oxy}-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol

Maltotetraose: (2R,3R,4S,5S,6R)-2-{[(2R,3S,4R,5R,6R)-6-{[(2R,3S,4R,5R,6R)-4,5-dihydroxy-2-(hydroxymethyl)-6-{[(2R,3S,4R,5R)-4,5,6-trihydroxy-2-(hydroxymethyl)oxan-3-yl]oxy}oxan-3-yl]oxy}-4,5-dihydroxy-2-(hydroxymethyl)oxan-3-yl]oxy}-6-(hydroxymethyl)oxane-3,4,5-triol

### 2.1.3 CAS Registry Number of the Main Constituents

Maltosyltrehalose: 25545-20-4

Maltotetraose: 34612-38-9

### 2.1.4 CAS Name of the Main Constituents

Maltosyltrehalose: α-*O*-Glucopyranoside, α-D-glucopyranosyl *O*-α-D-glucopyranosyl-(1->4)-*O*-α-D-glucopyranosyl-(1->4)- (9CI)

Maltotetraose: D-Glucopyranose, *O*-α-D-glucopyranosyl-(1->4)-*O*-α-D-glucopyranosyl-(1->4)-*O*-α-D-glucopyranosyl-(1->4)- (9CI)

### 2.1.5 Synonyms, Other Common Names, and Trade Names

- HALLODEX™ (Trade name)
- TG4 Syrup
- Reaction mass of maltosyltrehalose and maltotetraose

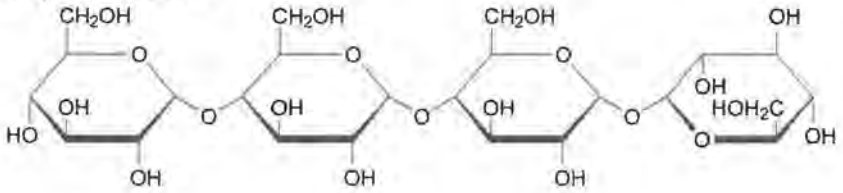
## 2.2 Chemical Formula, Structure and Molecular Weight

### 2.2.1 Empirical Formula of the Main Constituents

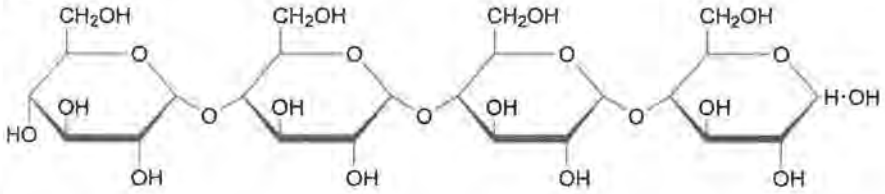
Maltosyltrehalose: $C_{24}H_{42}O_{21}$
Maltotetraose: $C_{24}H_{42}O_{21}$

### 2.2.2 Structural Formula of the Main Constituents

Maltosyltrehalose:



Maltotetraose:



### 2.2.3 Molecular Weight of the Main Constituents

Maltosyltrehalose: 666.58
Maltotetraose: 666.58

## 2.3 Raw Materials and Processing Aids

For the manufacturing of Maltosyltrehalose Syrup (TG4 Syrup), the raw materials used meet applicable US regulations or are GRAS for their intended use as provided in the raw materials list below. These substances are also suitable for food production (food or food additive grade) and used in accordance with Food Sanitation Act (Act No. 233, 1947) in Japan. The enzymes meet the requirements for the FCC monograph for "Enzyme Preparations" (FCC, 2019).

| Raw Materials | Regulator status | References |
|---|---|---|
| Starch | Food grade<br>Substances Added to Food (formerly EAFUS) | FDA, 2018 |
| Activated carbon | LSRO SCOGS-II-6, 1981<br>Substances Added to Food<br>21CFR §170.3(o), Processing aid | LSRO, 1981<br>FDA, 2018<br>FDA, 2019(h) |
| Calcium carbonate | 21CFR §184.1191 | FDA, 2019(q) |
| Diatomaceous earth | LSRO SCOGS-61, 1979<br>Substances Added to Food<br>21CFR §170.3(o), Processing aid | LSRO, 1979<br>FDA, 2018<br>FDA, 2019(h) |
| Hydrochloric acid | 21CFR §182.1057 | FDA, 2019(j) |
| Ion exchange resin | 21CFR §173.25 | FDA, 2019(i) |
| Perlite | LSRO SCOGS-61, 1979 | LSRO, 1979 |
| Sodium bicarbonate | 21CFR §184.1742 | FDA, 2019(u) |
| Sodium hydroxide | 21CFR §184.1763 | FDA, 2019(v) |
| Sodium metabisulfite | 21CFR §182.3766 | FDA, 2019(k) |

The enzymes used and the organisms from which they are produced have all be used in products that are sold into the US and/or other countries. The following is a list of the regulatory basis for their safe use.

**α-Amylases (EC 3.2.1.1)**, including thermostable α-amylase, are generally used for starch processing to produce conventional types of starch hydrosylate products such as glucose syrup, corn syrup, HFCS, oligosaccharide, maltodextrin, and similar products. α-Amylase derived from microorganisms is listed in Substances Added to Food (FDA, 2018) and Title 21 of the U.S. Code of Federal Regulations (21CFR), and the following amylases are authorized in 21CFR;

i. §137.105 "Flour may contain α-amylase obtained from the fungus *Aspergillus oryzae*" (FDA, 2019(a)).
ii. §184.1012 "Alpha-amylase enzyme preparation from *Bacillus stearothermophilus* used to hydrolyze edible starch to produce maltodextrin and nutritive carbohydrate sweeteners" (FDA, 2019(n)).
iii. §184.1443 "Malt α-amylase and β-amylase from barley to hydrolyze starch" (FDA, 2019(s)).
iv. §172.892 "Alpha-amylase (E.C. 3.2.1.1)" for Food Starch - Modified" (FDA, 2019(c)).
v. §137.105 and §137.200 "Amylase from *Aspergillus oryzae*" (FDA, 2019(a),(b).

vi. §173.120 "Carbohydrase and cellulase derived from *Aspergillus niger*" (FDA, 2019(d)).
vii. §173.130 "Carbohydrase derived from *Rhizopus oryzae*" (FDA, 2019(e))
viii. §184.1027 "Mixed carbohydrase and protease enzyme product" (FDA, 2019(o)).
ix. §184.1148 "Bacterially-derived carbohydrase enzyme preparation" (FDA, 2019(p)).

The α-amylase from *Bacillus licheniformis* that is currently used for the production of TG4 is also incorporated and has an "ADI not specified" (WHO, 2004). In Japan, α-amylase is listed in List of Existing Food Additives (JMOH, 1996) and is monographed in Japan's Specifications and Standards for Food Additives, Ninth Edition (JSSFA) (JMOH, 1996; JMHLW, 2018). In Australia/New Zealand, α-amylases from *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus licheniformis* containing the gene for α-amylase isolated from *Geobacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus subtilis* containing the gene for α-amylase isolated from *Geobacillus stearothermophilus*, and *Geobacillus stearothermophilus* are listed as permitted enzymes of microbial origin in FSANZ STANDARD 1.3.3 "Processing aids" (FSANZ, 2016).

*Bacillus licheniformis* and *Bacillus amyloliquefaciens* are categorized as BioSafety Level "1" by the American Type Culture Collection (ATCC), and are not listed in the FDA Bad Bugs Book.

**Glucan 1,4-α-maltotetraohydrolase (EC 3.2.1.60)** is also known as maltotetraohydrolase, exomaltotetrahydrolase, or G4 producing enzyme, and is categorized as a glycosidase. In the USA and Australia/New Zealand, "maltotetraohydrolase enzyme preparation from *Bacillus licheniformis* expressing a modified maltotetraohydrolase gene from *Pseudomonas stutzeri*" is evaluated as GRAS and is listed as a permitted enzyme of microbial origin in FSANZ STANDARD 1.3.3 "Processing aids", respectively, which means that maltotetraohydrolase protein from *P. stutzeri* is safe (FDA, 2009). In Japan, maltotetraohydrolase is listed in the List of Existing Food Additives (Ministry of Health and Welfare Notification No. 120, 1996) and is monographed in JSSFA (JMOH, 1996; JMHLW, 2018). In Korea, exomaltotetrahydrolase is monographed in the Food Additives Code.

*P. stutzeri* is categorized in BioSafety Level "1" by ATCC, and is not listed in the FDA Bad Bugs Book.

**(1->4)-α-D-Glucan 1-α-D-glucosylmutase (EC 5.4.99.15)** from *Arthrobacter ramosus* is used also for the production of trehalose. Trehalose has GRAS status in the USA, has Novel Food status in the EU, Australia/New Zealand and Canada and is

approved/registered/listed in various countries (see Table 6-2; FDA, 2000; EU, 2001; FASANZ, 2003; Canada, 2005). No regulatory authorities or experts expressed any safety concerns regarding this, or any other enzymes used for the production of trehalose, during the many safety evaluations of trehalose. In Japan, (1->4)-α-D-glucan 1-α-D-glucosylmutase is listed as α-glucosyltransferase in the List of Existing Food Additives (Ministry of Health and Welfare Notification No. 120, 1996) and is monographed in JSSFA (JMOH, 1996; JMHLW, 2018).

*Arthrobacter* spp. is generally considered to be non-pathogenic and belongs consequently to risk group 1 of the German "Gentechnik-Sicherheitsverordnung" (GenTSV), i.e., Regulation on the Safety of Gentechnology. Further *Arthrobacter ramosus* is categorized as BioSafety Level "1" by ATCC and is not listed in the FDA Bad Bugs Book.

**Isoamylase (EC 3.2.1.68)** from *Pseudomonas amyloderamosa*, was tested using the Ames test, acute and subchronic (90-day) oral toxicity studies in animals. The results of Ames test demonstrated that the isoamylase preparation was not mutagenic at concentrations of up to 5,000 μg/plate (FDA, 2001). From the results of acute toxicity of isoamylase, the $LD_{50}$ was determined to be about 17 g/kg-bw (FDA, 2001). In a 90-day toxicity study of the isoamylase commercial preparation, the no-observed-adverse-effect level (NOAEL) was concluded to be the highest dose tested (10 mL (228 mg)/kg-bw) (FDA, 2001). In addition, the acute toxicity was tested on *P. amyloderamosa* (wet cells) and culture filtrate in mice. No mortalities or clinical signs were observed after administration of the wet cells and the culture (FDA, 2001).

Isoamylase from *P. amyloderamosa* has been already evaluated to be generally recognized as safe (GRAS), which issued a "no question letter" (FDA, 2001). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) allocated an acceptable daily intake (ADI) "not specified" for this enzyme (WHO, 2017). In Japan, isoamylase is listed in the List of Existing Food Additives (Ministry of Health and Welfare Notification No. 120, 1996) and is monographed in JSSFA (JMOH, 1996; JMHLW, 2018).

*P. amyloderamosa* is deposited at ATCC, which is categorized as BioSafety Level "1", and is not listed in the FDA Bad Bugs Book.

## 2.4 Product Specifications, Methods of Analysis, and Nutritional Analysis

### 2.4.1 Specifications and Analytical Methods

The Notifier, Hayashibara Co., Ltd., developed the specifications for TG4 Syrup which are shown in Table 2-1 below.

All analytical methods, except Composition, are from officially recognized sources in which the methods have been validated to test for the specification analyte. The Composition specification for glucose and Maltosyltrehalose in TG4 was developed by the Notifier, and has been validated for this purpose. In addition to the product specifications there are other analyses that have been performed on the substance. These include viable yeasts count, viable molds count, total aflatoxins, protein, arsenic and lead, and the results of the analyses are provided in Part 2.4.

**Table 2-1 Final Product Specifications for TG4 Syrup**

| Variables | Specifications | Analytical Methods |
|---|---|---|
| Dry solid | Not less than 72.0% | Refractive index method (Industrial Analytical Methods for Starch-derived Saccharides) |
| Total ash | Not more than 0.05% | Electric conductivity method (Industrial Analytical Methods for Starch-derived Saccharides) |
| pH | 3.5 – 6.5 | pH determination, 30% (Japanese Industrial Standards) |
| Color in solution | Not more than 0.100 | Absorption spectrophotometer, 30% (Japanese Agricultural Standards) |
| Turbidity | Not more than 0.050 | Absorption spectrophotometer, 30% (Japanese Agricultural Standards) |
| Sugar composition (on the dry basis) | | HPLC method (Industrial Analytical Methods for Starch-derived Saccharides) |
| Glucose | Not more than 6.0% | |
| Maltosyltrehalose | Not less than 50.0% | |
| Total aerobic microbial count | Not more than 300 CFU/g | Pour plate method, Standard agar (Standard Methods of Analysis for Hygiene Chemists) |
| Coliform organisms | Negative/g | BGLB method (Standard Methods of Analysis for Hygiene Chemists) |

### 2.4.2 Analysis of Multiple Lots

To demonstrate that the Notifier consistently manufactures TG4 Syrup that meets the specifications proposed above, 5 lots of the commercial products (HALLODEX™) were analyzed, with the exception of only three lots being analyzed for lead and arsenic. The results are shown in Table 2-2.

**Table 2-2 Five (5) Lot Analyses of TG4 Syrup**

| Variables | Specifications | Lot No. | | |
|---|---|---|---|---|
| | | **6A27471** | **6A27491** | **6B03461** |
| Dry solid | ≥ 72.0% | 72.9 | 73.1 | 72.8 |
| Total ash | ≤ 0.05% | 0.00 | 0.00 | 0.00 |
| pH | 3.5 – 6.5 | 5.0 | 5.3 | 5.1 |
| Color in solution | ≤ 0.100 | 0.008 | 0.007 | 0.007 |
| Turbidity | ≤ 0.050 | 0.000 | 0.000 | 0.000 |
| Sugar composition (on the dry basis) | | | | |
| Glucose | ≤ 6.0% | 4.6 | 4.5 | 4.2 |
| Maltosyltrehalose | ≥ 50.0% | 53.1 | 53.0 | 51.0 |
| Total aerobic microbial count | ≤ 300 CFU/g | 0 | 0 | 0 |
| Coliform organisms | Negative/g | Negative | Negative | Negative |

| Variables | Specifications | Lot No. | |
|---|---|---|---|
| | | **6B03491** | **6B09501** |
| Dry solid | ≥ 72.0% | 73.1 | 72.8 |
| Total ash | ≤ 0.05% | 0.00 | 0.00 |
| pH | 3.5 – 6.5 | 5.3 | 5.1 |
| Color in solution | ≤ 0.100 | 0.007 | 0.005 |
| Turbidity | ≤ 0.050 | 0.000 | 0.000 |
| Sugar composition (on the dry basis) | | | |
| Glucose | ≤ 6.0% | 4.5 | 4.1 |
| Maltosyltrehalose | ≥ 50.0% | 53.0 | 54.2 |
| Total aerobic microbial count | ≤ 300 CFU/g | 0 | 0 |
| Coliform organisms | Negative/g | Negative | Negative |

All the commercial lots tested meet the proposed specifications described in Part 2.4.1.

### 2.4.3 Microbial and Toxicant assays

The Notifier has examined TG4 Syrup for a number of substances in addition to those listed in the specifications and the results are shown in Table 2-3. These include viable yeasts count, viable molds count, total aflatoxins (aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$), protein, arsenic (as $As_2O_3$) and lead. Viable yeast and mold counts were both negative/0.1g and aflatoxins were not detected. Protein, arsenic and lead were not detected at the limits of detection.

**Table 2-3 Microbial and Toxicant Assays**

| Lot No. | **Viable yeasts count** | **Viable molds count** | **Total aflatoxins** |
|---|---|---|---|
| 4C05471 | Negative/0.1g | Negative/0.1g | Not detected |
| 4J28491 | Negative/0.1g | Negative/0.1g | Not detected |
| 5J28501 | Negative/0.1g | Negative/0.1g | Not detected |
| Lot No. | **Protein (μg/mL)** | **Arsenic (as $As_2O_3$) (ppm)** | **Lead (ppm)** |
| 7K04512 | < 20 | < 2 | < 0.1 |
| 7L05512 | < 20 | < 2 | < 0.1 |
| 8A13452 | < 20 | < 2 | < 0.1 |

### 2.4.4 Nutritional Analysis of TG4 Syrup

Table 2-4 shows the results of nutritional analysis on triplicate samples from 4 lots of TG4 Syrup. The mean values for the total carbohydrates and energy were 73.2 g/100-g and 293 kcal/100-g, respectively. The water content was from 26.5 to 27.1 g/100 g, and protein, lipid, ash, dietary fiber, sodium and $SO_2$ were undetectable in TG4 Syrup.

The determined energy of TG4 Syrup, 293 kcal/100-g, corresponds to 4.00 kcal/g on a dry basis. Essentially all starch syrups including glucose, HFCS, maltodextrin/dextrin syrups provide approximately 4 kcal/g (dwb) of food energy as do other digestible sugars (sucrose, maltose, glucose, lactose, trehalose), maltooligosaccharides and starches. Therefore, the energy of TG4 Syrup is the same as that of conventional sugars and starch-based syrups. TG4 Syrup as a substitute of starch syrup would not have a significant impact on overall dietary nutrient intakes for consumers.

**Table 2-4 Nutritional Components of TG4 Syrup (4 Lots)**

| Lot No. | 4C05471 | 4J28491 | 5J19959 | 5J28501 | Average |
|---|---|---|---|---|---|
| Water (g/100-g) | 26.7 | 26.5 | 27.1 | 26.8 | 26.8 ± 0.25 |
| Protein (g/100-g) | < 0.1 | < 0.1 | < 0.1 | < 0.1 | < 0.1 |
| Lipid (g/100-g) | < 0.1 | < 0.1 | < 0.1 | < 0.1 | < 0.1 |
| Ash (g/100-g) | < 0.1 | < 0.1 | < 0.1 | < 0.1 | < 0.1 |
| Carbohydrate (g/100-g) | 73.3 | 73.5 | 72.9 | 73.2 | 73.2 ± 0.25 |
| Dietary fiber (g/100-g) | < 0.5 | < 0.5 | < 0.5 | < 0.5 | < 0.5 |
| Energy (kcal/100-g) | 293 | 294 | 292 | 293 | 293 ± 0.5 |
| Sodium (mg/100 g)[1] | N.D. | N.D. | N.D. | N.D. | N.D. |
| $SO_2$ (g/kg)[2] | N.D. | N.D. | N.D. | N.D. | N.D. |

[1] Detection limit: 1 mg/100 g
[2] Detection limit: 3 mg/kg

### 2.4.5 Dextrose equivalent (DE)

The dextrose equivalent (DE) of TG4 Syrup on a dry basis was determined on 4 lots. The results are shown in Table 2-5.

**Table 2-5 Dextrose Equivalent (DE) of TG4 Syrup**

| | Lot No. | | | | Average ± S.D. |
|---|---|---|---|---|---|
| | 4C05471 | 4J28491 | 5J19959 | 5J28501 | |
| DE (g/100-g on a product basis) | 13.7 | 14.2 | 14.3 | 14.2 | 14.1 ± 0.3 |
| Solid content (%) | 73.3 | 73.5 | 72.9 | 73.2 | 73.2 ± 0.2 |
| DE (g/100-g on a dry basis) | 18.7 | 19.3 | 19.6 | 19.4 | 19.3 ± 0.4 |

**Summary**

It was demonstrated that the DE of TG4 Syrup is approximately 19 on a dry weight basis and approximately 14 on a product basis. For comparison, the DE of glucose syrup is specified to be not less than 20.0% calculated on a dry basis as expressed as D-glucose (FDA, 2019(f)). Further the table shows that the production process for this variable is fairly consistent. The reason that the DE (product basis) is lower are the fewer number of reducing ends where the trehalose moieties are found.

## 2.5 Manufacturing Process

### 2.5.1 Introduction

TG4 Syrup is manufactured from the hydrolysis and transglucosylation of starch by enzymes. To manufacture TG4 Syrup, starch is gelatinized by heat to prepare a starch slurry, liquefied by thermostable α-amylase, and then saccharified using isoamylase, glucan 1,4-α-maltotetraohydrolase (G4ase), (1->4)-α-D-glucan 1-α-D-glucosylmutase (MTSase) and α-amylase. The resultant mixture is purified and concentrated to make TG4 Syrup.

The manufacturing process of TG4 Syrup is similar to that of conventional starch syrup such as glucose syrup, corn syrup, starch hydrolysate, etc., except for the additional use of the enzyme, MTSase, for transglucosylation (Maruta, et al., 1995). Conventional starch syrup is generally recognized to be manufactured from the hydrolysis of starch by acid, and/or enzymes. What is not well-known is that during the production of 'conventional starch syrups' by enzymes, hydrolyzing enzymes cause transglycosylation (Nakajima, et al., 2004; Song, et al., 2013). In other words, hydrolysis of starch in the production of conventional starch syrup, which constitutes hundreds of millions of tons consumed per year, also involves transglycosylation. Therefore, there is no functional difference, except in the relative amount of tranglycosylation between the production methods of TG4 Syrup and conventional starch syrup.

TG4 Syrup is composed of glycosyltrehaloses, maltose, oligosaccharides and a small amount of glucose (≤ 6%). The main constituents (≥ 10% on a dry basis) are maltosyltrehalose (TG4; ≥ 50%) and maltotetraose (G4; approximately 15%). The dextrose equivalent (DE) of TG4 Syrup is about 20 on a dry basis. The reason that the DE is relatively lower than a similar convential starch syrup is because the main component, maltosyltrehalose (≥ 50%) has no reducing end. This results in the unique functional features of TG4.

Therefore, TG4 Syrup is essentially a type of starch syrup with a low DE.

### 2.5.2 TG4 Production Process

Maltotetraose (G4) rich syrup has been used in a similar manner as a conventional starch syrup in various food applications in Japan for several years. It is manufactured from starch using α-amylase, isoamylase and glucan 1,4-α-maltotetraohydrolase (G4ase). G4 rich syrup meets the definition and specifications of a glucose sirup, which is allowed for use in the US (FDA, 2019(f)).

Maruta *et al.* reported that a trehalose-producing microorganism isolated from soil, *Arthrobacter* sp. strain Q36, produces 2 enzymes that are commmercially used to produce trehalose (FDA, 2000; Maruta, et al., 1995). One of these is (1->4)-α-D-glucan 1-α-D-glucosylmutase (MTSase), which converts the terminal (reducing) maltosyl unit of oligosaccharides (i.e. the last two glucose molecules on the reducing end; minimum degree of polymerization (DP) > 3) to a trehalose unit. The generic name of this molecule is glycosyltrehalose. In trehalose production the terminal trehalose molecule is released from the remaining oligosaccharide by a second enzyme, 4-α-D-{(1->4)-α-D-glucano} trehalose trehalohydrolase (Maruta, et al., 1995). However, in TG4 production this second enzyme is not used, leaving the terminal trehalose attached to the glycosyltrehalose molecule.

Scientists at Hayashibara Biochemical Laboratories, Inc. (now Hayashibara Co., Ltd., the Notifier) developed a commercial production method of TG4 Syrup using MTSase based on the manufacturing process that first produces a G4-rich syrup.

The current process to produce TG4 Syrup is described in detail below. The Notifier's technology includes the use of two characteristic enzymes; G4ase producing G4 from the non-reducing end of polyglucoses by hydrolyzation. This substance, as noted previously, is allowed a glucose syrup (FDA, 2019(f). The second enzyme is MTSase producing a trehalose unit at the reducing end of G4 by intramolecular transglucosylation, as shown in Figure 2-1.

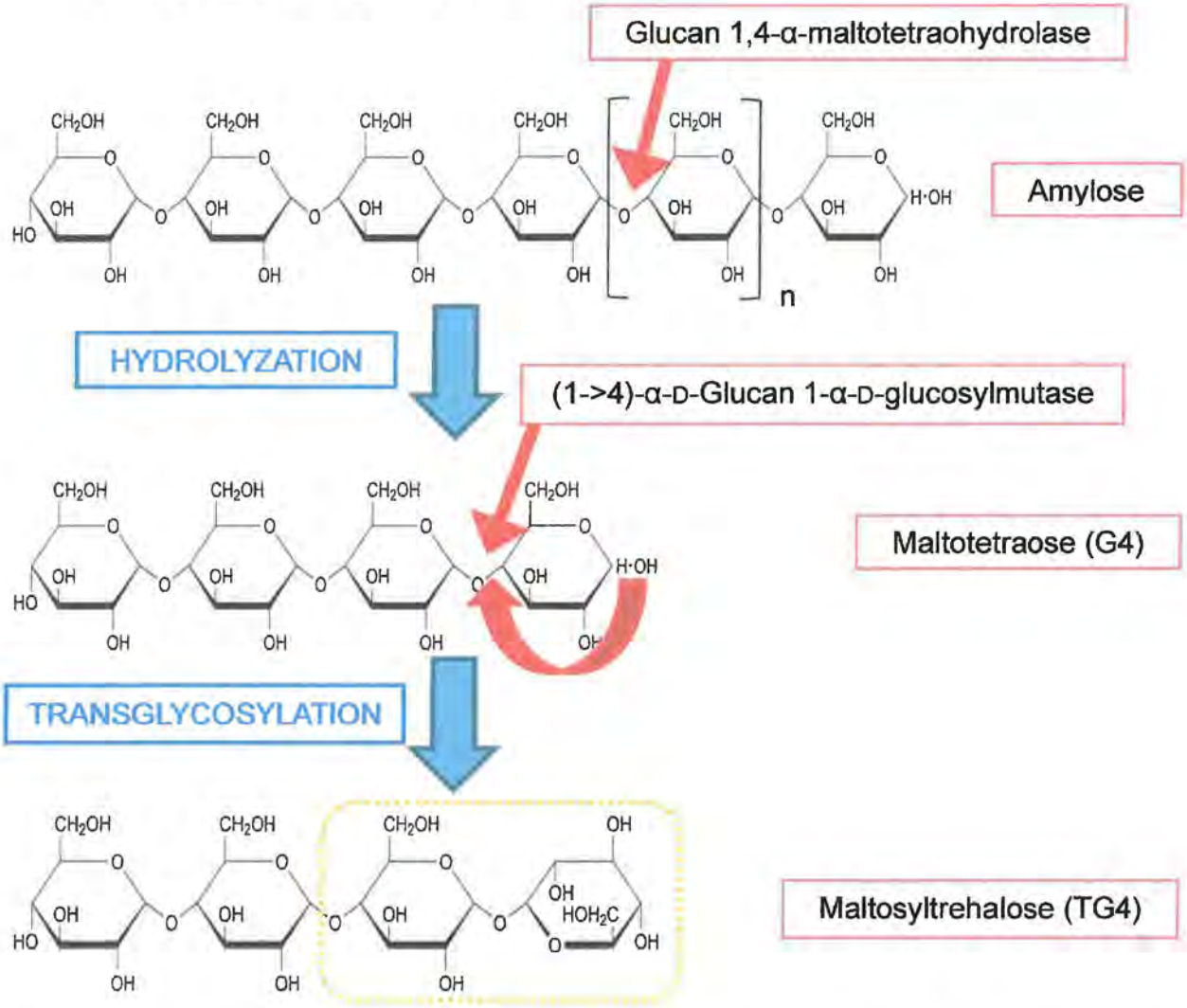



**Figure 2-1 Reactions of Glucan 1,4-α-Maltotetraohydrolase and (1->4)-α-D-Glucan 1-α-D-Glucosylmutase**

The following section describes the production process and the enzymes involved. It should be noted that all materials are suitable to food production (Part 2.3) and that there are a variety of process control points (Part 2.5.3) used to assure the wholesomeness of the finished product. The schematic diagram (Figure 2-2) shows the steps in the production process of TG4 Syrup.

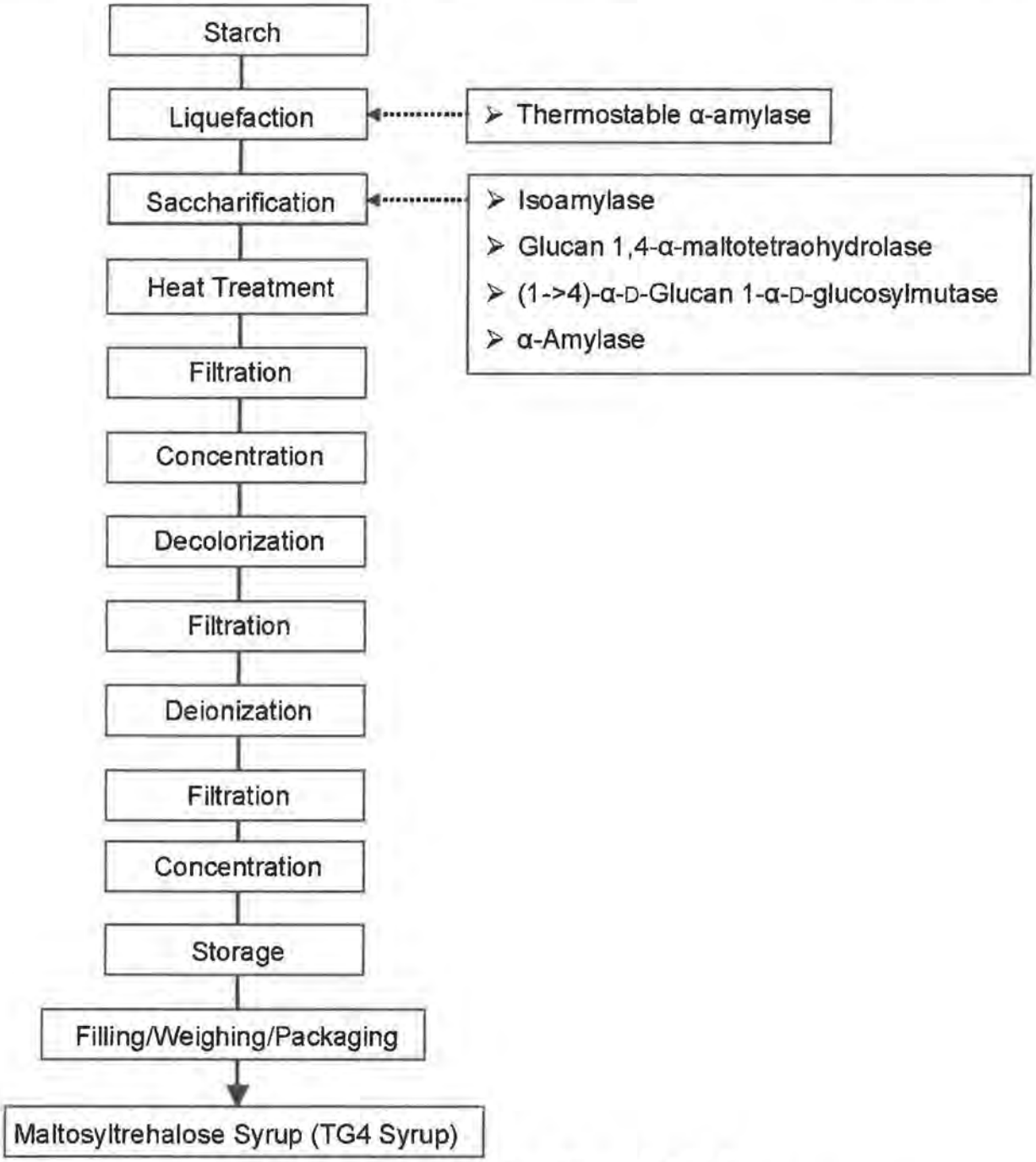



**Figure 2-2 Production Flow of TG4 Syrup**

The details of each step in the production process of TG4 Syrup are as follows:

Procedures

i. Starch preparation: The first step in the production process is suspension of starch to produce a slurry of a known concentration. The pH is adjusted. The starch slurry is heated for gelatinization.

ii. Liquefaction: The gelatinized starch is liquefied by the addition of thermostable α-amylase. This enzyme cleaves the amylose and amylopectin chains into shorter units to increase the saccharification efficiency. The liquefying enzyme is inactivated by increasing the temperature of the liquefied solution, and then the solution is cooled in preparation for the next production process.
iii. Saccharification: Isoamylase, Glucan 1,4-α-maltotetraohydrolase (G4ase), and (1->4)-α-D-Glucan 1-α-D-glucosylmutase (MTSase) are added under controlled conditions of temperature, pH and concentration. These enzymes participate in saccharification of the liquefied starch into TG4. Isoamylase hydrolyzes the 1->6 glycosidic bonds of amylopectin, which debrances it to amylose. G4ase hydrolyzes amylose to produce G4 units, and MTSase forms a trehalose unit at the reducing end of G4 by intramolecular transglucosylation. α-Amylase is then added under controlled conditions to cleave the remaining polysaccharides.
iv. Heat Treatment: The saccharifying enzymes are inactivated by heating, and then the solution is cooled for further processing.
v. Filtration: Insoluble, colored substances and proteins including degraded enzymes are removed from the saccharified solution by filtration using activated carbon and diatomaceous earth.
vi. Concentration: The filtrate is concentrated by evaporation.
vii. Decolorization: Activated carbon is added to the concentrated solution so colored substances are absorbed to activated carbon.
viii. Filtration: Activated carbon with adsorbed substances is removed with diatomaceous earth.
ix. Deionization: The filtrate is deionized using twin-bed ion exchange, followed by a mixed-bed procedure.
x. Filtration: The deionized solution is filtrated using activated carbon and diatomaceous earth and subsequently filtrated using a non-woven filter.
xi. Concentration: The filtrated solution is concentrated by evaporation.
xii. Storage: The intermediate product is stored until filling/weighing/packaging
xiii. Filling/Weighing/Packaging: After the syrup is filtered to remove insoluble matter before filling/weighing/packaging, 24 kg of TG4 Syrup is weighed and filled in tin-free steel cans. This is the standard packaging for this product; however, the packaging size might be changed depending on customer needs, with a subsequent change in the fill weight. Before any change is made the new containers will be tested to be sure they do not interact with the TG4 Syrup. For domestic transportation to Japanese food industry customers, TG4 Syrup might be filled into a tank truck or a bulk container.
xiv. Shipping: TG4 Syrup in the package is weight-checked and shipped.

### 2.5.3 Control Points in the Manufacturing Process

The Notifier maintains strict quality control by determining specified control points within the manufacturing process of TG4 Syrup. The critical control points and process control points are listed in Table 2-6.

**Table 2-6 Critical Control Points and Process Control Points in the Manufacturing Process of TG4 Syrup**

| Processing Steps | Control Points |
|---|---|
| Heat treatment | • Iodine reaction<br>• **Glucose concentration**<br>• **TG4 concentration** |
| Filtration after decolorization | • **Color**<br>• **Turbidity** |
| Concentration after deionization | • *Temperature of the deionized solution*<br>• *Holding time of the deionized solution*<br>• Solid content |
| Weighing / Filling / Packaging | • *Foreign matter in the sampled syrup* |

**Bold** and *Italic* print means that the variables are the Critical Control Points (CCP) and the Operational Prerequisite Programs (OPRP), respectively.

In addition, several monitored values are set in some of the manufacturing steps, such as pH, temperature, concentration, etc. to assure the successful progress of each step.

To manufacture TG4 Syrup with the constant high quality and safety, the CCP, CP and monitoring points are periodically reviewed in pursuant to the requirements of Food Safety System Certification (FSSC) 22000 and Hazard Analysis and Critical Control Point (HACCP) and might be changed according to the results of the review.

### 2.5.4 Tracking Program

The manufacturing process at is organized into units consisting of a series of liquefaction and saccharification steps. Each finished product is assigned its own lot number. The reference numbers and quantities of all materials and processing aids used in each step are recorded for each lot number and stored. Thus, it is possible for the Notifier to track not only the finished product, but also each raw material used in the processing of that specific finished product. All raw materials and finished products can be traced to a particular production lot.

## 2.6 Identity of Saccharides in TG4 Syrup

### 2.6.1 Quantitative Saccharide Composition

Due to the specificity and efficacy of the enzymes [Glucan 1,4-α-maltotetraohydrolase (G4ase) and (1->4)-α-D-Glucan 1-α-D-glucosylmutase (MTSase)] used for the production of TG4 Syrup, the main constituents of TG4 Syrup are TG4 and maltotetraose (G4). Kato, *et al.* reported that by HPLC analysis, α-anomers and β-anomers could be determined from each maltooligosaccharide (Kato, et al., 2003). Based on experience with various saccharides measurements, it was also observed by cation-exchange HPLC analysis, TG4 and maltopentaose (G5) are detected at almost the same retention time as G4 and glucosyltrehalose (TG3), respectively. In other words, only the sum of TG4 and G5, and the sum of G4 and TG3 could be determined. Therefore to arrive at the concentrations of the various saccharide substances in TG4 Syrup, a series of analytical methods needed to be employed. The following section provides the methods, results and the final conclusion of the results.

**Analytical Method 1**

Reverse-phase HPLC analysis was performed using the standards of glucose (G1), fructose (F), maltose (G2), maltotriose (G3), G4, G5, TG3, TG4, maltotriosyltrehalose (TG5), maltotetraosyltrehalose (TG6) and maltopentaosyltrehalose (TG7). It is expected that α- and β-anomers are separately determined on each oligosaccharide by the reverse-phase HPLC analysis. To determine the retention time of each of the standards, reverse-phase HPLC was performed on the standard solutions. The retention times of each standard by reverse-phase HPLC analysis are shown in TG3, TG4, TG5, TG6 and TG7 are shown in Table 2-7.

**Table 2-7 Retention Times of Saccharide Standards by Reverse-phase HPLC**

| Standard | G1 | F | G2 | G3 | | TG3 | G4 | |
|---|---|---|---|---|---|---|---|---|
| Retention Time (minutes) | | | | β | α | | β | α |
| | 6.339 | 6.486 | 7.053 | 7.986 | ***8.250*** | ***8.321*** | 9.211 | ***9.891*** |
| Standard | TG4 | G5 | | TG5 | TG6 | TG7 | | |
| Retention Time (minutes) | | β | α | | | | | |
| | ***10.126*** | 11.155 | 11.921 | 12.098 | 14.016 | 16.065 | | |

The results indicate that α-anomers of G3 and TG3 are observed at almost the same retention time by reverse-phase HPLC analysis and that α-anomers of G4 and TG4 are also essentially superimposed. The chromatograms are shown in Figure 2-3.

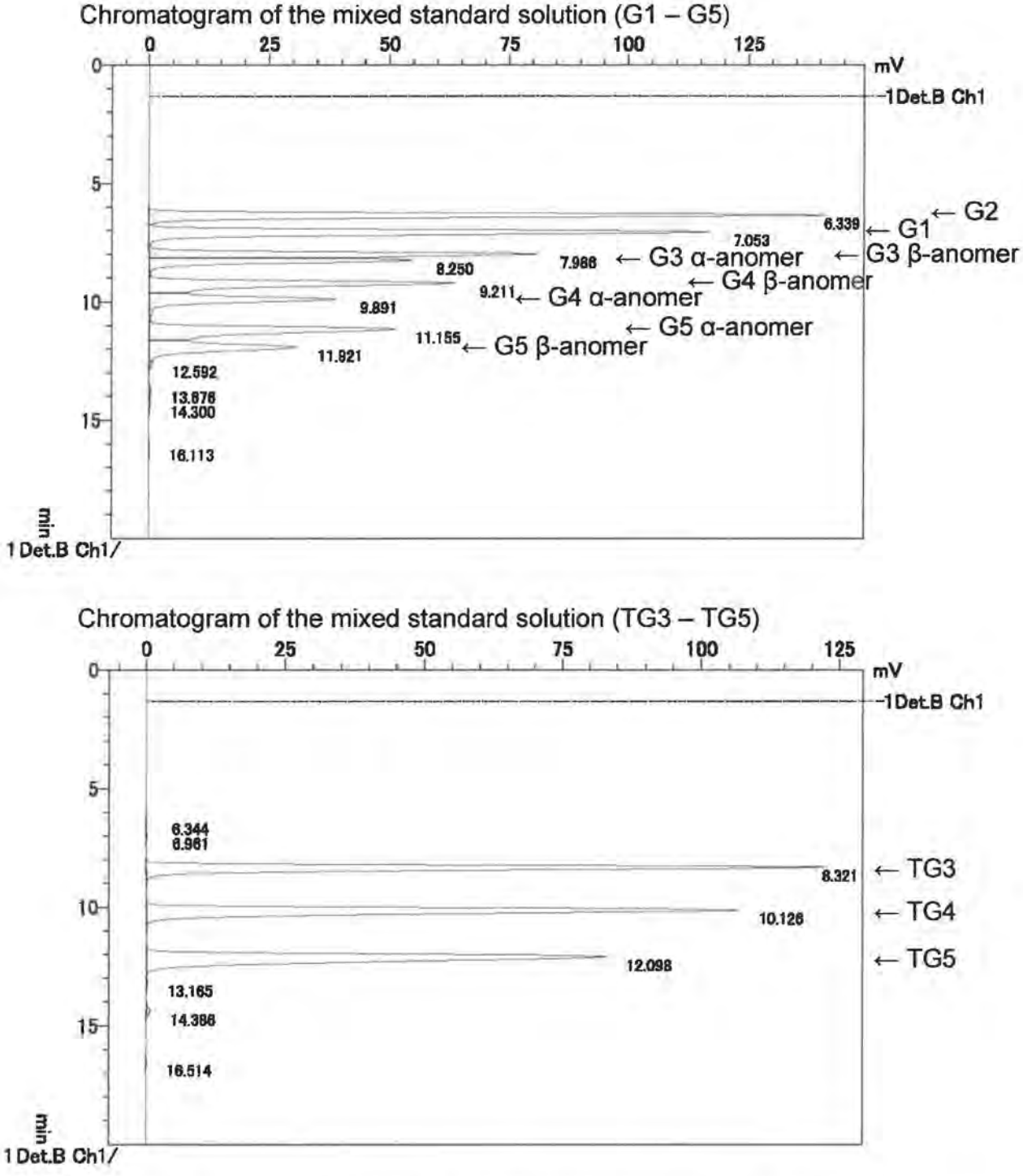



**Figure 2-3 Reverse-phase HPLC Chromatograms of the Standard Solutions**

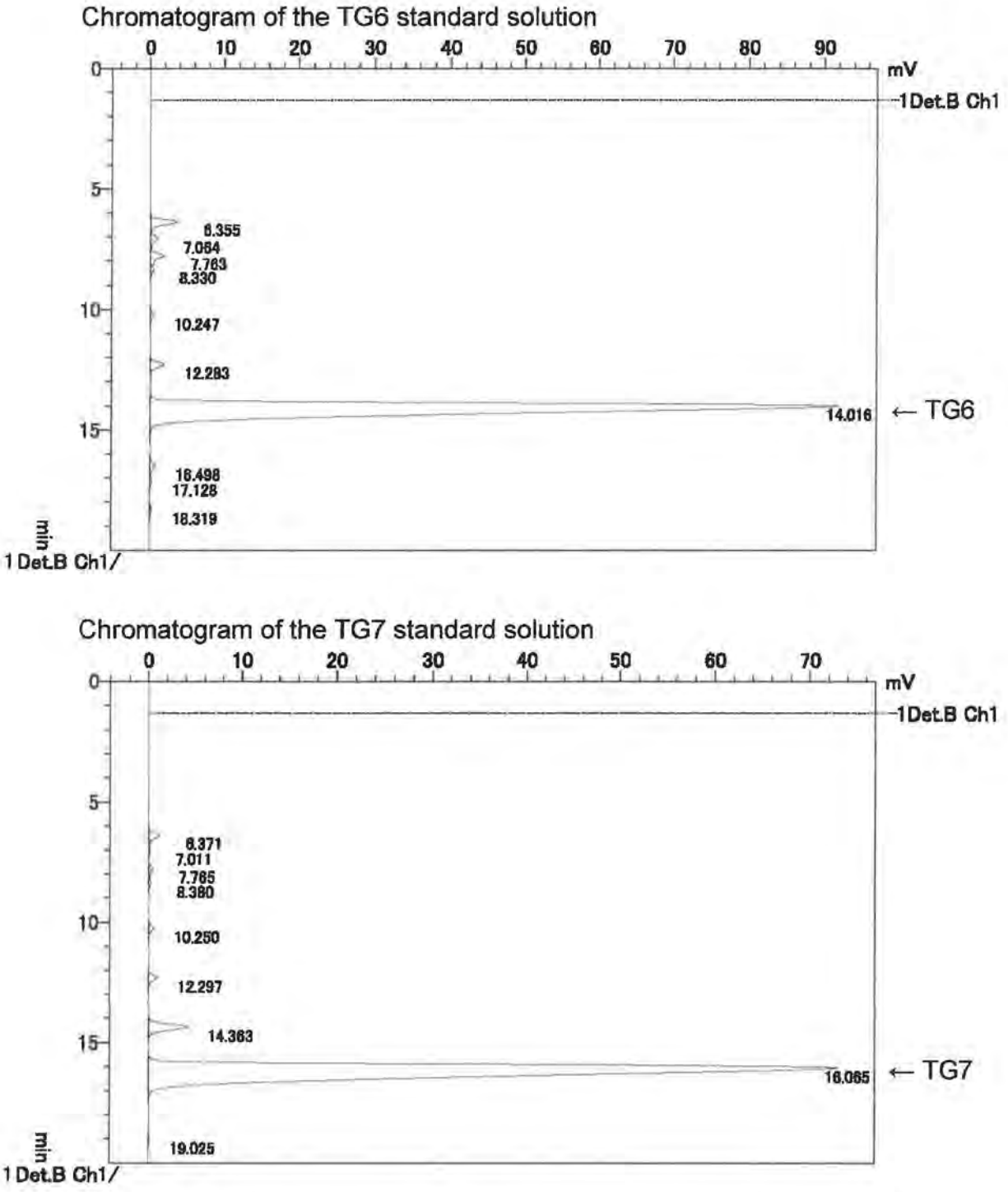



**Figure 2-3 Reverse-phase HPLC Chromatograms of the Standard Solutions (continued)**

To determine α-anomer content from β-anomer content, the ratios of each α/β-anomer of G3 – G5 were calculated based on the chromatograms of G3 – G5 standards. The ratios of α/β-anomers of G3 – G5 are shown in Table 2-8.

**Table 2-8 Ratios of α/β-Anomers of Oligosaccharide Standards**

| Standard | | Peak area | Ratio of α/β-anomers |
|---|---|---|---|
| G3 | α-Anomer | 3975049 | 0.76 |
| | β-Anomer | 5249483 | |
| G4 | α-Anomer | 3396197 | 0.64 |
| | β-Anomer | 5313373 | |
| G5 | α-Anomer | 3368907 | 0.66 |
| | β-Anomer | 5109028 | |

To determine the saccharide composition of TG4 Syrup, the reverse-phase HPLC was performed on the test solutions. The chromatograms are shown in Figure 2-4.

Analysis of the reverse-phase HPLC chromatograms of the test solutions shown in Figure 2-4, reveals β-anomers of each oligosaccharide and each "sum of α-anomer and glycosyltrehalose" in TG4 Syrup were determined by the area normalization method. Based on each determined β-anomer, each α-anomer could be calculated by the following formula using the ratios of α/β-anomers of each standard shown in Table 2-8:

$$\alpha\text{-Anomer} = \beta\text{-Anomer} \times (\text{Ratio of } \alpha/\beta\text{-anomers of the standard})$$

Then, each oligosaccharide is calculated as the sum of the calculated α-anomer and the measured β-anomer. And based on the calculated α-anomer, each glycosyltrehalose is calculated by subtracting the calculated α-anomer from the sum of the α-anomers and glycosyltrehalose determined in the chromatograms. The results are shown in Table 2-9.

Chromatogram of the test solution (Lot No. 6G08)

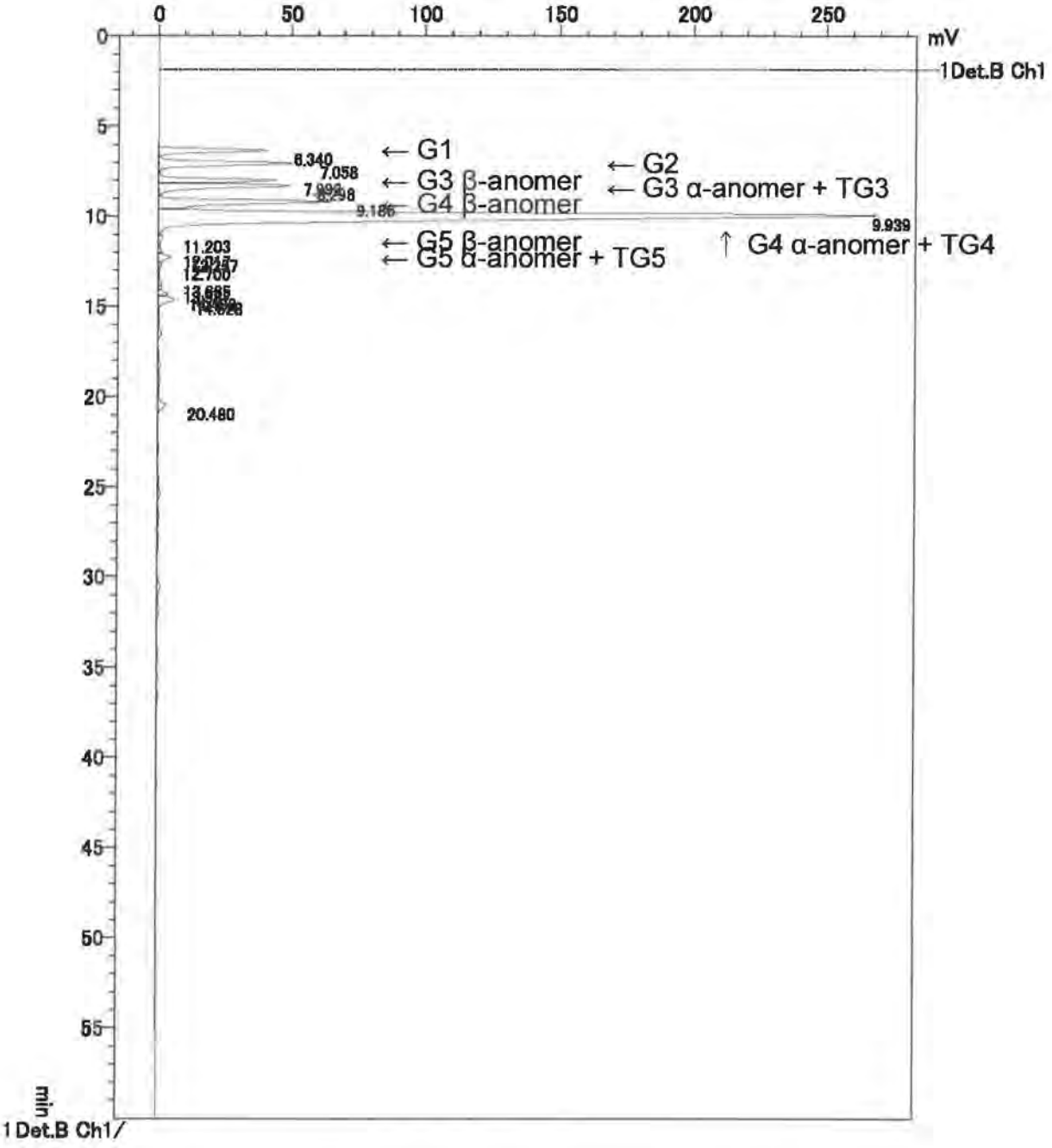



**Figure 2-4 Reverse-phase HPLC Chromatogram of the Test Solution**

**Table 2-9 Saccharide Composition of TG4 Syrup by Reverse-phase HPLC**

| Constituent (% on a dry basis) | | Lot No. 6G08 | | Lot No. 6H12 | | Lot No. 6H129 | | Average ± S.D. |
|---|---|---|---|---|---|---|---|---|
| G1 | | 4.5 | | 4.5 | | 4.5 | | 4.5 ± 0.0 |
| G2 | | 6.7 | | 6.4 | | 7.9 | | 7.0 ± 0.8 |
| β-Anomer | G3 | 5.1 | 8.9 | 5.1 | 9.0 | 5.5 | 9.7 | 9.2 ± 0.4 |
| α-Anomer | | 3.9 | | 3.9 | | 4.2 | | |
| TG3 | | 3.3 | | 3.4 | | 3.5 | | 3.4 ± 0.1 |
| β-Anomer | G4 | 9.2 | 15.1 | 9.2 | 15.1 | 8.3 | 13.6 | 14.6 ± 0.9 |
| α-Anomer | | 5.9 | | 5.9 | | 5.3 | | |
| TG4 | | 53.6 | | 54.3 | | 52.0 | | 53.3 ± 1.2 |
| β-Anomer | G5 | 0.3 | 0.5 | 0.3 | 0.4 | 0.3 | 0.4 | 0.5 ± 0.0 |
| α-Anomer | | 0.2 | | 0.2 | | 0.2 | | |
| TG5 | | 0.7 | | 0.7 | | 0.9 | | 0.7 ± 0.1 |
| Other saccharides | | 6.7 | | 6.2 | | 7.5 | | 6.8 ± 0.6 |

**Summary**

The results show that G5 in TG4 Syrup is only 0.9% of TG4, which is within the error range of TG4, and TG3 is 23.3% of G4.

**Analytical Method 2**

The saccharide composition of TG4 Syrup was determined by cation-exchange HPLC analysis. It is expected that by cation-exchange HPLC analysis, G4 and TG3 will be detected at the same retention time as TG4 and G5, respectively.

The cation-exchange HPLC chromatograms of the standard solutions are shown in Figure 2-5.

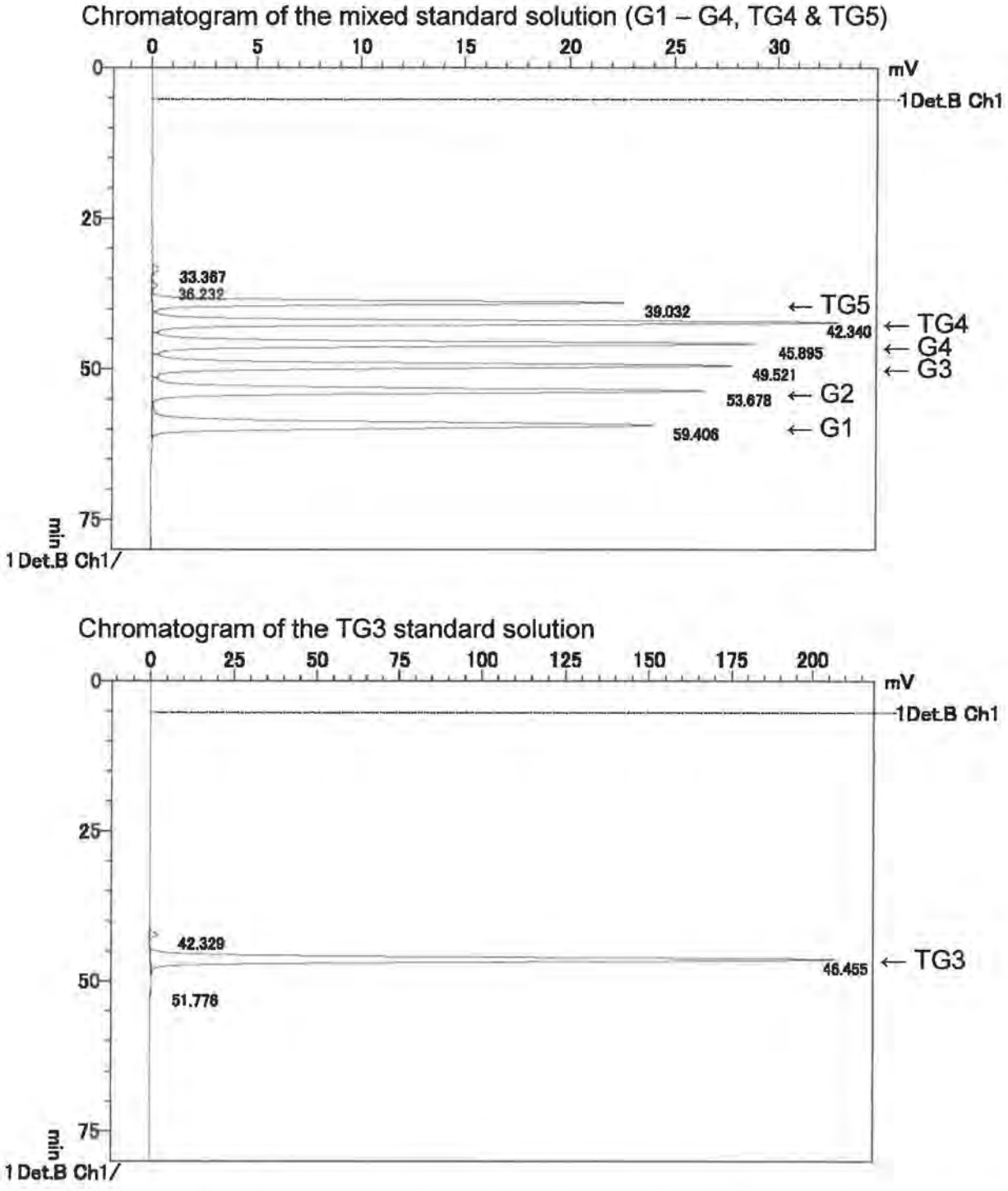



**Figure 2-5 Cation-exchange HPLC Chromatogram of the Standard Solutions**

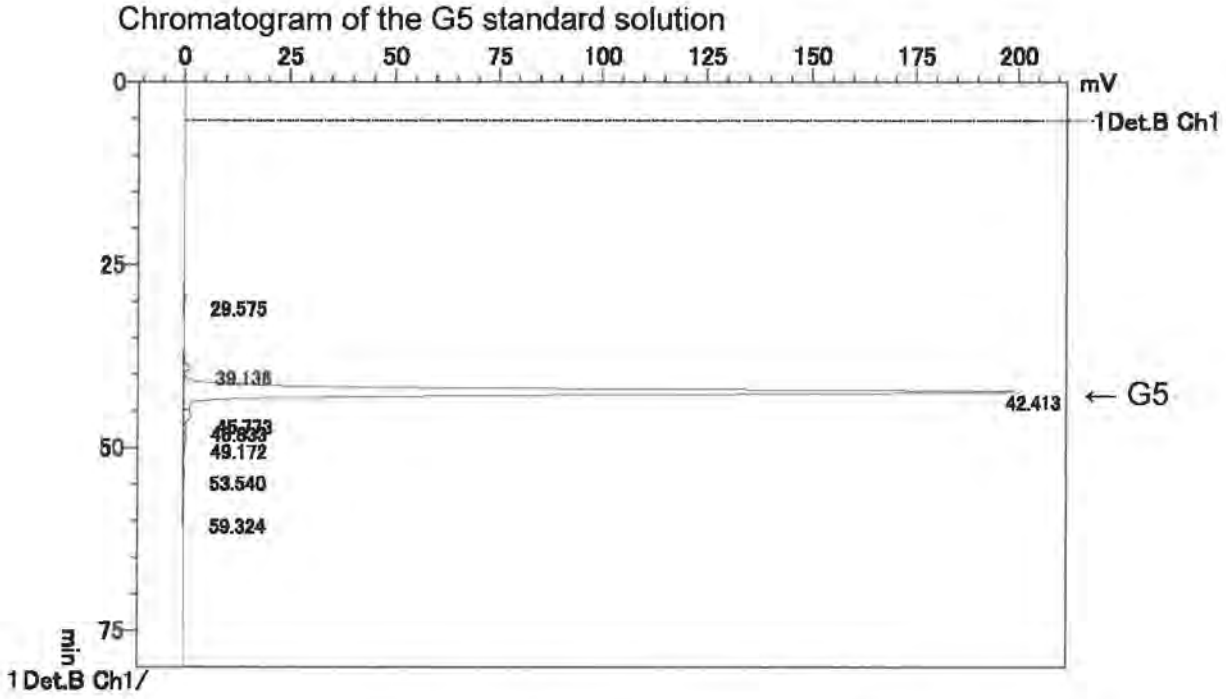



**Figure 2-5 Cation-exchange HPLC Chromatogram of the Standard Solutions (continued)**

The retention times of each standard by cation-exchange HPLC analysis are shown in Table 2-10.

**Table 2-10 Retention Times of Each Standard by Cation-exchange HPLC**

| Standard | F | G1 | G2 | G3 | TG3 | G4 |
|---|---|---|---|---|---|---|
| Retention Time (minutes) | 62.950 | 59.406 | 53.678 | 49.521 | ***46.455*** | ***45.895*** |
| Standard | G5 | TG4 | G6 | TG5 | TG6 | TG7 |
| Retention Time (minutes) | ***42.413*** | ***42.340*** | 38.056 | 39.032 | 36.232 | 33.367 |

The results indicate that TG4 and G5 are resolved at almost the same retention time by the cation-exchange HPLC analysis and that G4 and TG3 are also essentially superimposed. In other words, each peak from TG4 and G5, and G4 and TG3 cannot separately be determined by cation-exchange HPLC analysis.

The cation-exchange HPLC chromatograms of the test solutions are shown in Figure 2-6.

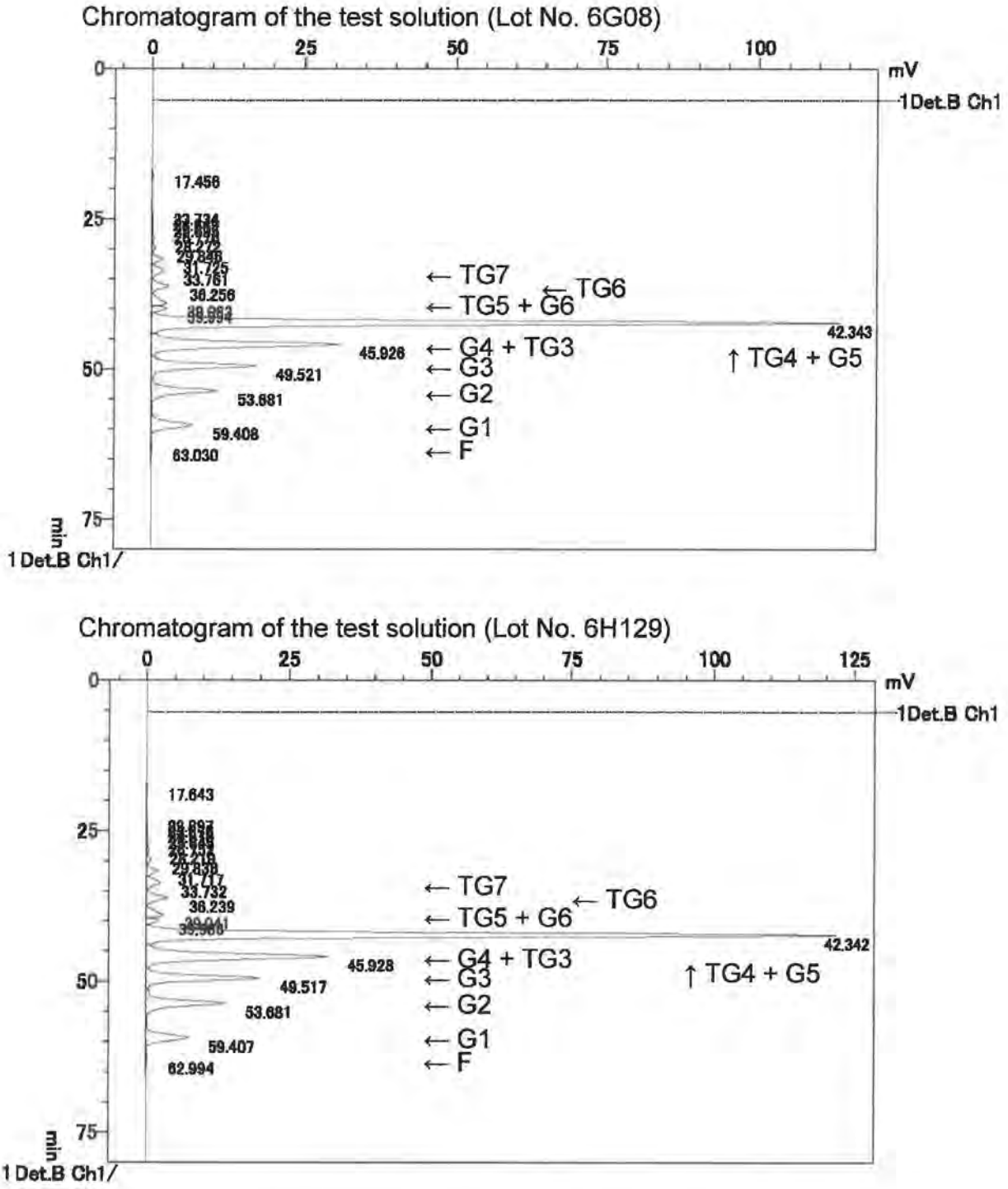



**Figure 2-6 Cation-exchange HPLC Chromatograms of Test Solutions**

Based on the chromatograms, the saccharide composition of TG4 Syrup was calculated by the area normalization method, and is shown in Table 2-11.

**Table 2-11 Saccharide Composition of TG4 Syrup**

| Constituent (% on a dry basis) | Lot No. | | | Average ± S.D. |
|---|---|---|---|---|
| | 6G08 | 6H12 | 6H129 | |
| G1 (Spec.: ≤ 6.0%) | 4.6 | 4.5 | 4.5 | 4.5 ± 0.0 |
| G2 | 6.8 | 6.6 | 8.0 | 7.1 ± 0.8 |
| G3 | 9.6 | 9.7 | 10.0 | 9.8 ± 0.2 |
| G4 + TG3 | 18.5 | 18.5 | 18.1 | 18.4 ± 0.2 |
| TG4 + G5 | 53.0 | 53.5 | 51.1 | **52.5 ± 1.2** |
| TG5 + G6 | 2.5 | 2.2 | 2.5 | 2.4 ± 0.2 |
| TG6 | 1.6 | 1.6 | 2.0 | 1.7 ± 0.2 |
| TG7 | 1.3 | 1.3 | 1.5 | 1.4 ± 0.1 |
| Other saccharides | 2.1 | 2.1 | 2.2 | 2.1 ± 0.1 |

**Summary**

As expected, G4 and TG3 were detected at the same retention time as are TG4 and G5 by the cation-exchange HPLC analysis. Based on the results, other saccharides than G4, TG3, TG4 and G5 would be considered impurities (< 10%). However, the 'impurities', as with the main ingredients, consist of only glucose containing molecules.

**Conclusion**

The results indicate that the amount of G5 in TG4 Syrup is negligible (0.9%) compared to TG4, and that the % of TG3 to G4 is about 23.3%. Therefore, in consideration that G5 in TG4 Syrup is negligible, and that the G4/TG3 ratio is 100/23, the saccharide composition of TG4 Syrup was calculated and is shown in Table 2-11.

Based on the results from analytical assays, it is concluded that the main constituents of TG4 Syrup (≥ 10%) are G4 and TG4. These two substances make up approximately 67% of TG4 Syrup. The glucose containing substances are 99.9% of the dry weight of the TG4 Syrup.

### 2.6.2 Comparison of Pilot and Commercial Lots

For some of the physico-chemical and toxicological tests described in this Notice, pilot lots of TG4 Syrup (Lot Nos. 020621 and 020907) were used. However, the pilot lots were not manufactured in an identical manner to the manufacturing process of TG4 Syrup described in Part 2.5.2. In detail, both pilot lots were manufactured using MTSase from *Arthrobacter pascens* instead of MTSase from *Arthrobacter ramosus*, which is currently used for commercial lot production. In addition, the production process (saccharification process) of the pilot lots was different from that of commercial lots as described in Figure 2-7.

Therefore, to confirm that the pilot lots could be used for the physico-chemical and toxicological tests, the saccharide composition of the pilot lots were determined and compared with that of commercial lots described in Part 2.6.1.

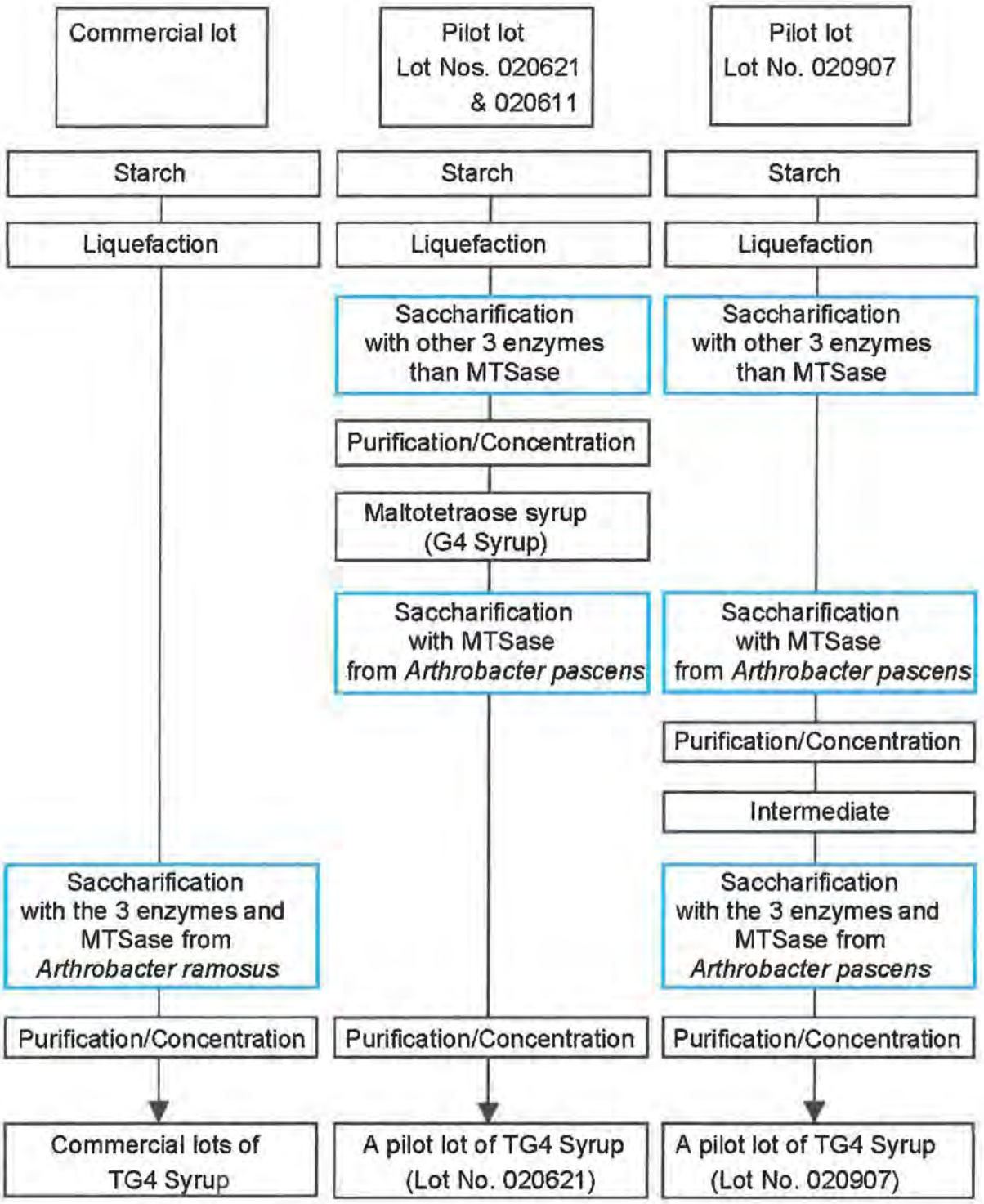



**Figure 2-7 Difference of Production Process among Commercial and Pilot Lots of TG4 Syrup**

**Analytical Method 1**

The results in Part 2.6.1 show that G4 and TG4 could not be deferentiated from TG3 and G5 by the cation-exchange HPLC analysis because their retention times were essentially the same. Therefore, TG3 and G5 in a pilot lot (Lot No. 020621) were first determined by reverse-phase HPLC analysis under the conditions described in Part 2.6.1. The results are shown in Table 2-12.

**Table 2-12 Comparison of Saccharide Composition of Pilot and Commercial Lots of TG4 Syrup by Reverse-phase HPLC**

| Constituent (% on a dry basis) | | Pilot lot (Lot No. 020621) | | Average (± S.D.) of commercial lots* |
|---|---|---|---|---|
| β-Anomer | G3 | 6.9 | 11.3 | 9.2 (± 0.4) |
| α-Anomer | | 4.5 | | |
| TG3 | | | 0.9 | 3.4 (± 0.1) |
| β-Anomer | G4 | 9.6 | 15.9 | 14.6 (± 0.9) |
| α-Anomer | | 6.3 | | |
| TG4 (Spec.: ≥ 50.0%) | | | 56.9 | 53.5 (±1.2) |
| β-Anomer | G5 | 0.1 | 0.3 | 0.5 (± 0.0) |
| α-Anomer | | 0.2 | | |
| TG5 | | | 0.6 | 0.7 (± 0.1) |
| Other saccharides | | | 14.1 | 18.3 (± 1.4) |

*From the results in Table 2-9.

The results indicate that in the pilot lot (Lot No. 020621) as well as commercial lots, G5 is negligible as compared to TG4 (0.5%), which is within the error range of TG4, and that TG3 is much less than G4 (about 5.7%), which is slightly less than the commercial lots.

**Analytical Method 2**

Under the assumption that G5 in the pilot lots is negligible compared with TG4 based on the results shown in Part 2.6.1, the saccharide composition of pilot lots (Lot Nos. 020621 and 020907) was determined by the cation-exchange HPLC analysis under the conditions described in Part 2.6.1. The results are shown in Table 2-14 with the average saccharide composition of commercial lots described in Table 2-13.

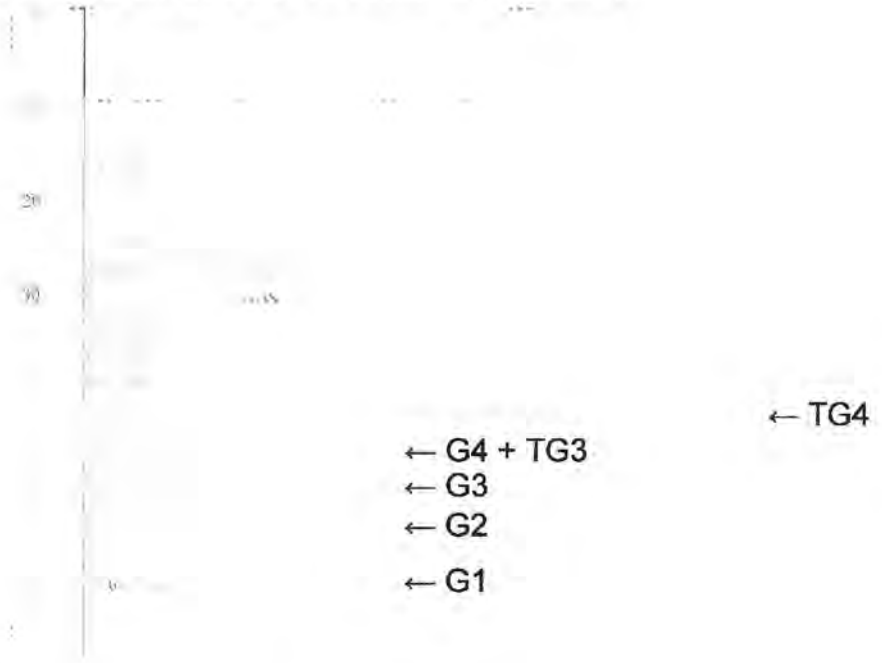
**Table 2-13 Comparison of Saccharide Composition of Pilot and Commercial Lots of TG4 Syrup by Cation-exchange HPLC**

| Constituent (% on a dry basis) | Pilot lots | | Commercial lots (Average ± S.D.)* |
|---|---|---|---|
| | Lot No. 020621 | Lot No. 020907 | |
| G1 (Spec.: ≤ 6.0%) | 3.5 | 1.9 | 4.5 ± 0.0 |
| G2 | 7.3 | 8.1 | 7.1 ± 0.8 |
| G3 | 11.1 | 10.8 | 9.8 ± 0.2 |
| G4 + TG3 | 16.4 | 17.5 | 18.4 ± 0.2 |
| TG4 (Spec.: ≥ 50.0%) | 54.8 | 52.1 | 52.5 ± 1.2 |
| Other saccharides | 6.9 | 9.6 | 7.7 ± 0.5 |

*From the results in Table 2-11.

The chromatograms of the pilot lot (Lot Nos. 020621 and 020907) are shown in Figure 2-8.

Chromatogram of the pilot lot (Lot No. 020621)




Chromatogram of the pilot lot (Lot No. 020907)

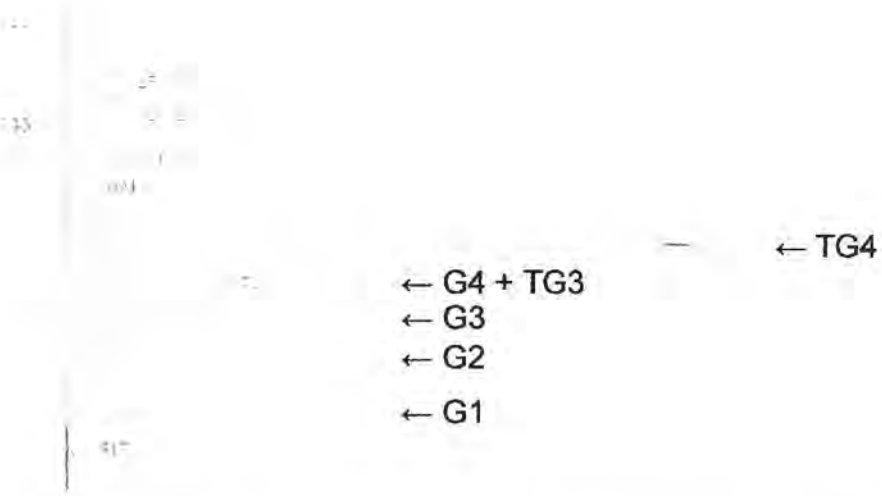



**Figure 2-8 Cation-exchanging HPLC Chromatograms of the Pilot Lots of TG4 Syrup**

**Summary**

The results in Part 2.6.2 indicate that the saccharide composition of the pilot lots (Lot Nos. 020621 and 020907) meet the proposed specifications (G1: ≤ 6%, TG4: ≥ 50%), and suggest that the pilot lots are substantially equivalent to commercial lots.

Therefore, the Notifier concluded that the results of the physico-chemical and toxicological tests on the pilot lots would reflect those of commercial lots. In addition, considering toxicological tests, 99.9% of the dry weight of TG4 Syrup consists of glucose oligosaccharides that are completely digested.

## 2.7 Product Stability

The results of the Stability test are shown in Table 2-14.

The stability study results showed that the product remained within the proposed specifications for the tested period (Lot Nos. 5D07451 and 5D07461 for 15 months, Lot No. 5A23949 for 8 months, and the pilot lot for 12 months) under the test conditions.

These results indicate that TG4 Syrup can maintain product quality for at least 12 months under the test conditions (unopened at 20 – 30°C).

## 2.8 Technical Effects of TG4 Syrup as Listed in US 21 CFR §170.3 (o)

In order to classify the various effects ingredients may have in food the FDA has published a list of 32 physical or technical functional effects for which direct food ingredients may be added to food. These are codified at 21 CFR §170.3 (o) (1-32), and applications for TG4 Syrup (Table 2-15) are covered under several of the following terms (FDA, 2019(h)).

**Table 2-14 Stability of TG4 Syrup**

| Variables | Spec. | Lot No. | Storage period (months) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Start | 1 | 3 | 6 | 8 (or 9) | 12 | 15 |
| Dry solid | ≥ 72.0% | 5D07451 | 73.4 | | | 73.4 | 73.1 | 73.3 | 73.3 |
| | | 5D07461 | 73.5 | | | 73.5 | 73.5 | 73.4 | 73.4 |
| | | 5A23949 | 73.5 | 73.2 | 73.4 | 73.8 | 73.4 | | |
| | | Pilot lot | 72.4 | 72.5 | 72.4 | 72.5 | 72.5 | 72.7 | |
| pH | 3.5 – 6.5 | 5D07451 | 4.8 | 4.5 | 4.3 | 3.9 | 4.3 | 4.2 | 4.1 |
| | | 5D07461 | 4.8 | 4.7 | 4.1 | 4.1 | 4.2 | 4.2 | 4.0 |
| | | 5A23949 | 4.9 | 4.9 | 4.8 | 4.7 | 4.7 | | |
| | | Pilot lot | 5.2 | 5.1 | 4.9 | 4.8 | 4.7 | 4.6 | |
| Color in solution | ≤ 0.100 | 5D07451 | 0.005 | 0.007 | 0.007 | 0.012 | 0.014 | 0.013 | 0.015 |
| | | 5D07461 | 0.006 | 0.008 | 0.008 | 0.012 | 0.013 | 0.013 | 0.015 |
| | | 5A23949 | 0.024 | 0.023 | 0.023 | 0.026 | 0.027 | | |
| | | Pilot lot | 0.007 | 0.021 | 0.005 | 0.009 | 0.010 | 0.009 | |
| Turbidity | ≤ 0.050 | 5D07451 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | | 5D07461 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | | 5A23949 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | |
| | | Pilot lot | 0.000 | 0.002 | 0.000 | 0.000 | 0.000 | 0.000 | |
| G1 | ≤ 6.0% | 5D07451 | 3.3 | 3.1 | 3.2 | 3.4 | 3.2 | 3.1 | 3.0 |
| | | 5D07461 | 3.4 | 3.4 | 3.3 | 3.5 | 3.2 | 3.3 | 3.1 |
| | | 5A23949 | 4.4 | 4.5 | 4.6 | 4.6 | 4.8 | | |
| | | Pilot lot | 4.2 | 4.2 | 4.2 | 4.3 | 4.2 | 4.6 | |
| TG4 | ≥ 50.0% | 5D07451 | 53.7 | 53.8 | 53.4 | 53.6 | 53.9 | 53.5 | 53.9 |
| | | 5D07461 | 53.2 | 53.4 | 53.1 | 53.2 | 53.6 | 53.1 | 53.6 |
| | | 5A23949 | 52.6 | 50.9 | 51.4 | 51.5 | 51.7 | | |
| | | Pilot lot | 52.2 | 52.2 | 52.4 | 52.1 | 51.9 | 52.1 | |
| TAMC | ≤ 300 cfu/g | 5D07451 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | | 5D07461 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 5A23949 | 0 | 0 | 0 | 0 | 0 | | |
| | | Pilot lot | 0 | 0 | 0 | 0 | 0 | 0 | |
| Coliform organisms | Negative | 5A23949 | Negative | Negative | Negative | Negative | Negative | | |
| | | Pilot lot | Negative | Negative | Negative | Negative | Negative | Negative | |

**Table 2-15 Technical Effects of TG4 Syrup in US 21CFR §170.3 (o)**

| | |
|---|---|
| (3) Antioxidants | Substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation. |
| (11) Flavor enhancers | Substances added to supplement, enhance, or modify the original taste and/or aroma of a food, without imparting a characteristic taste or aroma of its own. |
| (14) Formulation aids | Substances used to promote or produce a desired physical state or texture in food, including carriers, binders, fillers, plasticizers, film-formers, and tableting aids, etc. |
| (16) Humectants | Hygroscopic substances incorporated in food to promote retention of moisture, including moisture-retention agents and antidusting agents. |
| (21) Nutritive sweeteners | Substances having greater than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity. |
| (24) Processing aids | Substances used as manufacturing aids to enhance the appeal or utility of a food or food component, including clarifying agents, clouding agents, catalysts, flocculents, filter aids, and crystallization inhibitors, etc. |
| (27) Solvents and vehicles | Substances used to extract or dissolve another substance. |
| (28) Stabilizers and thickeners | Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc. |
| (29) Surface-active agents | Substances used to modify surface properties of liquid food components for a variety of effects, other than emulsifiers, but including solubilizing agents, dispersants, detergents, wetting agents, rehydration enhancers, whipping agents, foaming agents, and defoaming agents, etc. |
| (30) Surface-finishing agents | Substances used to increase palatability, preserve gloss, and inhibit discoloration of foods, including glazes, polishes, waxes, and protective coatings. |
| (32) Texturizers | Substances which affect the appearance or feel of the food. |

# Part 3. Dietary Exposure

## 3.1 Intended Effect

The intended effect of the use of Maltosyltrehalose Syrup (TG4 Syrup) in various foods was provided in Part 2.8.3, which included the Technical Effects given in 21CFR §170.3 (o)1-32 (FDA, 2019(h). The desired technical effects are not associated with the safety of the food, but rather to improve the quality of the products in which it is used.

## 3.2 History of Use

Since 2003, TG4 Syrup has been commercially sold in Japan and Taiwan (export to Taiwan 96 MT in 2016). The Notifier, Hayashibara Co., Ltd., manufactures TG4 Syrup (commercial name HALLODEX™) in accordance with the Food Sanitation Law in Japan and it is allowed for use in food as a starch syrup under the Food Sanitation Law. In Taiwan TG4 Syrup can be sold as a food ingredient. The types of foods in which TG4 Syrup has been used in Japan are numerous and diverse (Appendix A).

The annual sales of TG4 Syrup sold by the Notifier in Japan (including a small proportion in Taiwan) was approximately 10,000 metric tons (MT) each year in 2013, 2014, 2015 and 2016, which is equal to approximately 7,300 MT on a dry basis. In the last 14 years (2003 – 2016), approximately 110,000 MT of TG4 Syrup were sold at an average of 7,860 MT/year.

No adverse events have been reported from end users, food manufacturers, or from workers at the Notifier's manufacturing facilities.

## 3.3 Intended Use of TG4 Syrup

The range of the different types of foods in which TG4 Syrup can be used is as widespread as the food types in which starch (glucose) syrups can be used. However, that being said, it is not likely that it would directly replace all starch syrup as described in Part 4. The wide range of types of foods and beverages, and the technical effects for which it is being used in Japan are provided in Appendix A. The food categories include a wide variety of confectionaries, processed foods and beverages. While many of the commercial food products listed are not commonly consumed in large amounts

the US, it is felt that the functionalities they represent can be translated into useful applications in products more common to the US.

Further, from the years of experience in Japan, it is believed that TG4 Syrup will not be used in addition to existing starch syrups, but rather replace a portion or less likely all of the syrup normally used in a product. Additionally, one of the primary uses of starch syrups is to add sweetness to a product (i.e. HFCS), whereas TG4 Syrup is used in a number of products in Japan to reduce the sweetness (Appendix A). However, as with most food ingredients it will have other technical effects.

### 3.4 Estimated Use Levels

TG4 Syrup is generally used in Japan in foods following the *Quantum Satis* (QS) principle, *i.e.* at a level not higher than the necessary dosage to achieve the desired effects – according to current Good Manufacturing Practice. This is also how it would be used in the US. The amount of TG4 Syrup added in food/beverages by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions. As stated previously, theoretically, TG4 Syrup could be used in all food applications and at the same concentrations in which conventional starch syrups are used; however, this is highly unlikely (Part 4 – Self-limiting levels of use.)

Therefore, only estimated maximum and ordinary use levels of TG4 Syrup in food/beverages can be estimated and shown. Such use levels are the starting points for the individual food producer to 'fine-tune' their specific formulation and processes, and determine the lowest amount of TG4 Syrup that will provide the desired effect. Consequently, from a technological point of view, there are no 'normal or maximal' use levels for each food category. Manufacturers will need to test TG4 Syrup according to the good manufacturing practice (QS) principle. A food producer who would add much higher doses than that needed would experience untenable costs as well as negative technological consequences.

Based on the food categories in US FDA 21CFR §170.3 (n), Tables 3-1 shows the estimated maximum and "average" use levels for each food category where TG4 Syrup would be used, respectively (FDA, 2019(h)). It needs to be stressed that the estimated use levels of TG4 Syrup in every food category would be the same as conventional starch syrup because TG4 Syrup could theoretically, but not practically, be used in foods as a substitute for conventional starch syrup.

**Table 3-1 Estimated Maximum and Average Use Level of TG4 Syrup in US FDA 21CFR §170.3(n)**

| FDA 21CFR §170.3 (n) Food Category | Estimated Maximum Use Level (%) | Estimated Average Use Level (%) |
|---|---|---|
| (1) Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours, and mixes requiring preparation before serving. | 10 | 5 |
| (2) Beverages, alcoholic, including malt beverages, wines, distilled liquors, and cocktail mix. | 10 | 5 |
| (3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks. | 10 | 5 |
| (4) Breakfast cereals, including ready-to-eat and instant and regular hot cereals. | 10 | 5 |
| (5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses. | 10 | 5 |
| (6) Chewing gum, including all forms. | 10 | 5 |
| (7) Coffee and tea, including regular, decaffeinated, and instant types. | 10 | 5 |
| (8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs. | 10 | 5 |
| (9) Confections and frostings, including candy and flavored frostings, marshmallows, baking chocolate, and brown, lump, rock, maple, powdered, and raw sugars. | 40 | 10 |
| (10) Dairy product analogs, including nondairy milk, frozen or liquid creamers, coffee whiteners, toppings, and other nondairy products. | 10 | 5 |
| (11) Egg products, including liquid, frozen, or dried eggs, and egg dishes made therefrom, i.e., egg roll, egg food young, egg salad, and frozen multicourse egg meals, but not fresh eggs. | 15 | 5 |
| (12) Fats and oils, including margarine, dressings for salads, butter, salad oils, shortenings and cooking oils. | 10 | 5 |
| (13) Fish products, including all prepared main dishes, salads, appetizers, frozen multicourse meals, and spreads containing fish, shellfish, and other aquatic animals, but not fresh fish. | 10 | 5 |
| (14) Fresh eggs, including cooked eggs and egg dishes made only from fresh shell eggs. | 15 | 5 |
| (15) Fresh fish, including only fresh and frozen fish, shellfish, and other aquatic animals. | 10 | 5 |

**Table 3-1 Estimated Maximum and Average Use Level of TG4 Syrup in US FDA 21CFR §170.3(n) (continued)**

| FDA 21CFR §170.3 (n) Food Category | Estimated Maximum Use Level (%) | Estimated Average Use Level (%) |
|---|---|---|
| (16) Fresh fruits and fruit juices, including only raw fruits, citrus, melons, and berries, and home-prepared "ades" and punches made therefrom. | 30 | 5 – 10 |
| (17) Fresh meats, including only fresh or home-frozen beef or veal, pork, lamb or mutton and home-prepared fresh meat-containing dishes, salads, appetizers, or sandwich spreads made therefrom. | 10 | 5 |
| (18) Fresh poultry, including only fresh or home-frozen poultry and game birds and home-prepared fresh poultry-containing dishes, salads, appetizers, or sandwich spreads made therefrom. | 10 | 5 |
| (20) Frozen dairy desserts and mixes, including ice cream, ice milks, sherbets, and other frozen dairy desserts and specialties. | 10 | 5 |
| (21) Fruit and water ices, including all frozen fruit and water ices. | 10 | 5 |
| (22) Gelatins, puddings, and fillings, including flavored gelatin desserts, puddings, custards, parfaits, pie fillings, and gelatin base salads. | 10 | 5 |
| (23) Grain products and pastas, including macaroni and noodle products, rice dishes, and frozen multicourse meals, without meat or vegetables. | 10 | 5 |
| (24) Gravies and sauces, including all meat sauces and gravies, and tomato, milk, buttery, and specialty sauces. | 10 | 5 |
| (25) Hard candy and cough drops, including all hard type candies. | 40 | 10 – 20 |
| (26) Herbs, seeds, spices, seasonings, blends, extracts, and flavorings, including all natural and artificial spices, blends, and flavors. | 10 | 5 |
| (33) Plant protein products, including the National Academy of Sciences / National Research Council "reconstituted vegetable protein" category, and meat, poultry, and fish substitutes, analogs, and extender products made from plant proteins. | 10 | 5 |
| (34) Poultry products, including all poultry and poultry-containing dishes, salads, appetizers, frozen multicourse poultry meals, and sandwich ingredients prepared by commercial processing or using commercially processed poultry with home preparation. | 10 | 5 |

**Table 3-1 Estimated Maximum and Average Use Level of TG4 Syrup in US FDA 21CFR §170.3(n) (continued)**

| FDA 21CFR §170.3 (n) Food Category | Estimated Maximum Use Level (%) | Estimated Average Use Level (%) |
|---|---|---|
| (35) Processed fruits and fruit juices, including all commercially processed fruits, citrus, berries, and mixtures; salads, juices and juice punches, concentrates, dilutions, "ades", and drink substitutes made therefrom. | 30 | 5 – 10 |
| (36) Processed vegetables and vegetable juices, including all commercially processed vegetables, vegetable dishes, frozen multicourse vegetable meals, and vegetable juices and blends. | 10 | 5 |
| (37) Snack foods, including chips, pretzels, and other novelty snacks. | 10 | 5 |
| (38) Soft candy, including candy bars, chocolates, fudge, mints, and other chewy or nougat candies. | 40 | 10 – 20 |
| (39) Soups, home-prepared, including meat, fish, poultry, vegetable, and combination home-prepared soups. | 10 | 5 |
| (40) Soups and soup mixes, including commercially prepared meat, fish, poultry, vegetable, and combination soups and soup mixes. | 10 | 5 |
| (43) Sweet sauces, toppings, and syrups, including chocolate, berry, fruit, corn syrup, and maple sweet sauces and toppings. | 60 | 30 – 40 |

## 3.5 Estimated Daily Intake (EDI) using Modified Budget Method with Specific Factors

The human consumption was estimated using the so-called Budget Method as an appropriate and simple way for the estimation of the safety aspects of the proposed use levels (WHO, 2001). This method allows for the calculation of the estimated daily intake (EDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data. The Budget calculation should be performed on the ready-to-eat product for foods sold as concentrates or powders intended for reconstitution before consumption. The Budget Method was originally developed for food additives, and it should be noted that the EDI of food ingredients such as TG4 Syrup provides an overestimation (WHO, 2001).

As described above, because TG4 Syrup could be used in food as a substitute of conventional starch syrup, TG4 Syrup could be used in the same food applications at

the same concentrations as conventional starch syrup like glucose syrup, starch syrup, HFCS, etc. In other words, it is concluded that the EDI of TG4 Syrup calculated by the Budget Method corresponds to the EDI of all conventional starch syrups, which would lead to a very large overestimation of EDI of TG4 Syrup. The EDI of starch syrup is calculated first.

In the Budget Method, it is assumed that the upper physiological intakes from solid foods and non-milk beverages are 50 g/kg-bw/day and 100 mL/kg-bw/day, respectively, and that 12.5% of solid foods and 25% of non-milk beverages over the course of a lifetime contain the substance to be evaluated.

In the Budget Method, the maximum use levels in solid foods and non-milk beverage should be used in the calculation, but it is not realistic because it assumes that:

- ALL food manufacturers use the HIGHEST use level of starch syrup per application, and
- Solid foods and beverages containing starch syrup at the HIGHEST use level are consumed DAILY over the course of a lifetime.

Therefore, it is assumed that the mean use levels in solid foods and non-milk beverage are used for the EDI calculation of starch syrup in the Budget Method.

Because TG4 Syrup is used as a substitute of conventional starch syrup, it was assumed that the mean use levels of starch syrup in solid foods and non-milk beverages would be 7.5% and 12.5%, which are the same as those of TG4 Syrup as shown in Tables 3-1 and 3-2, respectively.

For the "worst-case" scenario, the EDI of starch syrup is calculated as follow.

| Food | Consumption of final food (g or mL/kg-bw/day) | Average consumption of final food over the course of a lifetime (%) | Mean use level of starch syrup (%) | EDI of starch syrup (g/kg-bw/day) |
|---|---|---|---|---|
| Solid food | 50 | 12.5 | 7.5 | 0.47 |
| Non-milk beverages | 100 | 25 | 12.5 | 3.12 |
| TOTAL | | | | 3.6 |

Using the value, 3.6 g/kg-bw/day (2.5 g/kg-bw/day on a dry basis, according to the solid content specification (not less than 70.0%) of "21CFR §168.120 Glucose sirup", this corresponds to 206 g/day of starch syrup on a dry basis in a 82-kg person (FDA,

2019(f)). This is the average weight of the US population aged 20 years and over according to the report of the US population in 2007 – 2010 by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC) of the U.S. Department of Health and Human Services (HHS, 2013). The Economic Research Service (ERS), United States Department of Agriculture (USDA) reported that the per capita corn sweeteners estimated consumption for domestic food and beverage use in the US is 56.5 pounds/year (25.6 kg/year) on a dry basis, corresponding to 70.2 g/day, according to the report updated on August 4, 2017 (Table 50 of U.S. Consumption of Caloric Sweeteners (USDA, 2019). Using the average 82 kg person in the US that would equal approximately 0.86 g/kg-bw/day. In other words, the EDI of starch syrup, 3.6 g/kg-bw/day, is 2.9 times greater than the current consumption of starch syrup. Therefore, it should be stressed that this calculated value is highly overestimated as the EDI of starch syrup.

Many kinds of starch syrup are in the market not only in Japan but also in the US. Each starch syrup has its own characteristic properties such as lower or higher sweetness, viscosity, colorization, glossing effect and other functions. Food producers use starch syrup or a combination thereof in foods and beverages to meet their individual characteristics, including the properties and cost of starch syrup (Hobbs, 2009). Therefore, it is totally impractical that all conventional starch syrup in the US market would be replaced with TG4 Syrup. To calculate the EDI of TG4 Syrup, it should be understood that the market share potential of TG4 Syrup in starch syrup is only a small proportion of the aforementioned calculation.

As mentioned in the 'History of Use' above the annual sales of TG4 Syrup sold by the Notifier (commercial name: HALLODEX™) in Japan (including a small proportion in Taiwan) was approximately 10,000 MT in 2013 - 2016, which is equal to approximately 7,300 MT on a dry basis, and a total of 110,00 MT (80,300 MT on dry basis) from 2003 to 2016.

The Ministry of Agriculture, Forestry and Fisheries in Japan (MAFF) reported that the annual sales of conventional starch syrup in Japan was approximately 622,500 MT in fiscal 2015 (JMAFF, 2017). Based on these data, the market share of TG4 Syrup in conventional starch syrup in Japan is less than 1.6% (10,000 / 622,500 × 100 = 1.6%).

In the US, the total estimated consumption of corn sweeteners for domestic food and beverage use in 2016 was approximately 9.1 million short tons on a dry basis, based on the report updated on August 4, 2017 by ERS (Table 49; USDA, 2019). If all TG4 Syrup manufactured by the Notifier (HALLODEX™) in 2016 was sold in the US, the market share of TG4 Syrup in starch syrup in the US would be less than 0.1% (7,300 / 9,100,000 × 100 = 0.08%).

Based on the current Japanese and estimated US market share of TG4 Syrup in starch syrup (1.6% and 0.08%, respectively), it was conservatively assumed that considering brand-loyalty, 5% of starch syrup at maximum is replaced with TG4 Syrup in the US as a "worst-case" scenario.

EDI of TG4 Syrup = 3.6 × 0.05
= 0.18 g/kg-bw/day (0.13 g/kg-bw/day on a dry basis)

Therefore, the EDI of TG4 Syrup is calculated to be **0.18 g/kg-bw/day as the "worst-case" scenario by the modified Budget Method, corresponding to 14.8 g/day of TG4 Syrup in a 82-kg person** (10.8 g/day in a 60-kg individual), which is the average weight of the US population aged 20 years and over according to the report by NCHS (HHS, 2013).

It must be stressed that this EDI of TG4 Syrup, 0.18 g/kg-bw/day, is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- it was assumed that approximately THREE (3) TIMES MORE quantity of starch syrup is used in foods and consumed in the US than the current consumption;
- it was assumed that FIVE PERCENT (5%) of conventional starch syrup is replaced with TG4 Syrup; whereas after approximately 10 years of marketing and sales in Japan less than 1% of starch syrup has been replaced by TG4 Syrup;
- it was assumed that TG4 Syrup is used in ALL solid foods and beverages as a substitute of conventional starch syrup.

**Estimated daily intake (EDI) based on data from the US surveys**

The National Center for Health Statistics (NCHS) have reported the mean number of calories consumed from added sugars (white sugar, brown sugar, raw sugar, corn syrup, corn syrup solids, high fructose corn syrup, malt syrup, maple syrup, pancake syrup, fructose sweetener, liquid fructose, honey, molasses, anhydrous dextrose, crystal dextrose, and dextrin) by males and females and by various age groups: US children and adolescents – 2005–2008; US adults 2005–2010 (NCHS, 2012; NCHS, 2013).

The Economic Research Service (ERS) at the USDA reported in Table 49 of U.S. Consumption of Caloric Sweeteners that almost 50% of the total consumption of caloric sweeteners for domestic food and beverage use in 2016 was from corn sweeteners (9,134,000 / 20,702,000 (short tons) = 44.1%) (USDA, 2019). Fifty (50) % will be used

in the calculations as a more conservative number.

As described in Part 2.4.5, the caloric and solid content of TG4 Syrup were determined to be 293 kcal/100-g and 72.9%, respectively. In addition, it is felt that the use of 5% of starch syrup is a great overestimation at a maximum that would be replaced with TG4 Syrup in the US as described above.

Further, the NCHS reported the distribution of body measurements, including body weight, in the US population in 2007 – 2010 (HHS, 2013). Based on these data, Table 3-2 shows the EDI of TG4 Syrup at the mean consumption by males and females and by age among the US population.

Accordingly, the EDI of TG4 Syrup at the mean consumption would be a maximum of 5.17 g/person/day in male adolescents aged 12–19 years and 0.149 g/kg-bw/day in boys aged 2–5 years, respectively.

The Office of Disease Prevention and Health Promotion (ODPHP) US Department of Health and Human Services reported the percentiles of usual intake of total sugars from food and beverages by Dietary Reference Intake age-gender groups in the US, 2007-2010, which was prepared by the Food Surveys Research Group, US Department of Agriculture in July 2013 (ODPHP, 2013). Based on this survey, Table 3-3 shows the EDI of TG4 Syrup at the 95$^{th}$ percentile of consumption by age-gender groups in the US.

The EDI of TG4 Syrup at the 95$^{th}$ percentile of consumption was calculated to be at a maximum of 9.29 g/person/day for men aged 19–30 years and 0.406 g/kg-bw/day for boys aged 1–3 years.

**Estimated daily intake (EDI) based on the sales in Japanese market**

As a comparator based on a more mature market, TG4 Syrup has been marketed and sold in Japan since 2003. In 2013, 2014, 2015 and 2016, the annual sales, mainly in Japan (a small portion in Taiwan), reached approximately 10,000 MT each year. In the last 14 years (2003–2016), a total of approximately 110,000 MT were sold and averaged 7,860 MT/year. If the Japanese population (approximately 127 million as of October 1, 2017), the Statistics Bureau of Ministry of Internal Affairs and Communications in Japan were to **consume the entire sales quantity for the 13 years in a 1-year period**, the mean yearly intake per person would equal to 866 g (JMIC, 2018). The EDI of TG4 Syrup would be 2.37 g/person/day, corresponding to 0.040 g/kg-bw/day in a 59-kg person which is the average body weight of Japanese population aged 20 years and over according to the National Health and Nutrition

Survey in Japan, 2014 (JMHLW, 2014).

### 3.6 Summary and Margin of Exposure

By the modified Budget Method, the EDI of TG4 Syrup was calculated to be 0.18 g/kg-bw/day (10.8 and 14.8 g/person/day in a 60- and 82-kg person, respectively).

Based on the survey data of US population, the EDI of TG4 Syrup for the mean and 95$^{th}$ percentile of consumption were calculated to be at maximum 5.2 g/person/day (0.15 g/kg-bw/day) and 9.3 g/person/day (0.41 g/kg-bw/day), respectively.

Based on the sales in the Japanese market, the EDI of TG4 Syrup was calculated to be 2.4 g/person/day, or 0.040 g/kg-bw/day.

Even given the factors used for each calculation, it is felt that it is reasonable to assume that the EDI for TG4 Syrup above is conservatively high and the realistic consumption would be substantially less than the EDI.

The Margin of Exposure for human consumption could be estimated as the worst-case scenario by dividing the No Observed Adverse Effect Level (NOAEL) by the EDI.

As described above, the worst-case EDI was calculated to be 0.41 g/kg-bw/day (for the 95$^{th}$ percentile of consumption for boys aged 1–3 years based on the data from the US surveys). And as shown in Part 6 of this GRAS Notice, the NOAEL of the 90-day oral consumption study in both female and male rats was determined to be 5,000 mg/kg-bw/day, corresponding to 3.65 g/kg-bw/day on a dry basis.

**Therefore, the Margin of Exposure is calculated to be 3.65/0.41 = 8.9.**

It should be stressed that this dietary intake assessment is based on conservative assumptions and represents a highly exaggerated value.

**Table 3-2 Estimated Daily Intake of TG4 Syrup at the Mean Consumption among US Populations (by Males and Females and by Age)**

| Gender | Age (years) | Average consumption from sugars (kcal/day) | Ratio of starch syrup in sugars (%) | Average consumption from starch syrup (kcal/day) | Estimated market share of TG4 Syrup in starch syrup (%) | Calorie of TG4 Syrup (kcal/100-g) | Solid content of TG4 Syrup (%) | EDI of TG4 Syrup (g/person/day) | Average body weight of US population (kg) | EDI of TG4 Syrup (g/kg-bw/day) |
|---|---|---|---|---|---|---|---|---|---|---|
| Male | 2 – 5 | 218 | 50 | 109 | 5 | 293 | 72.9 | 2.55 | 17.1 | **0.149** |
| | 6 – 11 | 345 | | 173 | | | | 4.04 | 34.3 | 0.118 |
| | 12 – 19 | 442 | | 221 | | | | **5.17** | 70.0 | 0.074 |
| | 20 – 39 | 397 | | 199 | | | | 4.65 | 87.1 | 0.053 |
| | 40 – 59 | 338 | | 169 | | | | 3.96 | 91.2 | 0.043 |
| | 60 ≤ | 224 | | 112 | | | | 2.62 | 87.1 | 0.030 |
| Female | 2 – 5 | 196 | | 98 | | | | 2.29 | 16.5 | 0.139 |
| | 6 – 11 | 293 | | 147 | | | | 3.43 | 34.7 | 0.099 |
| | 12 – 19 | 314 | | 157 | | | | 3.68 | 61.4 | 0.060 |
| | 20 – 39 | 275 | | 138 | | | | 3.22 | 75.1 | 0.043 |
| | 40 – 59 | 236 | | 118 | | | | 2.76 | 76.6 | 0.036 |
| | 60 ≤ | 182 | | 91 | | | | 2.13 | 74.1 | 0.029 |

**Table 3-3 Estimated Daily Intake of TG4 Syrup at the 95th Percentile of Consumption by Age-gender in the US**

| Gender | Age (years) | 95th Percentile of usual intake of total sugars (g/person/day) | Ratio of starch syrup in sugars | Estimated market share of TG4 Syrup in starch syrup (%) | Solid content of TG4 Syrup (%) | EDI of TG4 Syrup for 95th percentile (g/person/day) | Average body weight of US population (kg) | EDI of TG4 Syrup for 95th percentile (g/kg-bw/day) |
|---|---|---|---|---|---|---|---|---|
| Male | 1 – 3 | 160 | 50 | 5 | 72.9 | 5.49 | 13.5 | **0.406** |
| | 4 – 8 | 175 | | | | 6.00 | 24.5 | 0.225 |
| | 9 – 13 | 198 | | | | 6.79 | 45.9 | 0.148 |
| | 14 – 18 | 254 | | | | 8.71 | 73.4 | 0.114 |
| | 19 – 30 | 271 | | | | **9.29** | 82.7 (Age: 19 – 29)* | 0.112 |
| | 31 – 50 | 263 | | | | 9.02 | 90.7 (Age: 30 – 49)* | 0.099 |
| | 51 – 70 | 218 | | | | 7.48 | 90.9 (Age: 50 – 69)* | 0.082 |
| | 71 ≤ | 169 | | | | 5.80 | 83.9 (Age: ≥ 70)* | 0.069 |
| Female | 1 – 3 | 152 | | | | 5.21 | 13.0 | 0.401 |
| | 4 – 8 | 173 | | | | 5.93 | 24.4 | 0.243 |
| | 9 – 13 | 179 | | | | 6.14 | 45.8 | 0.134 |
| | 14 – 18 | 166 | | | | 5.69 | 63.2 | 0.090 |
| | 19 – 30 | 195 | | | | 6.69 | 72.8 (Age: 19 – 29)* | 0.092 |
| | 31 – 50 | 189 | | | | 6.48 | 76.4 (Age: 30 – 49)* | 0.085 |
| | 51 – 70 | 163 | | | | 5.59 | 77.3 (Age: 50 – 69)* | 0.072 |
| | 71 ≤ | 144 | | | | 4.94 | 71.1 (Age: ≥ 70)* | 0.069 |

* Because of no data of the average body weight of each age was available, these values were used for the calculation of EDI of TG4 Syrup for the 95th percentile of consumption (g/kg-bw/day).

# Part 4. Self-limiting Levels of Use

Maltosyltrehalose Syrup (TG4 Syrup) is intended to be used as a partial or full replacement for other starch syrups. The Estimated Daily Intake (EDI) provided in Part 3.5 was calculated using very conservative values and assumptions using a total replacement of all starch-based syrups, which is extremely unlikely for a number of technologic reasons.

Starch syrups are used in hundreds of types of food products to produce many technical effects. Some of the more common technical effects are to provide viscosity, browning reactions and color, fermentability, foam stabilization and gel strength, freezing point depression, boiling point elevation, gelatinization temperature, humectancy and hygroscopicity, crystallization, and sweetness (Hobbs, 2009; White, 2014).

The selection of starch-based sweeteners for food applications is similar to the use of most other food additives, namely the functional properties, availability, and cost are carefully considered. However, in many cases, the desired properties may be mutually exclusive in a given starch sweetener. This would be the case, for example, if a high degree of sweetness and high viscosity were required in the sweetener. In such cases, blends of sweeteners or the use of other food ingredients would be required (Hobbs, 2009).

This is certainly the case for TG4 Syrup. The trehalose moiety at the reducing end of approximately 56% of TG4 Syrup (TG4 & TG3; Table 2-12) results in a unique mix of properties. As mentioned above, one of the main technical functions of starch sweeteners is to provide browning for color and flavor, which are the result of the Maillard reaction (Whistler, BeMiller, 1997). The trehalose moiety does not participate in Maillard reactions (Figure 2-15), so it would not be used in the many applications where browning and this type flavor development would be desired.

Further, a large portion of the applications for starch syrups, especially high fructose corn syrup (HFCS) is for sweetening (White, 2014). In 1967 the per capita consumption of corn-based sweeteners was 14.2 lbs (6.4 kg), including 9.9 lbs (4.5 kg) of glucose syrup and 4.3 lbs (2.0 kg) of dextrose (glucose, corn sugar), but no HFCS. By 2016 the per capita consumption of the same group of sweeteners was 56.5 lbs (25.6 kg). This included 41.4 lbs (18.8 kg) of high fructose corn syrup (HFCS), 12.4 lbs (5.6 kg) of glucose syrup, and 2.7 lbs (1.2 kg) of dextrose (corn sugar), Table 50 of the U.S. Consumption of Caloric Sweeteners (USDA, 2019). HFCS represents 73% of

these three sweetener categories. While the HFCS rose from 0.0 lbs to 41.4 lbs (18.8 kg), the total of glucose syrup and dextrose stayed essentially the same (14.2 lbs (6.4 kg) in 1967, 15.1 lbs (6.8 kg) in 2016). Interestingly the per capita consumption of refined sugar in 1967 was 98.5 lbs (44.7 kg), while in 2016 it was 69.7 lbs (31.6 kg), and conversely the amount of HFCS, rose from 0.0 to 41.4 lbs (18.8 kg). The other caloric sweeteners, pure honey and other 'edible syrups' remained about the same over this period. Total per capita caloric sweetener consumption rose from 114.2 to 128.1 lbs (51.8 to 58.1 kg; on dry basis). This suggests that the increase in "corn sweeteners" and total caloric sweeteners was the result of the dramatic increase in the use of HFCS as a substitute sweetening agent in place of refined sugar, and not to replace corn or other starch-based sweeteners (i.e. corn sugar, syrups, honey or other edible syrups; Table 50 of the U.S. Consumption of Caloric Sweeteners) (USDA, 2019).

HFCS at 42, 55 and 90% fructose concentrations have sweetness compared to sucrose of 100, 100–110, and 120–160, respectively (Hobbs, 2009). In beverages, greater than 70% of the sweeteners used in the US are HFCS. This is because HFCS offers the same sweetness as sucrose but has other advantages, such as pH stability, easier logistical handling, and improved mouth feel (White, 2014). However, the sweetness of TG4 Syrup is only about 27% that of sucrose (at a 5% sucrose solution). Therefore, TG4 Syrup would not be acceptable as a substitute for sucrose or HFCS as a sweetener in many food categories, especially beverages. This alone would rule out TG4 as a complete substitution for HFCS in about 73% of the current applications. In support of this is the information shown in Table 3-1 where the known uses of TG4 Syrup are for the reduction in sweetness in several of the products. Further, several acid-converted syrup sweeteners with dextrose equivalences from 30 to 62 are reported to have sweetness relative to sucrose of 30–35, to 60–70, respectively (Hobbs, 2009).

As a quantitative measure, it was noted in Part 3 that in Japan TG4 Syrup has been sold (a small portion of that into Taiwan) since 2003. From 2003 to 2016 a total of 110,000 MT was sold. From 2013 to 2016 the amount of TG4 sold has plateaued at approximately 10,000 MT per year (7,300 MT on dry basis). This means that in the Japanese population of 127 million just under 58 g/person/year (on dry basis; 0.16 g/person/day) was consumed. While no specific analysis has been performed, the most likely explanation for this is simply the same reasons for the use limitations of any food product in a free market; functional properties, availability and cost (Hobbs, 2009). Additionally, as shown in Appendix A, and previously mentioned, many of the known uses of TG4 Syrup are for the reduction of sweetness in various products.

Finally, the cost is one of the most important constraints on the use of any ingredient (Hobbs, 2009). The cost of TG4 is higher than other corn based syrups, and therefore

the functional benefits must outweigh the added expense, which in many cases will not be possible.

The Notifier, Hayashibara Co., Ltd., believes that the same market principles will govern the sale of TG4 Syrup in the US, thereby limiting its use in the US well below the calculated EDI presented in Part 3.

# Part 5. Experience Based on Common Use in Food Before 1958

The statutory basis for the conclusion of GRAS status is not through experience based on common use in food before 1958, but rather on scientific procedures.

That being said, it can be stated that corn (starch) syrups, of which TG4 is an example, have been safely consumed in the US since the early 1900s (Hobbs, 2009). USDA records of shipments of corn starch-derived products, both syrups and dried solids, show that from 1910 to 1957 there was approximately 70 billion lbs (32 billion kg; dw) of corn sweeteners shipped in the US. These corn sweetener products are essentially identical to approximately 44% of the molecules of TG4 Syrup that are without the trehalose moiety at the reducing end of the molecules. These are completely reduced to glucose and absorbed as such in the small intestine. Additionally, the glycosyltrehalose fraction (TG4 & TG3; 56%) is also metabolized in a similar manner and completely assimilated as glucose in the small intestine (see Part 6).

The trehalose itself is found in a host of plants and lower animals, which have been consumed by humans for thousands of years (Richards, et al., 2002). The most common foods in the human diet that include trehalose include honey, products of brewer's and baker's yeast, mushrooms, and shellfish. The historical use and published studies strongly support the substantial equivalency, and safety of TG4 Syrup (FDA, 2019(f); FDA, 2019(r); FDA, 2019(t); FDA, 2019(w); Richards, et al., 2002; FDA, 2000; LSRO, 1976; LSRO, 1975).

# Part 6. Narrative Basis for the Conclusion of GRAS Status

## 6.1 General Introduction

The safety and GRAS status of Maltosyltrehalose Syrup (TG4 Syrup) is discussed as required by 21 CFR 170.250 (FDA, 2019(g)). The Notifier has performed an extensive review of safety/toxicity studies on TG4 Syrup, TG4, maltosyltrehalose and other related substances in PubMed/TOXLINE through December 15, 2020, and to the best of the Notifier's knowledge has included all known positive and negative information and data.

The GRAS conclusion of the Notifier is based on "scientific procedures" using two lines of reasoning. The first and most direct are published safety-related studies of TG4 Syrup. The second is supporting published data on the constituent components, which are "substantially equivalent" to many other saccharides which are common in the human diet all over the world, and known to be safe for human consumption, and have been GRAS. A corollary to the second is the fact that the components of TG4 Syrup are composed of only glucose, which is GRAS. The second rationale will be presented first.

As explained in the introduction to Part 2 TG4 Syrup is composed of two similar, but not identical, types of saccharides. The main components are maltotetraose (G4) and maltosyltrehalose (TG4), which have the same empirical formula and molecular weight (Part 2.2).

G4 is a simple tetraglucose molecule with the glucose molecules being bound by α-1,4 linkages (Part 2.2.2). This linkage is the most common in starch, starch syrups and maltodextrin/ dextrin, and are quickly and completely digested to individual glucose units in the small intestine (Richards, et al., 2002; Ao, et al., 2007). G4 comprises about 15% of the total composition of TG4 Syrup (dry solid basis). TG4 Syrup also contains glucose (G1; 4.5%), maltose (G2; 7.1%) and maltotriose (G3; 9.8%), for a total of 36.3%. All these molecules have α-1,4 linkages, except G1 of course.

The second and most common type of substance in TG4 Syrup is TG4, for which the product is named. TG4 constitutes approximately 52.5% of the TG4 Syrup. It is composed of four glucose units in which the terminal reduction end of the G4 has been inverted (Part 2.2.2). This results in the final bond between the 3$^{rd}$ and 4$^{th}$ glucose molecule being an α-1,1 linkage, hence the G4 molecule becoming a TG4. The structure of the two end glucoses is the same as that of the disaccharide trehalose

(Richards, et al., 2002; FDA, 2000). The change in linkage places the 4 carbon on the terminal glucose exposed without a reducing end, which is much more stable and less susceptible to reaction with other substances in the final product. The chemical structure results in functional and technical properties which are less or more exaggerated than those of a conventional α-1,4 linked oligosaccharide found in starch (glucose) syrups. In addition to TG4, TG4 Syrup also contains TG3 molecules at about 3.5%, making a total of about 56% with terminal α-1,1 bonds. As with G4, TG4 is also completely digested in the small intestine (see below), as has been shown for the trehalose molecule.

Finally there are approximately 7.7% of other saccharides in TG4 Syrup. These include larger molecules, like those that are produced by most other starch syrup production processes (Hobbs, 2009). This fraction would also contain some molecules with or without a trehalose end moiety. These molecules are also digested to glucose in the small intestine. Together the three types of saccharides constitute 100% of the dry weight of the TG4 Syrup, and only include glucose molecules.

The Notifier, Hayashibara Co., Ltd., has concluded that the essentially exclusive content of glucose and the glycosidic linkages that are present strongly indicates that the innate composition and structure of TG4 Syrup is safe. Further it is substantially equivalent to straight chain (α-1,4) oligosaccharides, and to trehalose (α,α-1,1), which are already GRASed or GRAS Noticed, respectively (FDA, 2000; FDA, 2019(r)(t)(w)).

## 6.2 Digestion and Absorption

To support the conclusion that TG4 Syrup is safe for consumption by humans for the intended uses, the Notifier offers the following information and data. This section provides safety information from other starch-based substances that the Notifier claims are "substantially equivalence" to TG4. Although the Notifier has determined that TG4 could be considered GRAS because of its substantial equivalence to other GRAS products, the Notifier has undertaken a number of safety-based studies, including pivotal 90-day studies which demonstrate the specific safety and tolerance of TG4, and its equivalence to the other previously identified commercial products. The data from the TG4 studies include the intrinsic safety of TG4 (composition, structure), the safety of the production process, and the results of *in vitro* and *in vivo* studies (animal and human).

As shown in Part 2 of this document, sugars (DP1 & 2) and oligosaccharides (DP3 & greater) that have α-1,4 linkages constitute a major portion (36.3%; Table 2-12) of the components of TG4 syrup. Oligosaccharides and sugars are a major component of

starch syrup (glucose sirup, corn syrup, maltodextrin, dextrin, and similar substances listed as GRAS in 21CFR (Hobbs, 2009; FDA, 2019(f)(r)(t)(w)). In the USA the amount of sweeteners made from corn, and other minor starch sources, over the past 10 years has been about 8.5 billion kg/year (USDA, 2019). This is considered an overestimation because it includes substances that are manufactured, but may not be eaten. However, this does not include the tremendous amount of dextrin and maltodextrin that is used by the food industry. These are all approved existing food ingredients or additives with very long histories of safe use in various foods not just in the USA, but worldwide.

These glucose-based substances are usually digested by α-amylase and α-glucosidase in the alimentary tract to glucose and readily absorbed and metabolized by well-known metabolic pathways in humans (Richards, et al., 2002; Butterworth, et al., 2011; Dahlqvist, Borgstrom, 1961; Dhital, et al., 2013; Ravich, Bayless, 1983).

A conventional starch syrup, G4-rich syrup, is manufactured from starch by enzymatic hydrolysis, consists of oligosaccharides (mainly G4: ≥ 50% on dry basis), is commercially used in various foods as a conventional starch syrup in Japan, Korea, Thailand, and other countries, and is categorized a glucose syrup in the USA as a Glucose sirup (FDA, 2019(f)).

### 6.2.1 Unpublished Human Digestion and Absorption Study of TG4 Syrup

Scientists at Hayashibara Biochemical Laboratories, Inc. performed a human cross-over study to determine the plasma glucose and serum insulin concentrations after oral administration of TG4 Syrup (HBL, 2003a). Fifty (50) g (dry basis) of TG4 Syrup (54.8% TG4 on dry basis) or an equal amount of glucose control in 200 mL of warm water was orally administered to 9 healthy male subjects after an overnight fast (1 subject was excluded due to common cold-like symptoms before the second cross-over administration). This dose is approximately 0.73 g/kg-bw. Immediately before ingestion and at 0.25, 0.5, 1, 1.5 and 2 hours after administration, approximately 5 mL of blood was collected. Plasma glucose and serum insulin were measured at third party laboratories using the glucose oxidase method, and the enzyme immunoassay, respectively. The results are shown in Figures 6-1 and 6-2.

Plasma glucose rapidly increased after administration of TG4 Syrup and reached the maximum level (150 ± 28 mg/dL) at 0.5 hour. There were no significant differences between the TG4 Syrup and glucose on the C-maximum, T-maximum, kinetic profile, and incremental area under the curve (IAUC) of plasma glucose. The IAUC of plasma glucose concentration for 2 hours after administration of TG4 Syrup and glucose control were 48.8 ± 20.0 and 40.1 ± 14.8 mg/dL·hour, respectively, which were not

significantly different. Based on the data, the glycemic index (GI) of TG4 Syrup was calculated to be 124.2 ± 46.8 in comparison with the GI of glucose as 100.

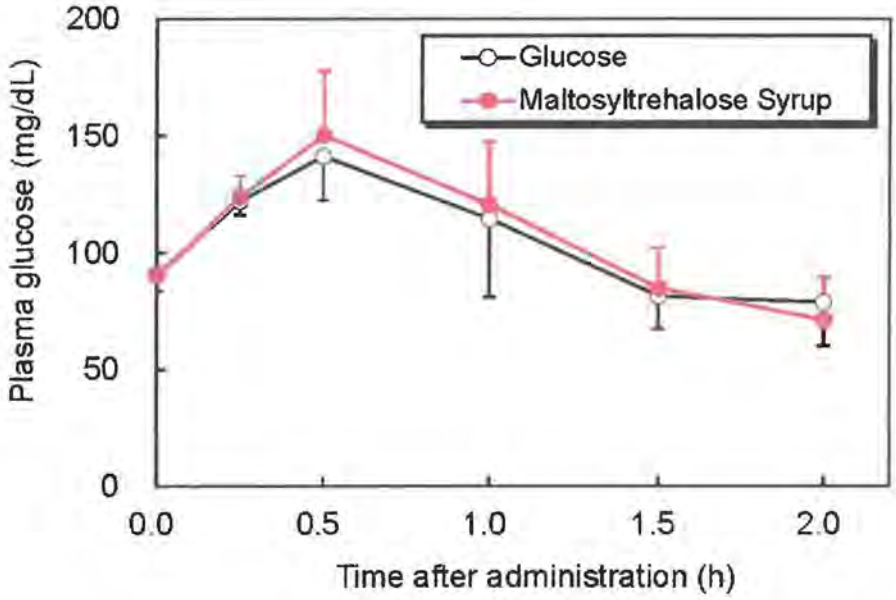



**Figure 6-1 Plasma Glucose after TG4 Syrup Ingestion**

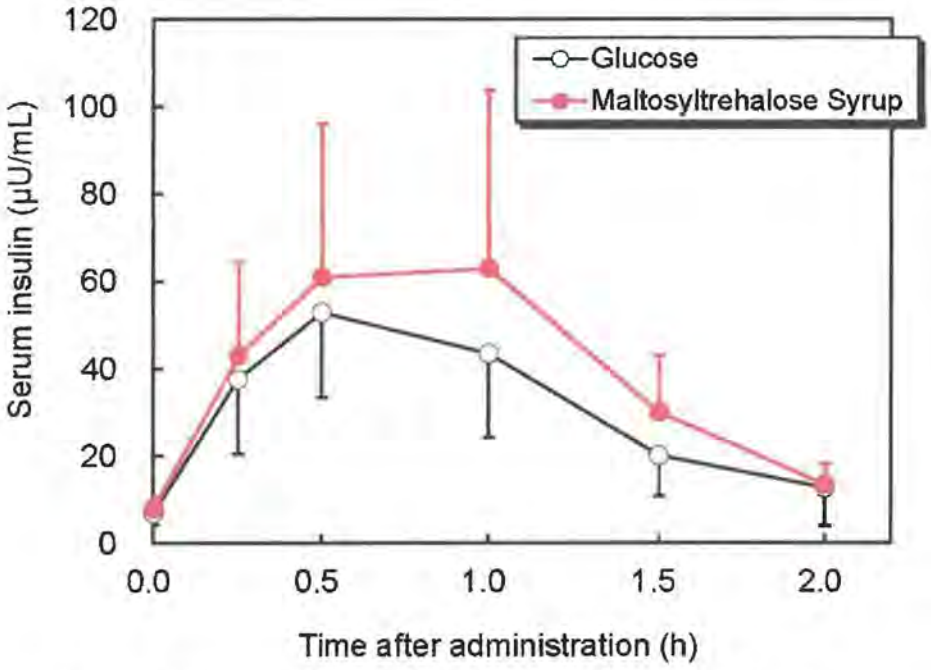



**Figure 6-2 Serum Insulin after TG4 Syrup Ingestion**

Serum insulin concentrations after administration of TG4 Syrup and glucose control

rapidly increased. The maximum concentrations of serum insulin of TG4 Syrup and glucose were 62.9 ± 40.8 (at 1.0 hour after administration), and 52.8 ± 19.4 μU/mL (at 0.5 hour after administration), respectively. Because of the similarities in the insulin values of the TG4 Syrup at the 0.5- and 1.0-hour measurements, it is possible that the actual peak of the serum insulin of the TG4 Syrup group was sometime between the two samples. The insulinogenic indexes of TG4 Syrup and glucose were calculated by the following formula to be 0.94 ± 0.50 and 0.99 ± 0.53, respectively. The two profiles were similar, and there were no significant differences between the administration of TG4 Syrup and glucose.

$$\text{Insulinogenic index} = \frac{\Delta AUC_{0\text{-}0.5h} \text{ of serum insulin concentration}}{\Delta AUC_{0\text{-}0.5h} \text{ of plasma glucose concentration}}$$

It was concluded that TG4 Syrup induces blood glucose and insulin concentrations to the same degree as glucose administration. These data demonstrate that TG4 Syrup is completely digested (enzymatically hydrolyzed) to glucose in the small intestines and absorbed into the circulation in a manner and with the same kinetics as glucose. Therefore, there appears to be no difference in how the human body digests and absorbs the glucose generated from the portion of TG4 Syrup that contains only α-1,4 bonds from that of saccharides containing the α,α-1,1 trehalose moiety. These data demonstrate that TG4 Syrup is "substantially equivalent" to the digestion and absorption of glucose, and by other common saccharides.

No adverse effects were reported by any of the subjects (HBL, 2003 (unpublished)).

### 6.2.2 Digestion and Absorption of Trehalose

As addressed in the preceding section, the digestion of glucose or TG4 Syrup with the trehalose moieties were shown to be similar. This subpart will address the issue of the mechanism of the effect digestion and absorption of trehalose-containing oligosaccharides (TG4 Syrup). When looking at the composition of TG4 Syrup, there are only approximately 20% of the total bonds that are the trehalose α,α-1,1 linkages, whereas the remaining 80% would be α-1,4 (the "other" 7.7% oligosaccharides were assumed to be an average of 13 glucose units in length and 50% of these molecules contained trehalose terminal units). The concern about digestion and absorption of trehalose is specifically addressed in GRAS Notice 000045 on trehalose, and in a review article on its properties, history of use, human tolerance and the results of multiple safety studies of trehalose (Richards, et al., 2002; FDA, 2000). The study reported above (Figure 6-1) also demonstrates the ability of humans to digest these trehalose-containing molecules. However, because of reports of the inability of a small percentage of some populations to digest trehalose this section is included. A more

complete discussion can be found in the publication by Richards *et al.* (Richards, et al., 2002).

Trehalose is composed of 2 glucose units bound in an α,α-1,1 configuration (Richards, et al., 2002; Birch, 1963; Elbein, 1974). In the review article (Richards et al., 2002) it is noted that trehalose, like most disaccharides cannot be directly absorbed into the body. Rather it must be enzymatically cleaved into 2 glucose molecules by the enzyme trehalase (EC 3.2.1.28). Trehalase is tightly bound to the enterocyte microvilli in the small intestine (Maestracci, 1976). The glucose formed is assimilated into the body by the exact same mechanisms as when glucose is consumed or glucose is produced by the hydrolysis of other common disaccharides (Dahlqvist, Borgstrom, 1961; Ravich, Bayless, 1983). While there have been a few reports of individuals that lack or have a reduced concentration of trehalase in the gut, these are quite uncommon (< 1%) and less than that observed for lactose (Richards, et al., 2002). The result of having a low or absent complement of trehalase is transient self-limiting laxation (osmotic loose stools), like that observed in lactose intolerance or excessive consumption of sugar alcohols (Richards, et al., 2002; Dahlqvist, Borgstrom, 1961). The only ethnic group that has been identified as having a high proportion of low or absent trehalase activity is the Greenlandic Inuit population. In one study of this ethnic group, of 97 individuals 14% had a low trehalase activity; however, over 60% had reduced lactase activity (Gudmand-Hoyer, et al., 1988).

### 6.2.3 Summary of Digestion and Absorption

TG4 Syrup consists of glycosyltrehaloses (mainly TG4), and oligosaccharides (mainly G4) with a relatively small amount of glucose, maltose, α-1,4 oligosaccharides and other trehalose moiety containing oligosaccharides.

This section demonstrates that in *in vivo* human studies sugars and oligosaccharides, as well as glycosyltrehaloses contained in TG4 Syrup are readily digested by the enzymes of the human digestive system. This includes trehalase, which hydrolyzes trehalose into two glucose molecules for absorption. Figures 6-3 and 6-4 provide a schematic representation of the digestion of TG4 (as a representative of glycosyltrehaloses) and G4 (as a representative of oligosaccharides).

The schematic shows that all the constituents of TG4 Syrup are digested to glucose, and is absorbed as glucose, supported by Figure 6-1 using standard pathways for all types of polyglucose molecules consumed in the diet (Richards, et al., 2002; Butterworth, et al., 2011; Dahlqvist, Borgstrom, 1961; Dhital, et al., 2013; Ravich, Bayless, 1983).

The Notifier has concluded that TG4 Syrup is of no safety risk to humans because TG4 Syrup is completely composed of D-glucose, and all TG4 Syrup components consumed are enzymatically hydrolyzed to glucose and assimilated into the human body as D-glucose. This is identical to all the other starch-based syrups, dextrin, and maltodextrin (except resistant carbohydrates that may only be partially digested) that have been consumed throughout the world for over 100 years (Hobbs, 2009).

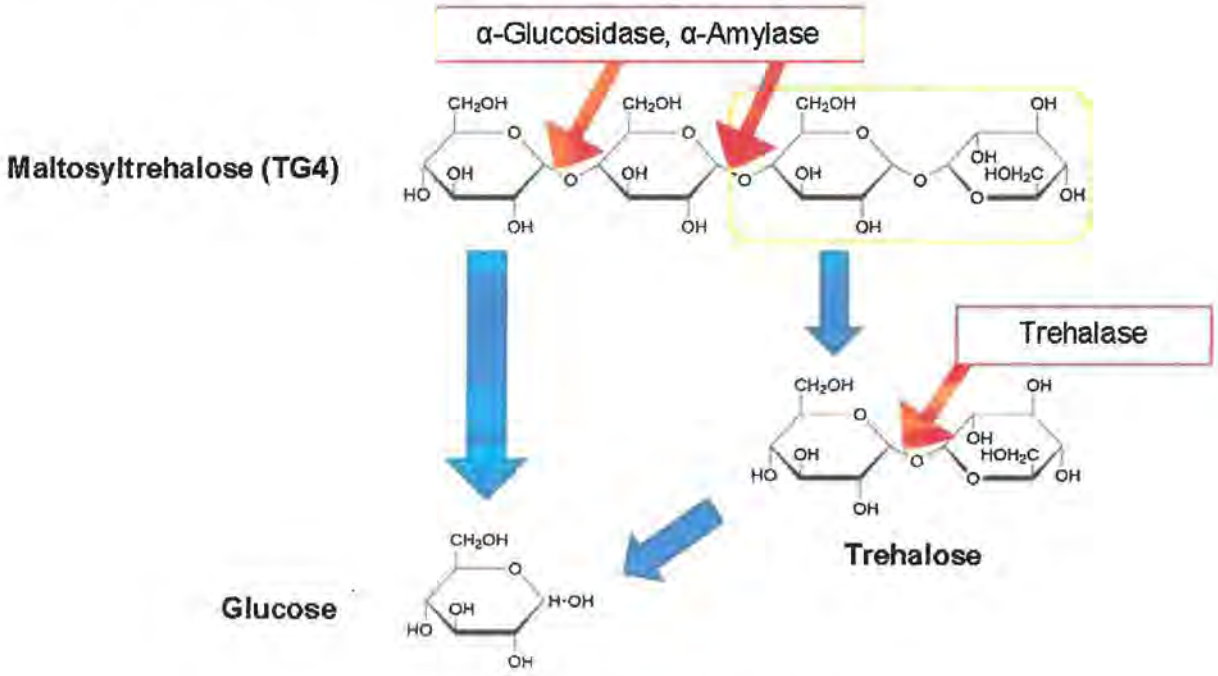



**Figure 6-3 Diagram of Digestion of TG4 in TG4 Syrup**

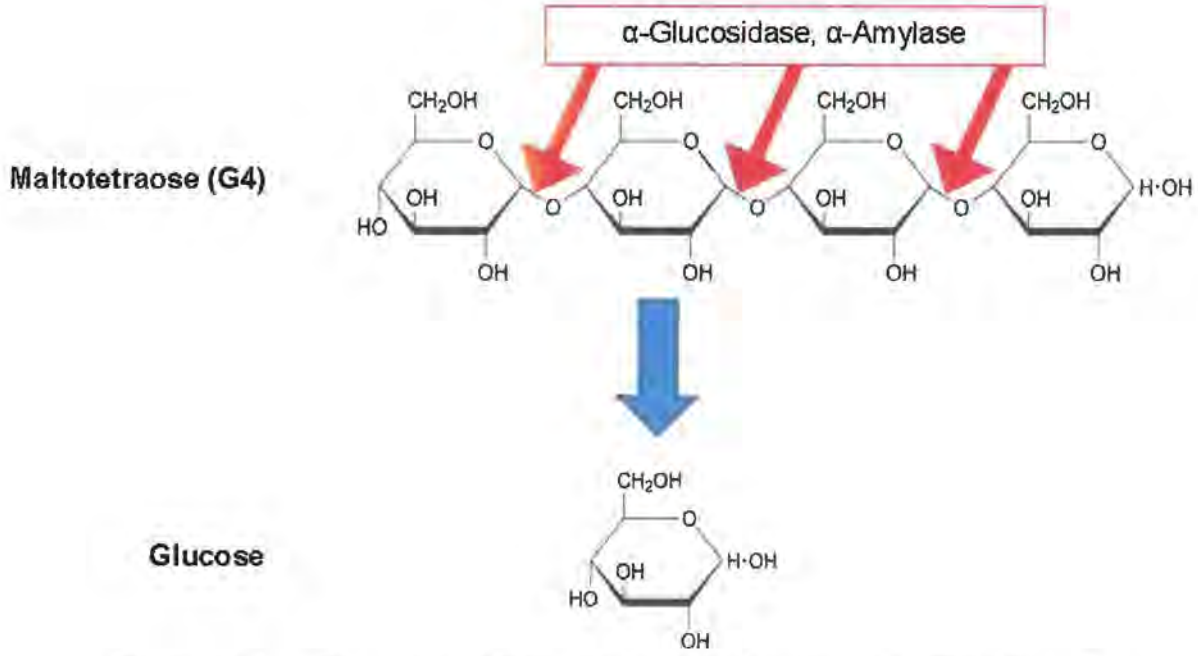



**Figure 6-4 Diagram of Digestion of Maltotetraose inTG4 Syrup**

## 6.3 Safety of Glucose Syrup, Corn Syrup, Maltodextrin, and Dextrin

As discussed in Parts 2 and 6.2, one of the component types of TG4 Syrup consists of glucose and other maltooligosaccharides. These are also components of glucose sirup, corn syrup (also known as glucose syrup), maltodextrin, and dextrin (FDA, 2019(f)(r)(t)(w)). In 21CFR §184.1(b)(1) it states that these substances can be used in foods with no limitation, other than current good manufacturing practice (FDA, 2019(m)). The one caveat is that in 21CFR 184.1(d) it does not allow for the additive use of "a combination of two or more ingredients listed in 21CFR184 to accomplish the same technological effect in any one food at a combined level greater than the highest level permitted for one of the ingredients". This is not an issue for this GRAS Notice because the intended use of TG4 Syrup is only as "a direct replacement for another starch-based syrup."

These food ingredients listed in 21 CFR 184 and 170 have long histories of safe use as existing food ingredients and/or additives in thousands of food products in the USA and worldwide. It is well established that maltose, and oligosaccharides are digested by α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) to glucose. These enzymes are found in the small intestine, and the glucose is absorbed into the body primarily by active transport and to a smaller portion by diffusion (Ravich, Bayless, 1983; Crane, 1960).

### 6.3.1 Safety of Glucose Syrup, Corn Syrup & Maltodextrin

Glucose syrup, corn syrup and maltodextrin are listed as GRAS substances in 21 CFR §168.120, §184.1865, and §184.1444, respectively (Hobbs, 2009; FDA, 2019(f)(r)(t)(w)). The basis for the GRAS determination was a report titled "Evaluation of the Health Aspects of Corn Sugar (Dextrose), Corn Syrup, and Invert Sugar as Food Ingredients" prepared for the FDA in 1976 by a Select Committee on GRAS Substances (SCOGS) of the Life Science Research Office (LSRO) of the Federation of American Societies for Experimental Biology (FDA, 1976). This was one report in a series of such evaluations that LSRO performed under contract to the FDA to review the safety of presumed GRAS substances or other prior sanctioned foods.

It should be noted that this LSRO (SCOGS) report is only a secondary source for the cited primary summarized reports. A number of these reports are not published in peer-reviewed journals. Many of the primary references could not be found. A few of the primary sources are in foreign language publications. In the references it states that the translations can be found in reference 1 of the LSRO report; however, the Notifier has attempted to find the original text with the cited references and translations upon which the LSRO report was based. Neither reference number 1 nor the original LSRO document could be found. Contact with LSRO resulted in a statement that the SCOGS files are no longer available.

The names 'glucose syrup' and 'corn syrup' have been used interchangeably over the years; however, glucose syrup can be produced from any number of other starch sources. If this is done, as with cornstarch, it can then more specifically be labeled 'corn syrup' or 'wheat', 'tapioca', 'potato' and so on syrups (FDA, 2019(f)). Alternatively all these can be called 'glucose syrup'. While 21 CFR provides no requirement for the production method of glucose syrup, only that it is obtained from edible starch, 21CFR does state that corn syrup is produced from partial hydrolysis of cornstarch using suitable acids or enzymes (FDA, 2019(f)(w)).

While maltodextrin is not listed on the title of the LSRO report, the report states that while it is not listed ".... in the identity standards for glucose syrup ...." it is manufactured by similar processes and represents nutritive saccharides obtained from starch resulting in a product having a "dextrose equivalent of less than 20." Further, "(T)he maltodextrins thus differ from glucose syrup in that the extent of the hydrolysis of starch is less. Because of their close relation to glucose syrup the Select Committee considers them as glucose syrup for the purpose of this report" (LSRO, 1976; Newton, 1970). As mentioned previously maltodextrin is also listed in 21 CFR as GRAS (FDA, 2019(t).

Corn sugar (dextrose) is simply crystallized D-glucose, and as noted all corn (glucose) syrup and maltodextrin is enzymatically converted to glucose and assimilated into the body (LSRO, 1976). Therefore the safety data on corn sugar reviewed in the LSRO report is applicable to the syrups and maltodextrins. As shown in Figures 6-1 and 6-2 TG4 is assimilated into the body as D-glucose (corn sugar or dextrose).

The following is a review of the safety studies used to establish the GRAS status of corn sugar (dextrose), corn syrup (including maltodextrin), and invert sugar. Because invert sugar is a mixture of glucose and fructose, and fructose is not found in TG4 Syrup it will not be mentioned unless it may be relevant to the safety of the study presented.

**Mutagenicity and Teratogenicity**

No mutagenicity or teratogenicity studies of dextrose were identified by the Select Committee (LSRO, 1976).

**Acute Toxicity**

"Chicks 18 to 20 hours old and weighing from 40 to 45 g were given 40 percent dextrose solution orally at a rate of 0.2 g of dextrose per hour (about 5 g per kg body weight per hour). The chick survived for 13 hours, with mortality being attributed to substantial water loss in the tissues due to high osmotic pressure of the treatment. In a second experiment an 8% dextrose solution was administered at 0.1 g per 30 minutes with no deaths occurring, and minimal water loss in the skin and muscles were noted (Kopfler, Wilkenson, 1963).

An $LD_{50}$ study was performed on rats given intravenous (IV) dextrose. The $LD_{50}$ was shown to be about 10 to 12 g/kg-bw. "Lethal doses produced changes in the liver, particularly in periportal hepatocytes. An $LD_{50}$ dose of glucose increased serum transaminase activity, sulfobromophthalein retention and serum concentrations aldolase pyruvic acid and lactic acid. The no-effect level on transaminase activity was about 2.5 g per kg (Orcell et al., 1970).

Studies on rabbits weighing about 2 kg showed that 30 g of dextrose did not produce any pathologic effects (Evans, 1933).

Charles River rats (142–190 g) were orally administered various doses of maltodextrin. The study reported that the $LD_{50}$ of the maltodextrin was greater than 34.6 kg/kg-bw (Industrial Bio-Test, 1969).

**Short-term Studies**

Rats were fed equicaloric diets of either dextrose or fructose. The amount approximated 50 g/kg-bw. Weight gain, feed efficiency, and average total glycogen and nitrogen content of the whole body was equivalent in the two groups. The total body fat content was greater for the dextrose group; however, "[N]o serious untoward effects were observed." (Bachmann et al., 1938).

Adult male rats were given a diet of about 81% carbohydrate (anhydrous dextrose, spray-dried liquid glucose, sucrose or fructose at about 40 g/kg-bw. Only the two former substances are report here) (Allen, Leahy, 1966). Control rats received a standard diet of 50% carbohydrate, mostly from starch. The spray-dried liquid glucose had a dextrose equivalence of 42.5 and contained 76.5% carbohydrate. It was a mixture of dextrose, maltose (G2), maltotriose (G3), maltotetraose (G4), and about 44% higher oligosaccharides (similar to a 42.5 dextrose equivalent commercial glucose syrup. The dextrose group had a lower weight gain than control; however, the glucose syrup group did not. Plasma cholesterol was significantly increased in the sucrose and fructose groups, and to a lesser extent in the dextrose group. The glucose syrup group showed no difference from control. Heart weight of the dextrose group increased, as did the sucrose and fructose groups, but not with the glucose syrup group. Liver protein was lower in all test groups when compared to controls, and the reductions were greatest for dextrose and sucrose.

Twenty (20)% dextrose solutions were given for 8 days to male Wistar rats with an average body weight of 217 g. It used the term "given exclusively" which appeared to mean without any other food source for the 8 days. After two days single ulcers developed in the upper part of the stomach. The ulcers increased in size and number until day 12. No intestinal ulcers were noted, and the ulcers healed rapidly when given a normal diet. The authors judged that the ulcers were caused by the gastric acid produced not needed for digestion, but cautioned that the upper stomach of rat was not similar to humans, so care was needed before any conclusions about this specific effect, as it might relate to humans was made (Rohm et al., 1964).

A 39-day feeding study, in which 9-week-old pigs were given a semi-purified diet of 50% carbohydrates, showed that dextrose at about 23 g/kg-bw provided sufficient weight gain and feed efficiency (Becker, Terrill, 1954).

Mature baboons were fed a diet containing 74% carbohydrates for 26 weeks. Animals fed liquid glucose showed no differences to those on a starch diet; whereas, a sucrose diet did result in some differences (Allen et al., 1966).
Thirty (30) pre-mature infants (humans) weighing between 1.42 and 2.08 kg were fed

glucose, sucrose or lactose. "The infants were initially fed dextrose at 4 to 6 hours of age at 0.5 g per kg body weight. Subsequently, the carbohydrates were fed as part of a soy isolate-based formula supplying 4.15 g of the carbohydrate per kg body weight per day, and were gradually increased to 11.5 to 12.2 g per kg body weight per day of life in 8 equal feedings." This feeding regimen was continued until the infants reached 5 pounds." No statistical differences were seen in percent weight loss, days to regain birth weight, weekly weight grains, or caloric efficiency. No adverse events were observed (Andrews, Cook, 1969).

**Special Studies**

A single dose (1 g; 5.5 g/kg-bw) of dextrose given to 180 g rats by gavage resulted in intense lymphopenia believed to be caused by an effect on ACTH. Treatment of weanling rats (1 g; 14 g/kg-bw) daily with dextrose for 14 days produced atrophy or the thymus. If adenine was given at the same time it suppressed both effects (Uebelin, 1953).

In two studies mice were given 30 to 35% dextrose solutions *ad libitum*. It was found that high intake of dextrose could increase the duration of barbiturate-induced sleep, and decreased the metabolism of these drugs (Peters, Strother, 1972; Strother et al., 1971).

Two studies on the carcinogenicity of dextrose were performed. In the first study daily subcutaneous injections of a 25% dextrose solution (2.5 to 5 g/kg-bw) were compared to a control group receiving physiologic saline in 5 to 6 month old rats. "Subcutaneous fusiform and polymorphous cellular sarcomata were observed in two of 55 animals. A third animal had a sarcoma in the abdominal cavity after 299 injections." (Cappellato, 1942). In the second study mice and rats were injected with 0.5 mL or 2.0 mL, respectively of 25% dextrose (5 g/kg-bw) two or three times per week for up to 2 years. No tumors were found at the injection site and no other signs of untoward effects were observed (Heuper, 1965).

A number of reports were reviewed regarding possible allergenicity to corn sugar and corn syrup. In one report, ingestion of corn sugar and corn syrup, and IV injection of corn sugar (dextrose) were reported to result in allergic symptoms in some individuals if they were highly allergic to corn (Randolph, Yeager, 1949; Randolph et al., 1950). In a "blindfold" study 25 subjects with histories' that suggested a corn allergy subjects were fed large amounts of starches of corn, tapioca and arrowroot, and corn sugar and corn syrup. A few cases of reaction to the starches were observed, but none to the corn sugar or syrup (Loveless, 1950). A third study showed no cases of sensitivity to ingested corn syrup in subjects that were sensitive to corn meal and cornstarch

(Bernton, 1952). A review concluded that there were no studies demonstrating that chemically pure glucose can act as a heptene or allergen in individuals that are sensitive to corn (Fisher, Carr, 1974).

In 4 studies on the action of fructose, a molecule of sucrose, as it might relate to the etiology of heart disease and diabetes, various animals were tested with glucose (dextrose). It should be noted that the untoward effects reported resulted from the consumption of fructose, which in not a constituent of TG4. White male rats (250 to 300 g) were fed a commercial diet supplemented with glucose or fructose as a 10% solution in the drinking water. The daily average intake was 8 g for glucose and 6 for fructose (about 30 and 20 g/kg-bw, respectively). Blood samples were collected at 2 and 4 weeks after start of treatment, at 2 hours after the start of fasting. Fructose caused a 160% increase whereas glucose caused only a 60% increase in plasma triglycerides. In another similar study with male and female rats glucose did not raise the serum triglycerides above the controls, while the fructose did after 6 and 19 days of treatment (Yudkin, 1964; Yudkin, 1968; Nikkila, Ojala, 1965; Bar-On, Stein, 1968).

A fat-free diet containing 7 g carbohydrate per kg-bw and 50 g calcium caseinate daily for 5 days was fed to men, and pre- and post-menopausal women. The carbohydrate was starch, 40 dextrose:60 starch; 40 fructose:60 starch; or 40 fructose:60 dextrose. Fasting serum lipid values were taken on the last two days, and was highest in the fructose/dextrose diet (61% in men; 120% in postmenopausal women), and least by dextrose/starch (-19% men and 27% in postmenopausal women). Serum lipids were decreased in premenopausal women in both fructose-containing groups (MacDonald, 1967). It has been shown that serum lipid values are determined by the sex of the person consuming, the type of protein and frequency of intake (MacDonald, 1972). Another report stated that measuring serum lipid after an overnight fast tends to overestimate the effects of carbohydrates in the diet (Aherns, 1974). As with the preceding paragraph the greatest effects appear to be related to fructose, and not to glucose-based substances.

The final group of studies were related to the effect of sugars, starches and foods on cariogenic activity. In one human study of adults, it appears that it is the frequent or excessive amount of confections consumed during and between meals that related to dental caries; whereas in pre-school and school aged children it is more the quantity of sugar consumed between meals (Stephan, 1966; Zita et al., 1959; Trithart, Weiss, 1957). A 5-year study of adult hospital patients on a restricted sugar diet showed that an increase in caries occurred when subjects were given a small portion of sugar between meals (Gustafsson et al., 1954). In brief, a number of studies using rats and hamsters showed that sucrose was more cariogenic than glucose, while other studies in rats have shown that there was no significant difference, and was dependent on the

strain of rats, or microflora of the mouth and strain (Green, Hartles, 1969; Grenby, Hutchinson, 1969; Frostell et al., 1967; Stephan, 1966). Carcinogenicity of fructose appears to be similar to glucose. There is evidence that more complex carbohydrates and natural foods promote caries activity in animal diets; however, sucrose was the most cariogenic (Stephan, 1966).

**Conclusion of LSRO Select Committee**

The conclusion of the Select Committee for corn sugar, and corn syrup is quoted below. This may include comments about sucrose and fructose; however, these are not thought relevant to the current Notification because the notified substance does not contain fructose, which appears to be metabolized and result in some different reactions in the body. It was also GRASed as based on the current LSRO report (LSRO, 1976). It should be remembered that maltodextrin was also included with corn syrups.

The Select Committee has weighed all of the foregoing and concludes that:

> "Other than the contribution made to dental caries, there is no evidence in the available information on corn sugar (dextrose) corn syrup, and invert sugar that demonstrates a hazard to the public when they are used at levels that are now current and in the manner now practiced. However, it is not possible to determine without additional data [sic] whether an increase in consumption - that would result if there were a significant increase in the total of corn sugar, corn syrup, invert sugar and sucrose added to foods - would constitute a dietary hazard.

**GRAS Conclusion**

It is the opinion of the Notifier that the data in this Part of the GRAS Notice supports the safety of the Notified substance, Maltosyltrehalose Syrup (TG4 Syrup), as GRAS. While, as the LSRO report concludes, the consumption of corn-based sweeteners may expand from 1976, most of that has been in the doubling of the per capita consumption of high fructose corn syrup, which is not felt to be directly related to the safety of non-fructose starch-based syrup. In 1976 the per capita amount of corn-based sweeteners shipped in the USA was 25.2 lbs (11.4 kg). Of that 7.2, 13.9 and 4.1 lbs were of HFCS, corn (glucose) syrup and dextrose (corn sugar), respectively (USDA, 2019). In 2016 the per capita consumption of these sweeteners increased to 56.5 lbs (25.6 kg); however, this included 41.4 HFCS, 12.4 glucose syrup, and 2.7 lbs dextrose. This demonstrates that the per capita consumption of substances substantially equivalent to TG4 Syrup is less than when the LSRO report was written.

As mentioned above and in Part 4, TG4 Syrup would not likely be substituted for HFCS, because one of the primary technical properties of HFCS is its greater sweetness than sucrose or glucose. In Table 2-23 it showed that TG4 Syrup has approximately 27% of the sweetness of a 5% sucrose solution. HFCS at 42, 55 and 90% fructose concentrations have sweetness' compared to sucrose of 100%, 100-110%, and 120-160%, respectively.

### 6.3.2 Safety of Dextrin and Corn Dextrin

The GRAS citation for Dextrin is found at 21 CFR 184.1277 (FDA, 2019(r)). It has also been evaluated by a Select Committee on GRAS Substances of the LSRO in 1975 (FDA, LSRO. 1975). The classification of dextrin and maltodextrin in the USA is not clear-cut; however, as noted maltodextrin was included in the other LSRO report reviewed above. The LSRO report defines dextrin as polymers of α-D-glucose, and are prepared from various starches, which contain anhydroglucose units that are mainly linked through the 1,4 carbons. As mentioned previously this makes the molecules essentially and entirely digestible in the small intestine. However, other linkages may be formed depending on the type of starch used and the method of preparation (FDA, LSRO. 1975). In many instances the production method produces bonds that cannot be digested in the small intestine (dietary fibers) but rather are fermented in the colon by resident bacteria. The definition used in §184.1277 of 21 CFR is that "Dextrin ($(C_6H_{10}O_5)_n \cdot H_2O$, CAS RN® 9004-53-9) is an incompletely hydrolyzed starch (FDA, 2019(r)). It is prepared by dry heating corn, waxy maize, waxy milo, potato, arrowroot, wheat, rice, tapioca, or sago starches, or by dry heating the starches after: (1) Treatment with safe and suitable alkalis, acids, or pH control agents and (2) drying the acid or alkali treated starch. More specific names used for dextrin manufactured by this process are pyrodextrin, pyrofaction and torrefraction dextrin (FDA, LSRO. 1975).

The LSRO Select Committee recognized that the term "dextrin" has been applied to a wide variety of products that are produced by enzymatic or acid hydrolysis, or a combination of both. Therefore the LSRO report on dextrin and corn dextrin does not include any products identified in the literature as dextrin that was not produced by the process in §184.1277 (FDA, 2019(r)). The LSRO report regarded the dextrin produced by enzymes and/or acid hydrolysis as "maltodextrin" and considers them as "corn syrup", as mentioned in Part 6.4.1.

Although products such as TG4 Syrup are therefore considered a type of maltodextrin, because the structure and composition of dextrin is substantially equivalent to TG4 Syrup, the conclusion of the Select Committee will be provided below:

"The Select Committee concludes that:

There is no evidence in the available information on dextrin and corn dextrin that demonstrates or suggests reasonable grounds to suspect, a hazard to the public when they are used at levels that are now current or that might be reasonably expected in the future."

**GRAS Conclusion**

The LSRO report concluded that dextrin manufactured by dry heating or roasting unmodified starch with or without acid or alkaline catalyst were GRAS. The Notifier has concluded from years of experience as an active and internationally recognized manufacturer of starch hydrolysate products that TG4 Syrup has structure and composition substantially similar to dextrin, which is GRAS.

### 6.3.3 Safety of Trehalose

As noted several times previously, the major constituents of TG4 Syrup are glycosyltrehaloses. These are glucose chains bound by α-1,4 linkages, except that the two terminal reducing end glucoses are bond by an α-1,1 linkage. Figure 6-3 provides a schematic of the hydrolysis (digestion) of these units. As shown in Figure 6-1, all TG4 Syrup consumed by humans resulted in digestion of all molecules to glucose, even though there are trehalose moieties at the reducing end of approximately 56% of the molecules. As shown in Figure 6-3 the trehalose moiety is released from the glucose chain and the further digested by trehalase (EC 3.2.1.28) in the intestine to glucose, which is then absorbed into the body. Therefore, the safety of TG4 Syrup is closely related to both non-resistant starch hydrolysates and to trehalose.

Trehalose occurs in bacteria, yeast (e.g., *S. cerevisiae*), a wide variety of fungi, a few higher plants, and many species of insects (Birch, 1963; Elbein, 1974). Through foods related to the above organisms, humans have consumed trehalose since ancient times.

Various safety studies have been performed on purified trehalose as shown in Table 6-1. These studies were the basis upon which trehalose, submitted by the Notifier of this TG4 GRN, was evaluated as a GRAS substance by a GRAS Panel under its intended uses in foods, and was subsequently notified to the FDA (Richards, et al., 2002). The Agency issued a "no question letter" (GRN 00045) in 2000. Further, trehalose has been evaluated by a number of international and national agencies and declared to be safe. The 55$^{th}$ Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated the trehalose safety as a miscellaneous food additive and allocated an "ADI not specified". In Japan, trehalose is listed in the List of Existing Food Additives. According to the EU, Canadian, and Australia/New Zealand (FSANZ) food authorities trehalose is approved as a novel food/novel food ingredient. The regulatory status of

trehalose for food use in various countries is shown in Table 6-2. Trehalose is allowed for use in food in essentially all countries in the world. In addition to the common classification as a food/novel food/food ingredient in most countries, trehalose manufactured by the Notifier (TREHA$^{TM}$), is registered as a food additive, raw material, and food ingredient by importers in Thailand, Philippines and Vietnam, respectively.

Since the "no questions" Agency response letter to the trehalose GRAS 00045 Notice in 2000, an additional paper has been published on the toxicity of trehalose (Liu, et al., 2013). This publication demonstrated that trehalose showed no genotoxicity, male reproductive toxicity, and general toxicity in mice (Liu, et al., 2013). In brief the Liu et al. study reported on a bone marrow micronucleus test, and sperm abnormality test in which 5 groups of 10 randomly selected male mice were intragastrically administered with one of the following substances: 1.25, 2.5, or 5.0 g trehalose/kg body weight; 40 mg cyclophosphamide (CP) as a positive control; or distilled water as a negative control. In the micronucleus study the animals were treated for 3 consecutive days with trehalose, the negative control or positive control. On the 4$^{th}$ day the animals were sacrificed and examined. For the sperm abnormality test the mice were given the same doses of each treatment for 5 days, and then examined on the 36$^{th}$ day. The final study using 10 males mice per group was a 40-day gavage toxicity study using a distilled water control, and, as with the genotoxicity studies, 1.25, 2.5, or 5.0 g trehalose/kg body weight. Both the micronucleus and male reproductive studies showed no significant effects ($p < 0.05$) of the trehalose at any concentration as compared to the control; however, the positive CP control had a significant effect ($p < 0.05$) in both study in comparison to the negative and trehalose treatment groups. The 40-day toxicity study examined hematology, including WBC, RBC, HGB, PLT, MCV, red cell volume distribution, lymphocytes, lymphocyte ratio, platelet distribution width, and neutrophils. The paper mentions clinical biochemistry but no mention is made of it in the results or discussion. Additionally daily clinical evaluations were performed, with a gross pathologic examination and ophthalmological examinations were made at necropsy between control and the high dose groups; organ weights and relative weights were reported; and body weight, and food consumption and feed efficiency were calculated every 4$^{th}$ day. The authors stated that there were no treatment-associated effects on the hematologic variables, or the relative organ weights. No treatment-related adverse events were in clinical or ophthalmological variables were noted. Finally, no statistically significant ($p < 0.05$) differences were observed in body weight, feed consumption, and feed efficiency. The authors concluded that trehalose is safe for use in the food industry, and the NOAEL was 5 g/kg bw/day for 40 days (Liu, et al., 2013). The NOAEL for trehalose from the publication by Richards and coworkers was 9.3 g/kg bw/day for females and 7.3 g/kg bw/day for male rats (Richards, et al., 2002).

The Notifier has sold trehalose in Japan and Taiwan since 1995, and the USA and most countries of the world beginning in the early 2000s. Despite the long history Notifier is unaware of any reports as to trehalose intolerance from food products. Also, trehalose has been sold in the USA without any reported laxation (osmotic intolerance), therefore it is not expected that the use of TG4 Syrup would cause these types of untoward events.

**Table 6-1 Safety Studies of Trehalose for Food Use**

| Study | Test Subject | Administration Route & Procedure | Dose | Result |
|---|---|---|---|---|
| **Acute Oral Toxicity** | Rat | Oral, Single dose | 16.0 g/kg | Acute Lethal Oral Dose: > 16 g/kg |
| **Subchronic 13-Week Oral Toxicity** | Mouse | Oral feeding, 13 weeks | 5,000, 15,000, 50,000 ppm | No-Toxic-Effect-Level: 50,000 ppm |
| **Mutagenicity** Bacterial Mutation Assay | *Salmonella typhimurium* (TA1535, TA1537, TA98, and TA100), *Eschelichia coli* (WP2 uvrA) | Direct method | ≤ 5,000 µg/plate | No evidence of mutagenic activity |
| | | Metabolic activation method | | |
| **Mutagenicity** Chromosome Aberration Assay | Chinese Hamster Ovary (CHO) cell | Direct method | ≤ 5,000 µg/mL | No evidence of mutagenic activity |
| | | Metabolic activation method | | |
| **Mutagenicity** Micronucleus Assay | Mouse | *i.p.*, approximately 24 or 48 hours | ≤ 5,000 mg/kg | Meets the criteria for a negative response (not mutagenic) |
| **Embryotoxicity / Teratogenicity** | Rabbit | Oral feeding, 29 days | 2.5, 5, 10% | Does not induce maternal nor developmental toxicity |
| | Rat | Oral feeding, 21 days | 2.5, 5, 10% | Does not induce maternal nor developmental toxicity |
| **Two-Generation Reproduction** | Rat | Oral feeding, two successive generations | 2.5, 5, 10% | NOAEL (g/kg-bw/day):<br>7.09 (males premating)<br>7.61 (females premating)<br>6.16 (gestation)<br>14.09 (lactation) |

**Table 6-2 Regulatory Status of Trehalose for Food Use**

| International Agency / Country / Area | Regulatory status |
|---|---|
| JECFA | ADI "not specified" as a miscellaneous food additive. |
| CAC | Adopted the specifications established by JECFA.<br>No INS number allocated because of a disaccharide. |
| Japan | Listed in the List of Existing Food Additives. |
| USA | Evaluated as GRAS (FDA notified, GRN 000045).<br>Monographed in Food Chemical Codex. |
| Canada | Approved as a novel food. |
| Brazil | Listed in the New Ingredients List. |
| EU | Approved as a novel food/novel food ingredient. |
| China | Approved as a novel food and changed the categorization to a general food. |
| Korea | Listed as a raw material used in food products. |
| Taiwan | Listed in the Food Ingredient List. |
| Thailand | Listed in the Food Additives List of Thai FDA. |
| Singapore | Listed as a permitted general purpose food additive. |
| Indonesia | Approved as a food ingredient. |
| Malaysia | Approved as a sweetening substance. |
| India | Approved as a food ingredient. |
| Australia / New Zealand | Gazetted as a novel food (FSANZ No. FCS 9). |

**Possible Inconsistent data**

Because the Notifer is including data on trehalose to support the safety of TG4 Syrup, the following published information is discussed as a requirement given in 21 CFR 170.250 Part 6(c)(1) regarding data that might be inconsistent with the conclusion of GRAS. This is the only data that is known by the Notifier.

In 2018, Collins and workers published an article linking the consumption of trehalose with *Clostridium difficile* infection (CDI) epidemics by two strains (ribotypes) with

enhanced virulence in the USA (2000-2003) and other locations around the world (Collins, et al., 2018). An additional paper was published by the same group identifying a third strain (ribotype; Collins, et al., 2019). A third paper essentially discusses the Collins et al. data without adding additional data (Abt, 2018). Conversely there have been three publications that discount the proposed role of trehalose in these CDI epidemics (Abbasi, 2018; Eyre, et al., 2019; Saud, et al., 2020). One basis for the claim of an association is the juxtaposition of trehalose regulatory approvals with disease outbreaks and trehalose metabolizing genes in the specific *C. difficile* strains (Collins, et al., 2018). On closer review the correlation of trehalose regulatory approvals with consumption is a misunderstanding by the authors (i.e., regulatory approval equals consumption). Consumption of trehalose added to the diet in the USA, UK, German and the EU at the time of the *C. difficile* outbreaks was less than 1 g/capita/year and less than 9 g/capita/year during 2000–2006 and 2007–2012, respectively; whereas the dietary consumption of trehalose from natural foods was about 100 g/capita/year (Eyre, et al., 2019). This is supported by the report of Abbasi, which states that the association of trehalose added to the diet and the cause of CDIs is not resolved (Abbasi, 2018). Further, the current Notifier was the company that had obtained all the regulatory approvals for trehalose in the world, and was the only company selling trehalose during that time. The sales of trehalose in Japan starting in 1995 and were substantial for 5 years before the US GRAS and 6 years before the Novel Food approval in the EU. Further, the incidence of CDI in England and the USA shows a rise peaking in mid-2002s followed by a fall in the UK and stabilization in the USA (Eyre, et al., 2019). Abbasi also states that even at the time of the Collins et al. publication, according to recent surveillance by other workers, new ribotypes are being identified in growing numbers than the ones reported in their publication (Abbasi, 2018). No *C. difficile* epidemics were recorded in Japan during this time. The Canadian *C. difficile* outbreak in over 30 hospitals in late 2002 as noted in the Collins et al. paper occurred 2-3 years before sales of trehalose were allowed in Canada. A number of other countries having outbreaks of *C. difficile* identified in the Collins et al. publication occurred well before approval and sales into these countries (Collins, et al., 2018).

A mouse model was used by Collins et al.; however, the animals were fed various antibiotics, which would have greatly changed the microbiome of the gut, and no other glucose containing carbohydrates were used as a control carbon source (Collins, et al., 2018). However, the mice experiments showed i) that small amounts of trehalose can be found in the mouse large intestine, and ii) there was increased virulence of the two highly virulent ribotypes of *C. difficile* when trehalose was fed to the mice (Collins, et al., 2018). Mice, like humans have trehalase in small intestine. This would reduce essentially all the trehalose to glucose, which is absorbed through the small intestine, leaving at most minute amounts to enter the large intestine (Richards, et al., 2002).

Although it is not impossible for a very small amount of a trehalose to cause changes in the make-up of the gut microbiome, without other gut microbes competing for the trehalose, the conclusion drawn is suspect. The Eyre group used a validated and clinically relevant *in vitro* gut model of CDI using clindamycin with the suspect ribotype (Eyre, et al., 2019). Toxin production was observed in the glucose and saline groups at day 40, but not in the trehalose group. Trehalose did not increase the concentration of *C. difficile* or spores differently than the glucose or saline groups. Finally other important microbial populations recovered faster in the trehalose and glucose groups as compared to saline. (Eyre, et al., 2018).

The Collins et al. paper had a large amount of molecular data using gene recombination and deletions (Collins, et al., 2018). The theory is that there are two or three strains of *C. difficile* that have evolved novel genes to metabolize low concentrations of trehalose. One ribotype has a single point mutation, while the second had a four gene-cluster. These are unique to the ribotypes. Eyre and coworkers reviewed 5,232 whole genomes of *C. difficile* to investigate the correlation of these two metabolic systems to clinical isolates. It was noted that both of these trehalose metabolic mutations were identified among a wide range of differing clinically important *C. difficile* "clades" (monophyletic groups) and not simple the ribotypes described by Collins and coworkers (Eyre, et al., 2018). The four-gene mutation Further that some isolates (sequence types) have both types of trehalose metabolic mutations, without being clinically important. Further, many isolates with the four-genes are not clinically relevant. Finally, it was concluded that from sequence analysis of thousands of *C. difficile* sequence types and evolution rates of *C. difficile*, suggest that these genes have been stably present for many years, and well before the introduction of added dietary trehalose (Eyre, et al., 2018).

Eyre and coworkers examined the presence or absence of the four-genes in closely related strains isolated from CDI patients. They adjusted the data for sex, age the sequence type. No evidence was observed of any association between 30-day all-cause mortality and the presence of the four-genes (Eyre, et al., 2018). Supporting this conclusion is a study in which a number of CDI cases were reviewed in which 898 patients with severe outcomes and complete clinical information (Saund, et al., 2020). The end points were clinical outcomes (admission to an intensive care unit; intro-abdominal surgery; or death) within 30 days of diagnosis of CDI. Ribotyping was done with 137 ribotypes identified. In this study no statistically significant evidence of an association between severe *C. difficile* outcomes and the presence of trehalose metabolic ribotypes were noted. The authors concluded that their results do not support the conclusions of Collins (Saund, et al., 2020).

While the above studies are directly related to the consumption of trehalose, the safety

of trehalose is felt to be relevant to TG4 Syrup because the Notifier considers trehalose to have 'substantial equivalence'. Taken together these data suggests that the assertion that the dietary addition of trehalose results in a specific increase in highly virulent *C. difficile* is not supported by the actual timing, the amounts of the consumption of added trehalose, the ribotypes proposed to be involved, or data from clinical patient studies.

**GRAS Conclusion**

The Notifier had determined that the fraction of TG4 Syrup that includes trehalose is hydrolyzed in the small intestine during human consumption to glucose, and absorbed and metabolized in an identical process as glucose obtained from any other of the common glucose sources consumed by humans. Therefore it is concluded that the trehalose portion of TG4 Syrup is GRAS by its substantial equivalence to consumption of trehalose as reviewed in GRN 00045 (FDA, 2000).

## 6.4 Safety Studies on TG4 Syrup

As required in 21 CFR 170.250 Part 6 a comprehensive literature search was made using PubMed/TOXLINE including the terms "maltosyltrehaose" "maltosyltrehalose syrup", "glycosyltrehalose", "safety" and "toxicity". No publications appeared to discuss safety or toxicity of TG4 Syrup (TG4) other than the publication summarized below (Matsumoto et al., 2020). In addition the Notifier researched any additional safety studies related to "trehalose", which as stated previously, is closely related to maltosyltrehalose. There are a few related publications, which are summarized above in Part 6.3.3.

In considering the general safety of TG4 Syrup it should be kept in mind that the source ingredient is food grade starch. This same ingredient is used for the production and consumption of hundreds of millions of metric tons of various starch-based syrups around the world. The enzymatic production process is also essentially identical to that used to produce a large proportion of these starch-based syrups. Further, the only constituent in the starch and subsequently in the TG4 Syrup is glucose, which is primarily formed from the most common and digestible glycosidic linkage found in nature (1->4), and the α,α-1,1 linkage of trehalose. The trehalose linkage and molecule is not uncommon in the natural human diet, and has been concluded to be GRAS in a Notice to the US FDA (FDA, 2000). Although the Notifier believes that the constituent molecules of TG4 fall under a number of GRAS classifications, because the specific structure of the glycosyltrehalose fraction has not be consumed in large quantities by a US population, the Notifier is submitting this GRAS Notice with specific safety data

related to TG4 Syrup. This section therefore includes the summary of published *in vitro*, and animal studies, while the next section provides unpublished *in vitro*, animal and human study information and data regarding both TG4 Syrup as well as other substances that contain TG4 molecules. *In vivo* data provided in Part 6.2.1 demonstrate that both oligosaccharides (mainly G4 and G3) and the glycosyltrehaloses (mainly TG4) are digested to glucose and trehalose molecules. Taken together the Notifier has concluded that Maltosyltrehaose Syrup (TG4 Syrup) qualifies for GRAS status.

### 6.4.1 Published Mutagenicity -- Ames Test

In accordance with Guidelines under the Japanese Industrial Safety and Health Law (1988 and revised in 1997), TG4 Syrup (54.8% TG4 on dry basis) was examined for mutagenicity in a standardized bacterial assay by the pre-incubation method using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2 *uvrA* in the presence and absence of S9 mix (Matsumoto et al., 2020). The first experiment (dose-finding test) was the plate incorporation assay and the second study (main test) was a pre-incubation assay. Six (6) concentrations (5–5,000 µg/plate at a common ratio of 4), and 5 concentrations (313–5,000 µg/plate at a common ratio of 2) of TG4 Syrup diluted in sterilized distilled water were used for the dose-finding and main tests, respectively. In both tests the number of revertant colonies induced by TG4 Syrup, even at 5,000 µg/plate, was less than twice that in the corresponding control value for all test strains, both in the presence and absence of the S9 mix. No microbial growth inhibition, or test substance precipitation was observed. It was concluded that TG4 Syrup is not mutagenic under the test conditions employed (Matsumoto et al., 2020).

### 6.4.2 Published Preliminary 90-day Oral Feeding Study

A 90-day oral feeding study with rats, modified from OECD #408, using only a control and high dose, was performed by an independent third-party laboratory using a pilot lot of TG4 Syrup (Matsumoto, et al., 2020; Appendix B). Rats were given TG4 Syrup (52.1% TG4 dry basis) mixed in feed at a 10% concentration (dry basis), which is equivalent to a mean consumption of 7,141 ± 1,165 mg/kg-bw/day for 91 consecutive days. All animals survived during the study. No significant differences were noted in the FOB, hematologic parameters, and ocular changes due to TG4 Syrup consumption. At necropsy some gross findings were noted but were determined sporadic, not seen in both sexes, and not treatment associated. These findings were not noted in the pivotal 90-day study (6.4.3). Further, no treatment-related changes were revealed by microscopic examination. Though female animals consumed significantly more of the test diet during some study weeks, and had a significantly greater mean body weight

measurement at Day 36, all effects were transient and did not persist to the end of the study. At study termination, the blood sodium concentration in male animals was significantly less in the treated group than controls. This was considered biologically insignificant given the lack of corroborating histological and related chemical data. Summary data provided in Appendix B.

Based on the findings, it was concluded that the no observed adverse effect level (NOAEL) was 10% TG4 Syrup (dry basis) which is equal to 6,818 and 7,464 mg/kg-bw/day in male and female rats, respectively. This would equal 409 and 448 g/day for 91 days in human males and females weighing 60 kg.

### 6.4.3 Published 90-day Gavage Repeated Oral Dose Toxicity Study

A 90-day gavage feeding study was performed on rats using multiple doses of a commercial lot of TG4 Syrup (53.9% TG4 dry basis) to evaluate the repeated oral dose toxicity of TG4 Syrup according to OECD #408 (Matsumoto, et al., 2020; Appendix B). TG4 Syrup (doses: 0, 1,000, 3,000 or 5,000 mg/kg-bw/day dry basis) was administered daily by gavage for 90-consecutive days. None of the animals died during the study. There were no treatment-related findings in any of the dose groups in either sex.

Trauma to the neck was observed from Day 15 to 21 in one male in the 1,000 mg/kg-bw/day group, and from Day 57 to 81 in one female in the 3000 mg/kg-bw/day group. However, this was judged by the investigators not to be attributed to the test substance, because it only occurred in two individual animals with no dose effect.

There were no noteworthy abnormalities on each experimental day in regard to response to removal from cage, condition during hand-held observation, behavior in open-field or sensorimotor reactivity. Furthermore, there were no significant differences in any of the quantitative data between the control group and each of the test substance-treated groups.

There were no significant differences in the body weights of the animals of either sex between the control group and each of the test substance-treated groups during the administration period. Moreover, there were no significant differences between the control group and each of the test substance-treated groups in the overall body weight gains from Day 1 to 90.

In males, the mean daily food consumption from Day 36 to 43 and from Day 50 to 90 of the 5,000 mg/kg/day group were significantly lower ($p < 0.05$) than those in the control group. In the females, the mean daily food consumption from Day 8 to 15 and from Day 22 to 29 of the 5,000 mg/kg/day group were significantly lower than those in the

control group.

The MCV in the males of the 5,000 mg/kg/day group was significantly higher than that in the control group. In females, there were no significant differences in any variables between the control group and each test substance-treated group. The MCV values were not thought to be clinically significant in spite of it being observed in the highest dose group because no other related hematologic changes were observed, and the mean value was within the mean value ± 2SD (based on 19 studies at the testing laboratory) for these animals.

Examination of the clinical chemistry values showed that the AST and ALT values of females in the 3,000 and 5,000 mg/kg/day groups were lower than those in the control group. However, these were very minor changes, and decreases in enzyme activity are not considered to be toxicologically significant. The mean values were within the normal range observed for this strain. In males, there were no significant differences in any parameters between the control group and each test substance-treated group.

The number of animals with protein-positive urine (more than 30 mg/dL) was increased slightly in the males of the 3,000 and 5,000 mg/kg/day groups, and in the females of 5,000 mg/kg/day group. The number of males with ketone body-positive urine was increased slightly in the 3,000 mg/kg/day group. Moreover, the number of animals with urobilinogen-positive urine was increased slightly in the males of the 3,000 mg/kg/day group, and in the females of 5,000 mg/kg/day group. However, these changes were considered to be incidental because there was no dose-dependent relationship.

There were no ophthalmological findings related to test substance-treatment in the 5,000 mg/kg/day group in either sex. All the observed findings were judged to be spontaneous in consideration of their types, grades, and incidence.

Post mortem analysis of the animals showed that in males, the relative weight of the kidneys in the 5,000 mg/kg/day group was lower than that in the control group. Moreover, the absolute and relative weights of the thymus in the 1,000 mg/kg/day group were significantly higher than those in the control group. Higher thymus weight was considered to be incidental because there was no dose-dependent relationship.

In females, the absolute weight of the ovaries in the 1,000 and 3,000 mg/kg/day groups and the relative weight of the ovaries in the 3,000 mg/kg/day group were significantly higher than those in the control group. However, these changes were considered to be incidental because there was no dose-dependent relationship.

There were no gross necropsy findings related to test substance-treatment in either sex.

All lesions observed were sporadic and focal, and often observed in this strain of rats, and these were considered to be spontaneously occurring.

There were no histopathological findings related to the test substance-treatment in either sex. The incidences of these histopathological changes in 5,000 mg/kg/day group were comparable to those in the control group, and these changes were often observed in this strain of animal. Therefore, these were considered to be spontaneously occurring lesions.

In conclusion, the No Observed Adverse Effect Level (NOAEL) of TG4 Syrup was 5,000 mg/kg-bw/day in male and female rats under the conditions of this study. The equivalent consumption in a 60 kg human would be 300 g/day for 90 days. The title page, compliance statement and summary data are provided for examination in Appendix B.

## 6.5 Unpublished Human Tolerance Study of TG4 Syrup and Hydrogenated TG4 Syrup

As described previously in Part 6.2.1 information was given on the digestibility of TG4 Syrup. In Part 6.2.1, 8 human male subjects consumed 50 g of TG4 Syrup in 200 mL of warm water, which corresponds to 0.73 g/kg-bw. No adverse effects (tolerability issues) were reported, and all the TG4 Syrup was digested and assimilated as glucose (HBL, 2003a).

In addition to the information in Part 6.2.1, there is another unpublished human study that reports data that is directly related to the tolerability of TG4 Syrup (HBC, 2003b). It was performed to determine the laxative threshold of TG4 Syrup. In addition it also includes laxation of hydrogenated TG4 Syrup, which is another commercial product sold by the Notifier.

To produce hydrogenated TG4 Syrup, the first step is to use the same enzymatic process that is used to form TG4 Syrup. As noted previously this results in over 50% of the molecules having a trehalose moiety at the reducing end. The reducing terminals of the remaining glucose and oligosaccharides that were not converted to trehalose moieties can then be hydrogenated, producing polyols. With the trehalose and hydrogenation at the reducing ends of glucose and oligosaccharides, a product is formed in which all the reducing ends are modified and no longer reactive. Hydrogenated TG4 Syrup is commercially available in many parts of the world, including the USA, as an ingredient in cosmetic products where reactivity with other ingredients is not wanted. The amount of TG4 in the hydrogenated TG4 Syrup is essentially identical to that in TG4 Syrup. Table 6-3 provides a comparison. These

results demonstrate that the TG4 (and TG3) in TG4 Syrup is not changed by hydrogenation and remains in hydrogenated TG4 Syrup essentially as is.
Therefore, the human tolerance study of hydrogenated TG4 Syrup can be used to demonstrate the safety of the maltosyltrehalose portion of TG4 Syrup.

**Table 6-3 Compositional analysis of TG4 Syrup and Hydrogenated TG4 Syrup**

| (% on a dry basis) | TG4 Syrup (Data from 3 lots) | Hydrogenated TG4 Syrup (Average from 3 lots) |
|---|---|---|
| Mono- and disaccharides | 11.6 | 10.4 |
| Maltotriose (G3) | 9.8 | – |
| Maltotriitol (G3OH) | – | 8.6 |
| Glucosyltrehalose (TG3) | 3.5 | 2.3 |
| Maltotetraose (G4) | 14.9 | – |
| Maltotetraitol (G4OH) | – | 15.3 |
| Maltosyltrehalose (TG4) | 52.5 | 54.2 |
| Other higher saccharides | 7.7 | 9.2 |

Scientists at Hayashibara Biochemical Laboratories, Inc. performed a blind study to test for the laxation threshold of TG4 Syrup (54.8% TG4 dry basis) and hydrogenated TG4 Syrup (Internal report of Hayashibara Biochemical Laboratories, Inc., 2003, unpublished).

In earlier studies, the laxative effect of trehalose and a sorbitol/maltitol mix was calculated to be 0.65 and 0.17–0.24 g/kg-bw, respectively in a Japanese population (Oku, Okazaki, 1998). In this study subjects (30 males and 10 females) were given 30, 40, 50 and 60 g (dry basis) of TG4 Syrup or "hydrogenated" TG4 Syrup in 100–200 mL of warm water. Subjects orally consumed the test substances over a 2-hour period in the order of lowest to highest dose after eating lunch. Each test substance was consumed at intervals of greater than 72 hours. If laxation resulted within 5 hours after administration, further tests of higher amounts were not performed, and it was assumed that further laxation would be caused by administration of an increased dose. Within 24 hours after administration, the subject verbally reported on the following conditions;

i. time of bowel movement after administration,
ii. fecal condition (hard, soft, pasty, muddy, liquid), condition and odor compared to normal,
iii. gastrointestinal symptoms (pain in upper or lower part of the abdomen, urge to defecate without defecation, borborygmus, abdominal distention, flatus, feeling of weakness around the waist, vomiting, feeling of discomfort, feeling of nausea

or general sickness, others, and no symptoms),
iv. fever, and
v. other comments.

If a subject defecated with muddy or liquid feces within 5 hours after administration, it was concluded that the condition was the result of the test substance and dose. The laxative threshold was calculated in accordance with a published method (Oku, Okazaki, 1998). Two (2) subjects were excluded from the TG4 Syrup group because of having symptoms of a cold, and diarrhea before administration of the 60 g dose of TG4 Syrup. Further 2 subjects were excluded from the hydrogenated TG4 Syrup group because of their discomfort caused by the sweetness of the hydrogenated TG4 Syrup.

As a result of consumption of TG4 Syrup, some subjects reported abdominal pain, abdominal distention, flatus, etc. as shown in Table 6-4, but the investigators reported that dose-dependency was not observed. Consumption of 50 g of TG4 Syrup caused loose stools in only 1 subject, and the frequency of loose stools at the 50 and 60 g doses of TG4 Syrup were calculated to be 2.6% (1/38). Therefore, the laxative threshold of TG4 Syrup could not be calculated because of the lack of intolerance to the treatment. The investigators concluded that TG4 Syrup is highly digestible.

**Table 6-4 Numbers of Subjects with Adverse Symptoms due to Consumption of TG4 Syrup**

| Number of subjects with a specific symptom / Number of tested subjects | Consumed amount of TG4 Syrup | | | |
|---|---|---|---|---|
| | 30 g | 40 g | 50 g | 60 g* |
| Abdominal pain | 1 / 38 | 1 / 38 | 2 / 38 | 1 / 38 |
| Tenesmus | 1 / 38 | 1 / 38 | 2 / 38 | 2 / 38 |
| Borborygmus | 3 / 38 | 1 / 38 | 2 / 38 | 4 / 38 |
| Abdominal distention | 1 / 38 | 9 / 38 | 8 / 38 | 5 / 38 |
| Flatus | 6 / 38 | 7 / 38 | 5 / 38 | 6 / 38 |
| Vomit / Discomfort | 0 / 38 | 0 / 38 | 0 / 38 | 0 / 38 |
| Loose stool | 0 / 38 | 0 / 38 | 1 / 38 | 1 / 38 |

*Two (2) subjects were excluded because of cold symptoms and diarrhea before the administration of 60 g TG4 Syrup.

As a result of consumption of hydrogenated TG4 Syrup, some subjects reported abdominal pain, abdominal distention, flatus, etc. as shown in Table 6-5, but the investigators reported that dose-dependency was not observed. The number of subjects that reported abdominal symptom increased over that observed for TG4 Syrup

and was dependent on the amount of hydrogenated TG4 Syrup consumed. Frequency of loose stools at the 40, 50 and 60 g dose of hydrogenated TG4 Syrup were calculated to be 7.9% (3/38), 18.4% (7/38), and 21.1% (8/38), respectively. The laxative threshold of hydrogenated TG4 Syrup was calculated to be 0.58 g/kg-bw. The increase in incidence in relation to consumption of TG4 Syrup was not unexpected because of the well-known laxation effect of sugar alcohols.

**Table 6-5 Number of Subjects with Adverse Symptoms due to Consumption of Hydrogenated TG4 Syrup**

| Number of subjects with a specific symptom / Number of tested subjects | Consumed amount of hydrogenated TG4 Syrup | | | |
|---|---|---|---|---|
| | 30 g | 40 g | 50 g | 60 g* |
| Abdominal pain | 2 / 38 | 4 / 38 | 3 / 38 | 4 / 38 |
| Tenesmus | 0 / 38 | 0 / 38 | 2 / 38 | 1 / 38 |
| Borborygmus | 1 / 38 | 5 / 38 | 6 / 38 | 6 / 38 |
| Abdominal distention | 2 / 38 | 5 / 38 | 7 / 38 | 5 / 38 |
| Flatus | 3 / 38 | 10 / 38 | 15 / 38 | 12 / 38 |
| Vomit / Discomfort | 0 / 38 | 0 / 38 | 0 / 38 | 0 / 38 |
| Loose stool | 0 / 38 | 3 / 38 | 7 / 38 | 8 / 38 |

*Two (2) subjects were excluded because of discomfort caused by sweetness.

## 6.6 Human Exposure

TG4 Syrup was developed in 2002, and has been manufactured, packaged, stored, transported and sold as a commercial product in Japan since 2003 (commercial name: HALLODEX™). Human exposure can result from the handling of production enzymes, raw materials, intermediates and finished products. In all that time, no employee has reported any untoward adverse events related to regular and direct contact to any substances involved in the manufacture of TG4 Syrup or to TG4 Syrup itself.

TG4 Syrup has been sold into the food industry and consumed by humans in Japan and to a lesser extent in Taiwan since 2003. The annual sales amounts of TG4 Syrup in Japan (and to a lesser extent in Taiwan) has been approximately 10,000 metric tons in each of the fiscal years 2013, 2014, 2015 and 2016. The cumulative sales of TG4 from 2003 to 2016 is approximately 110,000 metric tons. Although there are a number of methods to report untoward reactions to food products in Japan, there have been no known consumer reports of any adverse events, including allergenic reactions, related

to consumption of TG4 Syrup.

## 6.7 Unpublished Safety Studies Using a Substance Closely Related to TG4 Syrup, Hydrogenated TG4 Syrup and Hydrogenated TG4 Syrup Powder

As described in Table 6-3, the amount of glycosyltrehalose in the hydrogenated TG4 Syrup, including TG4, is essentially identical to that in TG4 Syrup. In addition, hydrogenated TG4 Syrup powder was used for some safety studies according to a requirement of European List of Notified Chemical Substances (ELINCS) to register hydrogenated TG4 Syrup as a cosmetic ingredient in ELINCS in 2006. It was demonstrated that this hydration did not change the quantification of the composition in hydrogenated TG4 Syrup.

### 6.7.1 Unpublished *in vitro* Mutagenicity Study Using Hydrogenated TG4 Syrup Powder - Ames Test -

Hydrogenated TG4 Syrup powder (Water content: 5.03%; TG4 content on a dry basis: 54.2%) was examined for mutagenic activity in the standardized bacterial reverse mutation tests (OECD #471; Commission Directive 2000/32/EC, L1362000); the plate incorporation test and the pre-incubation test using the *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, and the *Escherichia coli* strain WP2 uvrA (Sokolowski, 2005).

The data demonstrated that hydrogenated TG4 Syrup powder is not mutagenic in the *S. typhimurium* and *E. coli* bacterial reverse mutation study, and therefore TG4 and the other glycosyltrehaloses in hydrogenated TG4 Syrup are not mutagenic at a dose of 2,570 μg/plate.

### 6.7.2 Unpublished *in vitro* Mutagenicity Study Using Hydrogenated TG4 Syrup - Chromosomal Aberration Test -

To examine hydrogenated TG4 Syrup (Water content: 27.5%, TG4 content on a dry basis: 54.3%) for its potential to induce chromosomal aberration in cultured mammalian cells, a standard *in vitro* chromosomal aberration test (OECD #473; Notification No. 1604, PMSB, MHLW, Japan, 1991; Guide to Quasi-drug and Cosmetic Regulations in Japan, 4$^{th}$ Edition) was performed using the CHL/IU cell line derived from fibroblasts from the lungs of newborn female Chinese hamsters (Saigo, 2004).

The frequencies of cells having structural and numerical aberrations at any doses of hydrogenated TG4 Syrup, regardless of a metabolic activation system and treatment time length, were below 5%. It was therefore concluded that hydrogenated TG4 Syrup

did not induce chromosomal aberrations in CHL/IU cells, regardless of a metabolic activation system or treatment time length, under the conditions of this study (Saigo, 2004). Therefore it follows that TG4 and the other glycosyltrehaloses in hydrogenated TG4 Syrup did not induce chromosomal aberrations at a concentration of 1,970 µg/mL.

### 6.7.3 Unpublished Acute Oral Toxicity Study Using Hydrogenated TG4 Syrup

An acute oral toxicity study using hydrogenated TG4 Syrup (Water content: 27.5%, TG4 content on a dry basis: 54.3%) was performed (OECD #423; Directive 96/54/EEC, B.1) on 6 HanBrl:WIST (SPF) rats (3 females and 3 males) 11-12 weeks of age (Ott, 2004). Each animal received a single 2,759-mg/10 mL/kg-bw dose of hydrogenated TG4 Syrup (calculated to correspond to 1,086 mg/kg of TG4) by oral gavage after fasting for approximately 17-20 hours. Animals were examined for mortality, viability and clinical signs four times during study day 1 and once daily for 14 days after administration. Body weights were recorded 1 day before treatment and at days 8 and 15. At the end of the observation period, all animals were sacrificed, and then the organs or tissues were macroscopically examined.

No deaths were noted for either sex. Changes in body weight for both males and females were within normal range for rats of this strain and age. No abnormalities were observed in the general physical condition of the animals, and no macroscopic findings were observed in any organs at necropsy. The median lethal dose ($LD_{50}$) of hydrogenated TG4 Syrup after a single oral administration to rats of both sexes, observed over a period of 14 days, could not be estimated as no deaths occurred during the study. Therefore, the $LD_{50}$ of hydrogenated TG4 Syrup was concluded to be greater than 2,000 mg/kg on a dry basis for both male and female rats, using the OECD limit dose (Ott, 2004). This corresponds to a $LD_{50}$ of TG4 and the other glycosyltrehaloses in hydrogenated TG4 Syrup of greater than 1,090 mg/kg.

### 6.7.4 Unpublished 28-day Oral Toxicity Study Using Hydrogenated TG4 Syrup Powder

Using hydrogenated TG4 Syrup powder (Water content: 5.03%; TG4 content on a dry basis: 54.2%), a 28-day oral toxicity (gavage) study (OECD #407; Directive 96/54/EC, B.7) was conducted to investigate the toxicity of daily administration of hydrogenated TG4 Syrup powder in HanBrl:WIST (SPF) rats (Damme, 2005). Twenty (20) males and 20 females that were 6 weeks old were received 10 mL of 0, 50, 200 and 1,000 mg/kg-bw of hydrogenated TG4 Syrup powder (calculated to correspond to 0, 26, 103 and 515 mg/kg-bw of TG4) by oral gavage daily for 28 days. Then all animals were sacrificed after blood sampling under anesthesia for clinical laboratory data. Mortality/viability (twice daily), general cage side observations (daily), clinical observations (daily and

weekly for details), food consumption (weekly), body weights (weekly), functional observational battery (week 4) were recorded before the start of treatment, during and at the end of the study. Consistent with the guidelines, hematologic variables were examined, and selected organs were weighed and appropriate organs and tissues were histologically examined.

There were no deaths during the study. All survivors were euthanized after 4 weeks of treatment and subjected to hematological examination and necropsy. There were no consistent, treatment-related, dose-dependent changes of toxicological relevance reported on any variables evaluated including mortality/viability, clinical signs, food consumption, clinical laboratory investigations and macroscopic/microscopic findings. Test item-related findings were generally restricted to increased total locomotor activity and single run locomotor activity in test item-treated males and in decreased spleen-to-body weight and spleen-to-brain weight ratios in males treated with 200 mg/kg-bw/day of hydrogenated TG4 Syrup powder. The investigators concluded that the no-observed-adverse-effect-level (NOAEL) was 1,000 mg/kg-bw/day of hydrogenated TG4 Syrup powder (the highest dose on a dehydration basis), corresponding to the NOAEL of TG4 and the other glycosyltrehaloses in hydrogenated TG4 Syrup of 515 mg/kg-bw/day (Damme, 2005).

### 6.7.5 Conclusion

The preceding unpublished experiments demonstrate that the glycosyltrehaloses fraction of hydrogenated TG4 Syrup including TG4, which is in the same concentration as that found in TG4 Syrup, appears to have no untoward safety effects when tested in standard model systems for food safety. While it is possible that the hydrogenated glucose, maltose and other oligosaccharides present in hydrogenated TG4 Syrup might have the effect of reducing the safety of the TG4 fraction of hydrogenated TG4 Syrup, this conclusion would appear to be highly unlikely because of the other data provided on the safety of TG4 Syrup.

## 6.8 Safety of the TG4 Manufacturing Process

Scientists at Hayashibara Biochemical Laboratories, Inc. (now Hayashibara Co., Ltd., the Notifier) developed TG4 Syrup (commercial name: HALLODEX™) manufactured from starch using raw materials, and 4 enzymes that are commonly used in the US and around the world as processing aids. As provided in Part 2 of this GRAS Notice the manufacturing process is standard for the industry, and in fact, the Notifier was the first company in the world to commercially develop an all enzymatic process to produce high glucose and high maltose syrups in an essentially pure form. The Notifier has

continued to use this enzyme-based system to produce a variety of starch derived sweeteners. The company uses 4 enzymes, that are commonly employed to produce similar starch based foods to produce TG4 Syrup. These enzymes include α-amylases (EC 3.2.1.1) from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa*, glucan 1,4-α-maltotetraohydrolase (EC 3.2.1.60) from *Pseudomonas stutzeri*, and (1->4)-α-D-glucan 1-α-D-glucosylmutase (EC 5.4.99.15) from *Arthrobacter ramosus* (Part 2.3). All are obtained from wild type soil microorganism, which have undergone standard traditional mutation techniques, and are not derived from genetically modified microorganisms (non-GMO).

All raw materials, equipment and processes used to manufacture TG4 Syrup are suitable for food production and, as mentioned previously are very common to the food and dietary supplement industry. The production of TG4 Syrup by the Notifier is consistent with current Good Manufacturing Practices (cGMP) and the Specifications and Standards for Food, Food Additives, etc. under the Food Sanitation Act in Japan.

Further, as given in Part 2, assays from 3 lots of TG4 Syrup were tested and have demonstrated that the amount of protein in the final product is less than 20 µg/mL. This suggests that very little, if any, of the enzymes used to produce the end product remain after production. Finally in the production process the production lot is heated which inactivates and degrades the enzymes. There is no indication of allergenicity to TG4 Syrup or its constituents.

**GRAS Conclusion**

After review of the manufacturing process, equipment, food grade raw ingredients and processing aids, including the 4 enzymes, the Notifier has concluded that TG4 Syrup should be recognized as GRAS for its intended use because the raw materials, processing aid, manufacturing processes, and final product are substantially equivalent to most other starch-hydrolysate products, which have been consumed for many decades as one of the most common sources of food in the USA and worldwide.

## 6.9 Possible Inconsistent Data, Exempt Information, and Non-public Information

The data that may appear to be inconsistent is presented in Part 6.3.3, which is associated with trehalose, a GRAS substance that is considered to be substantially equivalent to constituents of TG4 Syrup. The relevance is discussed in this section.

The information that the Notifier is requesting to be exempt from disclosure under the

FOI Act is in regards to commercially privileged sales data that is provided in Parts 3.2, 3.5, 3.6, 4.0, and 6.6. The specific values are highlighted for greater ease of identification.

The non-public information included in this Notice that relates to the safety of TG4 Syrup is identified as "unpublished" in preceding sections. This information is intended only to support the publically available data that is provided. An example of this is Part 6.7 where unpublished studies of hydrogenated TG4 Syrup are provided. It is believed that the determination of GRAS for TG4 Syrup is not dependent on its availability to qualified experts. However, this non-public information is included because they are known to the Notifier and contain information on the use of TG4 Syrup or substances that are "substantially equivalent" in *in vitro*, animal and human studies.

## 6.10 General Conclusion of GRAS

In conclusion publically available information has been provided on the structure, composition, manufacturing and safety of TG4 Syrup (including a mutagenicity test, a preliminary 90-day feeding study, and a 90-day repeated oral dose toxicity study). Further, supporting evidence from a number of commonly consumed GRAS substances that are considered to be "substantially equivalent" to TG4 Syrup (i.e. corn [glucose] syrup, maltodextrin, dextrin and trehalose) have been provided. It is believed that this information and data provide a sufficient basis for the Notifier's conclusion that TG4 Syrup would be GRAS under conditions of intended use in foods, through scientific procedures, and that qualified experts would concur with this conclusion.

# Part 7. Supporting Information and Data

Part 7 includes the reference citations listed in the text. All unpublished data and information that is discussed in Part 6 is title as such, while publically available (published) safety data is titled as published. The unpublished sections include Parts 6.2.1, 6.5, and 6.7.1-6.7.4. The citations for these are underlined in the list of references below.

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## 7.2 Appendices

The Appendices include an Appendix A that includes information on the food classes and technical effects of the commercial used of TG4 Syrup in Japan.

Appendix B includes summary information/data regarding the published 90-day preliminary oral feeding safety study (Part 6.4.2), and the published 90-day Gavage Repeated Oral Dose Toxicity Study (Part 6.4.3).

### 7.2.1 Appendix A

**Commercial Foods and Drinks Containing TG4 Syrup in Japan**

| Food Category | | Commercial Food | Functionality |
|---|---|---|---|
| Confectionery | Sweets | Sweet bean paste | Reduction of total sweetness, Retention of moisture |
| | | Sweet potato cake | Reduction of total sweetness, Retention of moisture |
| | | Rice cake with sweet bean paste | Maintenance of Softness |
| | | Rice dumpling | Maintenance of Softness |
| | | Rice cake (Gyuhi) | Maintenance of Softness |
| | | Bracken-starch dumpling | Retention of moisture |
| | | Steamed bread | Retention of moisture |
| | | Sponge cake | Retention of moisture |
| | | Pancake | Retention of moisture |
| | | Doughnut | Retention of moisture |
| | | Soft cookie | Retention of moisture |
| | | Crape | Retention of moisture |
| | | Baumkuchen | Retention of moisture |
| | | Pound cake | Retention of moisture |
| | | Madeleine | Retention of moisture |
| | | Financier | Retention of moisture |
| | | Bean cake | Reduction of total sweetness, Retention of moisture, Glazing |
| | | Sweet bean jelly | Reduction of total sweetness, Suppression of dripping |
| | | Sauce for rice cake | Reduction of total sweetness, Glazing, Modification of viscosity |
| | | Hard and soft candies | Reduction of total sweetness, Improvement of texture, Suppression of moisture absorption |
| | | Caramel | Reduction of total sweetness, Improvement of texture, Suppression of moisture absorption |
| | | Gummi candy | Reduction of total sweetness, Improvement of texture, Suppression of moisture absorption |
| | | Pate de fruits | Reduction of total sweetness, Improvement of texture, Suppression of moisture absorption, Suppression of trehalose recrystallization |

**Commercial Foods and Drinks Containing TG4 Syrup in Japan (continued)**

| Food Category | | Commercial Food | Functionality |
|---|---|---|---|
| Confectionery | Sweets | Chocolate | Reduction of total sweetness, Suppression of unpleasant odor |
| | | Ganache | Reduction of total sweetness, Suppression of unpleasant odor |
| | | Cookie | Improvement of texture (crispy) |
| | | Sabouret | Improvement of texture (crispy) |
| | | Puff pastry | Glazing |
| | | Puff pastry, Popcorn | Glazing |
| | | Chocolate cake | Retention of moisture, Suppression of unpleasant odor |
| | | Cheese cake | Retention of moisture, Suppression of unpleasant odor |
| | | Custard cream | Reduction of total sweetness, Retention of moisture, Suppression of unpleasant odor |
| | | Whipped cream | Suppression of dripping |
| | Dessert | Pudding | Reduction of total sweetness, Suppression of dripping |
| | | Bavarois | Reduction of total sweetness, Suppression of dripping |
| | | Mousse | Reduction of total sweetness, Suppression of dripping |
| | | Jelly | Reduction of total sweetness, Suppression of dripping |
| | | Tiramisu | Reduction of total sweetness, Suppression of unpleasant odor |
| | | Yogurt | Addition of viscosity |
| | Ice cream | Ice cream | Improvement of texture (smoothness) |
| | | Sherbet | Improvement of texture (smoothness) |
| | | Gelato | Improvement of texture (smoothness) |
| Processed food | Seasoning agent | Seasoning agent for simmered foods | Reduction of total sweetness, Glazing |
| | | Liquid preparation for dried sardines | Reduction of total sweetness, Glazing, Suppression of fishy odor |
| | | Liquid preparation for salmon caviars | Improvement of workability (easy loosening), Suppression of fishy odor |
| | | Liquid preparation for fishy egg pickles | Suppression of fishy odor |
| | | Liquid preparation for fishes | Suppression of fishy odor |
| | | Sauce for broiled eel | Suppression of fishy odor, Glazing |

**Commercial Foods and Drinks Containing TG4 Syrup in Japan (continued)**

| Food Category | | Commercial Food | Functionality |
|---|---|---|---|
| Processed food | Seasoning agent | Liquid preparation for Japanese-style rolled omelets | Suppression of unpleasant odor, Suppression of protein denaturation |
| | | Flavored liquid preparation for dried laver seaweeds | Glazing |
| | | Sauce | Addition of richness |
| | | Mayonnaise | Addition of richness |
| | | Dressing | Addition of richness |
| | | Sweet cooking sake-style condiment | Addition of richness |
| | | Soy sauce | Addition of richness |
| | | Noodle soup base | Enhancement of flavor |
| | | Japanese miso (fermented soybean paste) | Addition of richness, Suppression of coloration |
| | | Instant miso soup | Enhancement of flavor |
| | | Flavored liquid preparation for dried laver seaweeds | Glazing |
| | | Sauce | Addition of richness |
| | | Mayonnaise | Addition of richness |
| | | Dressing | Addition of richness |
| | | Sweet cooking sake-style condiment | Addition of richness |
| | | Soy sauce | Addition of richness |
| | | Noodle soup base | Enhancement of flavor |
| | | Japanese miso (fermented soybean paste) | Addition of richness, Suppression of coloration |
| | | Instant miso soup | Enhancement of flavor |
| | | Green horseradish paste | Retention of moisture, Maintenance of viscosity, Maintenance of flavor |
| | Spreads | Jam, Fruit sauce (filling) | Reduction of total sweetness, Suppression of coloration during manufacturing |
| | Chinese food | Chinese noodle | Retention of moisture |
| | | Dumpling skin | Retention of moisture |
| | | Spring roll skin | Retention of moisture |
| | Processed meat products | Sausage, Bacon | Improvement of texture, Suppression of unpleasant odor |
| | | Fried chicken | Retention of moisture, Suppression of unpleasant odor |

**Commercial Foods and Drinks Containing TG4 Syrup in Japan (continued)**

| Food Category | | Commercial Food | Functionality |
|---|---|---|---|
| Processed food | Processed meat products | Breaded pork cutlet | Retention of moisture, Suppression of unpleasant odor |
| | | Hamburger steak | Retention of moisture, Suppression of unpleasant odor |
| | | Innards stew | Moisture retention, Suppression of unpleasant odor |
| | Processed fish products | Fish paste | Improvement of texture, Suppression of fishy odor |
| | Pickles, Food boiled in soy sauce | Pickled Japanese horseradish | Improvement of texture, Suppression of unpleasant odor |
| | | Food boiled in soy sauce | Reduction of total sweetness, Glazing |
| | | Flavored enokitake mushroom | Suppression of unpleasant odor |
| | Bakery | Bread | Retention of moisture |
| | | Brioche | Retention of moisture |
| | | Steamed bread | Retention of moisture |
| | | Frozen cream bun (dough) | Retention of moisture |
| | | Pan cake | Retention of moisture |
| | Processed soy products | Bean curd | Suppression of soy odor |
| | | Deep-fried bean curd | Suppression of soy odor |
| | Prepared foods | Frozen Japanese-style rolled omelets | Suppression of unpleasant odor, Suppression of protein denaturation |
| | | Peanut dressed with miso | Reduction of total sweetness, Glazing |
| | | Flavored boiled bean | Reduction of total sweetness, Glazing |
| | | Fried sweet potato (candy-coating) | Glazing, Suppression of moisture absorption |
| Drinks | Beverages | Lactobacillus beverage | Addition of richness |
| | | Soymilk | Suppression of soy odor |
| | Soft drinks | Coffee and Coffee-like | Addition of richness |
| | | Tea | Addition of richness |
| | | Sport drink | Addition of richness |
| | | Jelly beverage | Addition of richness |
| | Alcoholic drinks (not included in GRAS Notice | Liqueur | Addition of richness |
| | | Beer | Carbon source for fermentation |

### 7.2.2 Appendix B

90-day Preliminary Feeding Study in Rats

90-day Repeated Oral Dose Toxicity Study of Maltosyltrehalose Syrup in Rats

# MB RESEARCH LABORATORIES

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| | | |
|---|---|---|
| **Study Title** | : | 90 Day Feeding Study in Rats |
| **Test Article** | : | TG4 (Maltosyl Trehalose), Lot/Batch #020907 |
| **Author** | : | Daniel R. Cerven, M.S., Study Director |
| **Study Completed On** | : | November 22, 2004 |
| **Performing Laboratory** | : | MB Research Laboratories<br>1765 Wentz Road<br>P.O. Box 178<br>Spinnerstown, PA 18968 |
| **MB Research Project #** | : | MB 02-10706.01 |
| **MB Research Protocol #** | : | 2062 RA-Hayashibara |
| **Sponsor** | : | Hayashibara International, Inc.<br>8670 Wolff Court, Suite 200<br>Westminster, CO 80031-6953 |
| **Citation** | : | Daniel R. Cerven, M.S. (2002)<br>Unpublished Report by<br>MB Research Laboratories |

## Table 3 (cont'd): Mean Body Weights (g) - MALES

**GROUP #: 1 (Control 0%)**

| | Pre | Day 8 | Day 15 | Day 22 | Day 29 | Day 36 | Day 43 | Day 50 | Day 57 | Day 64 | Day 71 | Day 78 | Day 85 | Day 91 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 265 | 311 | 349 | 375 | 396 | 415 | 437 | 451 | 464 | 476 | 484 | 492 | 500 | 509 |
| S.D. | 15.5 | 20.1 | 22.7 | 23.5 | 26.8 | 25.9 | 26.3 | 27.6 | 20.1 | 31.2 | 31.6 | 31.3 | 31.7 | 33.5 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

**GROUP #: 2 (10%)**

| | Pre | Day 8 | Day 15 | Day 22 | Day 29 | Day 36 | Day 43 | Day 50 | Day 57 | Day 64 | Day 71 | Day 78 | Day 85 | Day 91 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 262 | 304 | 342 | 373 | 397 | 417 | 439 | 453 | 466 | 478 | 488 | 494 | 501 | 512 |
| S.D. | 10.5 | 16.9 | 23.9 | 27.8 | 30.0 | 30.6 | 35.0 | 37.1 | 37.9 | 40.7 | 38.1 | 42.7 | 43.4 | 48.2 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

## Table 3 (cont'd): Mean Body Weights (g) - FEMALES

**GROUP #: 1 (Control 0%)**

| | Pre | Day 8 | Day 15 | Day 22 | Day 29 | Day 36 | Day 43 | Day 50 | Day 57 | Day 64 | Day 71 | Day 78 | Day 85 | Day 91 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 193 | 216 | 236 | 256 | 258 | 266 | 281 | 291 | 294 | 296 | 304 | 309 | 319 | 317 |
| S.D. | 8.0 | 10.7 | 12.5 | 9.1 | 14.3 | 13.1 | 15.0 | 17.1 | 19.5 | 16.3 | 25.5 | 20.7 | 22.9 | 21.2 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

**GROUP #: 2 (10%)**

| | Pre | Day 8 | Day 15 | Day 22 | Day 29 | Day 36 | Day 43 | Day 50 | Day 57 | Day 64 | Day 71 | Day 78 | Day 85 | Day 91 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 190 | 210 | 237 | 253 | 261 | 280* | 282 | 291 | 289 | 304 | 307 | 310 | 316 | 315 |
| S.D. | 11.6 | 14.7 | 16.2 | 16.5 | 17.8 | 16.2 | 16.8 | 22.8 | 19.1 | 22.0 | 21.4 | 27.9 | 29.5 | 20.9 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

*significantly ($p \leq 0.05$) greater than control

## Table 4 (cont'd): Mean Food Consumption (g) - MALES

GROUP #: 1 (Control 0%)

| | WK 1 | WK 2 | WK 3 | WK 4 | WK 5 | WK 6 | WK 7 | WK 8 | WK 9 | WK 10 | WK 11 | WK 12 | WK 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 178.6 | 182.2 | 185.4 | 189.3 | 181.1 | 195.6 | 181.9 | 183.4 | 180.7 | 178.4 | 184.6 | 153.1 | 187.7 |
| S.D. | 27.7 | 11.6 | 11.8 | 17.6 | 21.7 | 36.6 | 13.5 | 20.5 | 25.5 | 29.6 | 37.4 | 24.6 | 32.7 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

GROUP #: 2 (10%)

| | WK 1 | WK 2 | WK 3 | WK 4 | WK 5 | WK 6 | WK 7 | WK 8 | WK 9 | WK 10 | WK 11 | WK 12 | WK 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 197.5 | 194.2 | 202.8* | 210.1* | 210.0 | 208.4 | 205.8 | 197.9 | 202.2 | 203.5 | 202.0 | 171.5 | 209.2 |
| S.D. | 12.1 | 20.4 | 17.0 | 26.8 | 35.6 | 31.6 | 38.5 | 28.8 | 32.2 | 29.8 | 44.6 | 33.0 | 39.8 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

*significantly ($p \leq 0.05$) greater than control

## Table 4 (cont'd): Mean Food Consumption - FEMALES

**GROUP #: 1 (Control 0%)**

| | WK 1 | WK 2 | WK 3 | WK 4 | WK 5 | WK 6 | WK 7 | WK 8 | WK 9 | WK 10 | WK 11 | WK 12 | WK 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 140.3 | 131.5 | 140.8 | 131.6 | 126.1 | 135.0 | 137.1 | 127.0 | 120.9 | 132.1 | 128.1 | 116.3 | 127.6 |
| S.D. | 15.5 | 12.8 | 12.5 | 19.3 | 16.9 | 11.9 | 18.6 | 11.0 | 14.0 | 16.1 | 8.7 | 18.8 | 11.7 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

**GROUP #: 2 (10%)**

| | WK 1 | WK 2 | WK 3 | WK 4 | WK 5 | WK 6 | WK 7 | WK 8 | WK 9 | WK 10 | WK 11 | WK 12 | WK 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 146.1 | 149.2* | 155.2* | 146.8 | 153.1* | 141.2 | 149.2 | 128.5 | 148.2* | 146.3 | 135.4 | 120.5 | 138.7 |
| S.D. | 13.4 | 10.8 | 13.1 | 15.7 | 12.0 | 15.8 | 20.9 | 12.7 | 9.7 | 14.1 | 22.9 | 14.6 | 18.4 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

*significantly ($p \leq 0.05$) greater than control

Summary of MB Research Laboratories
Project Number: MB 02-10706.01
Protocol Number: 2062 RA-Hayashibara

**Table 7 NECROPSY OBSERVATIONS**

| **Sex** | **Males (10/group)** | |
|---|---|---|
| **Dose** | Control (0%) | TG4 10% |
| **Observations** | 7 - Normal<br>1 - Forelimbs, alopecia<br>1 - Thymus, darker than normal<br>1 - Adrenals, smaller than normal | 6 - normal<br>1 - Front paws, alopecia<br>1 - Thymus, darker than normal and right side mottled<br>2 - Kidney and Adrenals, nodules on left one, smaller than normal<br>1 - Adrenal, one smaller than normal |
| **Sex** | **Female (10/group)** | |
| **Dose** | Control (0%) | TG4 10% |
| **Observations** | 10 - Normal | 7 - Normal<br>1 - Liver, penetrated through diaphragm<br>2 - Diaphragm, herniated by liver |

## Table 8 (cont'd): Mean Hematology (MALES)[1]

[1]There were no BANDS or ATLYMPH noted in any animal. Accordingly, these parameters are not included in this table.

**Group 1 - (Control 0%)**

| AN. # | WBC THSN/UL | RBC MILL/UL | HGB GM/DL | HCT % | MCV FL | MCH PG | MCHC % | PLATE THSN/UL | SEGS THSN/UL | LYMPH THSN/UL | MONO THSN/UL | EOSN THSN/UL | BASO THSN/UL | PROTH SEC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 6.81 | 8.712 | 15.85 | 45.82 | 52.7 | 18.17 | 34.57 | 864.8 | 1.035 | 5.258 | 0.384 | 0.065 | 0.07 | 17.3 |
| SD | 1.63 | 0.313 | 0.64 | 1.91 | 1.1 | 0.39 | 0.28 | 50.9 | 0.317 | 1.276 | 0.155 | 0.043 | 0.036 | 1.55 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

**Group 2 - (10%)**

| AN. # | WBC THSN/UL | RBC MILL/UL | HGB GM/DL | HCT % | MCV FL | MCH PG | MCHC % | PLATE THSN/UL | SEGS THSN/UL | LYMPH THSN/UL | MONO THSN/UL | EOSN THSN/UL | BASO THSN/UL | PROTH SEC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 6.89 | 8.633 | 15.42 | 44.28 | 51.3 | 17.87 | 34.79 | 829.2 | .933 | 5.433 | .391 | .066 | .067 | 16.27 |
| SD | 1.16 | 0.393 | 0.71 | 2.2 | 2.6 | 0.98 | 0.58 | 111.9 | 0.184 | 0.935 | 0.198 | 0.05 | 0.022 | 1.43 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

## Table 8 (cont'd): Mean Hematology (FEMALES)[1]

[1]There were no BANDS or ATLYMPH noted in any animal. Accordingly, these parameters are not included in this table.

**Group 1 - (Control 0%)**

| AN. # | WBC THSN/UL | RBC MILL/UL | HGB GM/DL | HCT % | MCV FL | MCH PG | MCHC % | PLATE THSN/UL | SEGS THSN/UL | LYMPH THSN/UL | MONO THSN/UL | EOSN THSN/UL | BASO THSN/UL | PROTH SEC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 4.57 | 7.987 | 15.58 | 43.23 | 54.2 | 19.49 | 36.03 | 889.2 | 0.514 | 3.753 | 0.196 | 0.069 | 0.04 | 17.0 |
| SD | 0.90 | 0.202 | 0.42 | 1.23 | 1.2 | 0.44 | 0.32 | 72.5 | 0.172 | 0.940 | 0.054 | 0.040 | 0.015 | 1.57 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

**Group 2 - (10%)**

| AN. # | WBC THSN/UL | RBC MILL/UL | HGB GM/DL | HCT % | MCV FL | MCH PG | MCHC % | PLATE THSN/UL | SEGS THSN/UL | LYMPH THSN/UL | MONO THSN/UL | EOSN THSN/UL | BASO THSN/UL | PROTH SEC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 4.8 | 7.998 | 15.19 | 42.47 | 53.1 | 19.02 | 35.8 | 819 | 0.607 | 3.915 | 0.186 | 0.05 | 0.04 | 16.06 |
| SD | 1.28 | 0.394 | 0.63 | 1.74 | 1 | 0.61 | 0.52 | 129.1 | 0.387 | 1.173 | 0.074 | 0.038 | 0.018 | 16.06 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

## Table 9 (cont'd): Mean Clinical Chemistry (MALES)

### Group 1 - (Control 0%)

| AN.# | $Na^+$ MEQ/L | $K^+$ MEQ/L | $Mg^{++}$ MEQ/L | $Cl^-$ MEQ/L | $Ca^{++}$ MG/DL | PHOS MG/DL | ALT U/L | AST U/L | AP U/L | GGT U/L | GLUC MG/DL | BUN MG/DL | CREAT MG/DL | CHOL MG/DL | TRIG MG/DL | TOT BILI MG/DL | ALB G/DL | TOT PROT G/DL | GLOB G/DL | SDH U/L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MEAN | 147.5 | 4.65 | 2.16 | 103.9 | 10.27 | 7.59 | 29.8 | 133.7 | 83.4 | 3.5 | 124.8 | 13.3 | 0.56 | 64.1 | 58.8 | 0.07 | 4.19 | 6.41 | 2.22 | 16.7 |
| SD | 0.7 | 0.24 | 0.16 | 1.3 | 0.23 | 0.33 | 9.6 | 14.2 | 19.8 | 0.5 | 17 | 1.6 | 0.05 | 8.7 | 25.1 | 0.05 | 0.19 | 0.21 | 0.16 | 8 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

### Group 2 - (10%)

| AN.# | $Na^+$ MEQ/L | $K^+$ MEQ/L | $Mg^{++}$ MEQ/L | $Cl^-$ MEQ/L | $Ca^{++}$ MG/DL | PHOS MG/DL | ALT U/L | AST U/L | AP U/L | GGT U/L | GLUC MG/DL | BUN MG/DL | CREAT MG/DL | CHOL MG/DL | TRIG MG/DL | TOT BILI MG/DL | ALB G/DL | TOT PROT G/DL | GLOB G/DL | SDH U/L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MEAN | 146.4** | 4.67 | 2.14 | 103.1 | 10.24 | 7.09 | 28.8 | 136 | 69.1 | 3.1 | 129.1 | 13.1 | 0.59 | 70.6 | 56.6 | 0.09 | 4.16 | 6.34 | 2.18 | 14.1 |
| SD | 1.1 | 0.21 | 0.10 | 0.10 | 0.27 | 0.72 | 4.5 | 26.8 | 13.4 | 0.9 | 17.2 | 1.4 | 0.09 | 15.2 | 32 | 0.03 | 0.21 | 0.25 | 0.16 | 4.6 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

**significantly ($p \leq 0.05$) less than control

## Table 9 (cont'd): Mean Clinical Chemistry (FEMALES)

### Group 1 - (Vehicle 0%)

| | $Na^+$ | $K^+$ | $Mg^{++}$ | $Cl^-$ | $Ca^{++}$ | PHOS | ALT | AST | AP | GGT | GLUC | BUN | CREAT | CHOL | TRIG | TOT BILI | ALB | TOT PROT | GLOB | SDH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AN.# | MEQ/L | MEQ/L | MEQ/L | MEQ/L | MG/DL | MG/DL | U/L | U/L | U/L | U/L | MG/DL | MG/DL | MG/DL | MG/DL | MG/DL | MG/DL | G/DL | G/DL | G/DL | U/L |
| MEAN | 145.6 | 4.54 | 2.39 | 104.3 | 10.30 | 7.26 | 26.9 | 142.6 | 58.0 | 3.5 | 115.0 | 15.9 | 0.66 | 67.9 | 28.5 | 0.14 | 4.48 | 6.27 | 1.79 | 19.1 |
| SD | 1.7 | 0.34 | 0.24 | 2.1 | 0.31 | 0.61 | 3.2 | 24.5 | 11.7 | 0.8 | 19 | 2.1 | 0.05 | 15.1 | 4.5 | 0.05 | 0.23 | 0.30 | 0.20 | 5 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

### Group 2 - (10%)

| | $Na^+$ | $K^+$ | $Mg^{++}$ | $Cl^-$ | $Ca^{++}$ | PHOS | ALT | AST | AP | GGT | GLUC | BUN | CREAT | CHOL | TRIG | TOT BILI | ALB | TOT PROT | GLOB | SDH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AN.# | MEQ/L | MEQ/L | MEQ/L | MEQ/L | MG/DL | MG/DL | U/L | U/L | U/L | U/L | MG/DL | MG/DL | MG/DL | MG/DL | MG/DL | MG/DL | G/DL | G/DL | G/DL | U/L |
| MEAN | 145 | 4.58 | 2.24 | 103.5 | 10.33 | 7.26 | 26.8 | 144.8 | 51.7 | 3.2 | 111.8 | 14.5 | 0.66 | 76.2 | 30.4 | 0.13 | 4.53 | 6.45 | 1.92 | 18.3 |
| SD | 1.6 | 0.38 | 0.13 | 1.1 | 0.25 | 0.43 | 6.4 | 21.4 | 11.4 | 1.2 | 10.3 | 2.2 | 0.05 | 15 | 5 | 0.08 | 0.23 | 0.28 | 0.22 | 3.7 |
| n | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

## Table 10 (cont'd): Summary Mean Organ Weights (g)-(MALES & FEMALES)

| | BODY WEIGHT | ADRENALS | KIDNEYS | LIVER | TESTES | EPIDIDYMIDES | THYMUS | SPLEEN | BRAIN | HEART |
|---|---|---|---|---|---|---|---|---|---|---|
| Grp#1 Males (0%) | 509 | 0.084 | 3.463 | 13.457 | 3.786 | 1.616 | 0.477 | 0.997 | 2.238 | 1.763 |
| Grp#2 Males (10%) | 512 | 0.081 | 3.374 | 13.535 | 3.690 | 1.702 | 0.504 | 1.051 | 2.267 | 1.827 |

| | BODY WEIGHT | ADRENALS | KIDNEYS | LIVER | UTERUS | OVARIES | THYMUS | SPLEEN | BRAIN | HEART |
|---|---|---|---|---|---|---|---|---|---|---|
| Grp#1 Females (0%) | 317 | 0.081 | 2.040 | 7.494 | 0.657 | 0.188 | 0.410 | 0.693 | 2.007 | 1.172 |
| Grp#2 Females (10%) | 315 | 0.084 | 2.084 | 7.632 | 0.733 | 0.200 | 0.421 | 0.738 | 2.020 | 1.186 |

**Table 10 (cont'd): Summary Mean Organ/Body Weight Ratios (%)-(MALES & FEMALES)**

| | ADRENALS | KIDNEYS | LIVER | TESTES | EPIDIDYMIDES | THYMUS | SPLEEN | BRAIN | HEART |
|---|---|---|---|---|---|---|---|---|---|
| Grp#1 Males (0%) | 0.016 | 0.681 | 2.6404 | 0.745 | 0.318 | 0.094 | 0.196 | 0.440 | 0.346 |
| Grp#2 Males (10%) | 0.016 | 0.662 | 2.645 | 0.727 | 0.332 | 0.098 | 0.206 | 0.447 | 0.358 |

| | ADRENALS | KIDNEYS | LIVER | UTERUS | OVARIES | THYMUS | SPLEEN | BRAIN | HEART |
|---|---|---|---|---|---|---|---|---|---|
| Grp#1 Females (0%) | 0.026 | 0.646 | 2.368 | 0.208 | 0.059 | 0.129 | 0.219 | 0.637 | 0.372 |
| Grp#2 Females (10%) | 0.027 | 0.662 | 2.425 | 0.235 | 0.063 | 0.134 | 0.235 | 0.644 | 0.377 |

# FINAL REPORT

## 90-DAY REPEATED-ORAL DOSE TOXICITY STUDY OF MALTOSYLTREHALOSE SYRUP IN RATS

Experiment No. G768 (499-047)

February 24, 2017

SPONSOR
Hayashibara Co., Ltd.

TESTING FACILITY
Public Interest Incorporated Foundation
Biosafety Research Center



I, the undersigned, hereby declare that this document is a photocopy of the original.
Date: February 24, 2017
SD: Sho Tanaka

Table 3 Body weight Exp. No. G768 (499-047)

Sex: Male

| Group No. Dose mg/kg/day | | Day of treatment 1 | 8 | 15 | 22 | 29 | 36 | 43 | 50 | 57 | 64 | 71 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Unit:g | | | | | |
| 01 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Control | Mean | 181 | 226 | 267 | 299 | 330 | 353 | 379 | 396 | 414 | 429 | 445 |
| 0 | S.D. | 8 | 17 | 26 | 29 | 33 | 35 | 37 | 39 | 42 | 44 | 46 |
| 02 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 182 | 229 | 272 | 308 | 337 | 360 | 384 | 402 | 419 | 434 | 451 |
| 1000 | S.D. | 8 | 12 | 16 | 20 | 25 | 30 | 35 | 42 | 46 | 47 | 50 |
| 03 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 180 | 220 | 261 | 296 | 327 | 353 | 379 | 400 | 419 | 435 | 452 |
| 3000 | S.D. | 9 | 14 | 21 | 29 | 35 | 38 | 42 | 46 | 49 | 53 | 56 |
| 04 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 181 | 227 | 273 | 313 | 346 | 373 | 396 | 417 | 434 | 448 | 463 |
| 5000 | S.D. | 8 | 13 | 18 | 24 | 26 | 28 | 32 | 32 | 34 | 34 | 34 |

No significant difference from the control.
MT-Syr.: Maltosyl Trehalose Syrup

Table 3 -continued Body weight Exp. No. G768 (499-047)

Sex: Male

| Group No. Dose mg/kg/day | | Day of treatment 78 | 85 | 90 | Gain ( 1-90) |
|---|---|---|---|---|---|
| | | Unit:g | | | |
| 01 | N | 10 | 10 | 10 | 10 |
| Control | Mean | 459 | 469 | 469 | 287 |
| 0 | S.D. | 45 | 47 | 46 | 42 |
| 02 | N | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 463 | 477 | 476 | 294 |
| 1000 | S.D. | 55 | 58 | 54 | 49 |
| 03 | N | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 467 | 478 | 479 | 299 |
| 3000 | S.D. | 57 | 60 | 58 | 53 |
| 04 | N | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 476 | 488 | 487 | 306 |
| 5000 | S.D. | 36 | 36 | 36 | 29 |

No significant difference from the control.
MT-Syr.: Maltosyl Trehalose Syrup

Table 3 -continued Body weight Exp. No. G768 (499-047)

Sex: Female

| Group No. Dose mg/kg/day | | Day of treatment 1 | 8 | 15 | 22 | 29 | 36 | 43 | 50 | 57 | 64 | 71 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Unit:g |
| 01 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Control | Mean | 173 | 193 | 215 | 231 | 242 | 252 | 262 | 271 | 279 | 285 | 292 |
| 0 | S.D. | 6 | 9 | 11 | 15 | 18 | 20 | 22 | 23 | 24 | 22 | 25 |
| 02 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 173 | 196 | 220 | 236 | 252 | 262 | 275 | 284 | 294 | 301 | 308 |
| 1000 | S.D. | 6 | 9 | 11 | 13 | 18 | 20 | 21 | 22 | 25 | 24 | 24 |
| 03 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 173 | 196 | 223 | 234 | 249 | 259 | 273 | 279 | 284 | 292 | 299 |
| 3000 | S.D. | 6 | 6 | 12 | 12 | 12 | 12 | 16 | 19 | 21 | 23 | 25 |
| 04 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 173 | 195 | 213 | 228 | 243 | 254 | 265 | 276 | 283 | 292 | 296 |
| 5000 | S.D. | 6 | 8 | 12 | 11 | 9 | 13 | 14 | 17 | 18 | 17 | 19 |

No significant difference from the control.
MT-Syr.: Maltosyl Trehalose Syrup

Table 3 -continued Body weight Exp. No. G768 (499-047)

Sex: Female

| Group No. / Dose mg/kg/day | | Day of treatment 78 | 85 | 90 | Unit:g Gain ( 1-90) |
|---|---|---|---|---|---|
| 01 | N | 10 | 10 | 10 | 10 |
| Control | Mean | 296 | 302 | 298 | 125 |
| 0 | S.D. | 24 | 26 | 26 | 25 |
| 02 | N | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 314 | 317 | 315 | 142 |
| 1000 | S.D. | 24 | 26 | 25 | 25 |
| 03 | N | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 304 | 308 | 307 | 135 |
| 3000 | S.D. | 26 | 25 | 25 | 25 |
| 04 | N | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 301 | 307 | 304 | 132 |
| 5000 | S.D. | 19 | 18 | 18 | 20 |

No significant difference from the control.
MT-Syr.: Maltosyl Trehalose Syrup

Table 4 Food consumption Exp. No. G768 (499-047)

Sex: Male

| Group No. / Dose mg/kg/day | | Day of treatment 1 => 8 | 8 => 15 | 15 => 22 | 22 => 29 | 29 => 36 | 36 => 43 | 43 => 50 | 50 => 57 | 57 => 64 | 64 => 71 | 71 => 78 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Unit:g/animal/day | | | |
| 01 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Control | Mean | 23 | 22 | 23 | 23 | 23 | 24 | 23 | 23 | 23 | 23 | 23 |
| 0 | S.D. | 2 | 3 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 2 |
| 02 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 22 | 22 | 22 | 22 |
| 1000 | S.D. | 2 | 2 | 1 | 2 | 2 | 2 | 3 | 2 | 2 | 2 | 2 |
| 03 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 22 | 21 | 22 | 22 | 22 | 22 | 22 | 21 | 21 | 21 | 21 |
| 3000 | S.D. | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 2 | 2 | 3 |
| 04 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 21 | 22 | 22 | 22 | 22 | 21* | 21 | 20* | 20* | 20* | 20* |
| 5000 | S.D. | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

Significant difference from the control; *: P ≤ 0.05 (Dunnett's multiple test)
MT-Syr.: Maltosyl Trehalose Syrup

Table 4 -continued Food consumption Exp. No. G768 (499-047)

Sex: Male

| Group No. / Dose mg/kg/day | | Day of treatment 78 => 85 | 85 => 90 | Unit:g/animal/day |
|---|---|---|---|---|
| 01 Control 0 | N | 10 | 10 | |
| | Mean | 22 | 20 | |
| | S.D. | 2 | 2 | |
| 02 MT-Syr. 1000 | N | 10 | 10 | |
| | Mean | 22 | 19 | |
| | S.D. | 3 | 1 | |
| 03 MT-Syr. 3000 | N | 10 | 10 | |
| | Mean | 21 | 18 | |
| | S.D. | 3 | 2 | |
| 04 MT-Syr. 5000 | N | 10 | 10 | |
| | Mean | 20* | 17** | |
| | S.D. | 1 | 1 | |

Significant difference from the control; *: P ≤ 0.05 , **: P ≤ 0.01 (Dunnett's multiple test)
MT-Syr.: Maltosyl Trehalose Syrup

Table 4 -continued Food consumption Exp. No. G768 (499-047)

Sex: Female

| Group No. / Dose mg/kg/day | | Day of treatment 1 => 8 | 8 => 15 | 15 => 22 | 22 => 29 | 29 => 36 | 36 => 43 | 43 => 50 | Unit:g/animal/day 50 => 57 | 57 => 64 | 64 => 71 | 71 => 78 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 01 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Control | Mean | 18 | 19 | 19 | 19 | 18 | 18 | 18 | 18 | 17 | 17 | 17 |
| 0 | S.D. | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 1 | 1 | 1 | 2 |
| 02 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 18 | 18 | 18 |
| 1000 | S.D. | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 2 |
| 03 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 18 | 19 | 19 | 19 | 19 | 18 | 18 | 17 | 17 | 17 | 16 |
| 3000 | S.D. | 1 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 3 | 3 | 2 |
| 04 | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| MT-Syr. | Mean | 18 | 17* | 17 | 17* | 17 | 17 | 17 | 16 | 16 | 16 | 16 |
| 5000 | S.D. | 1 | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 1 |

Significant difference from the control; *: $P \leq 0.05$ (Dunnett's multiple test)
MT-Syr.: Maltosyl Trehalose Syrup

Table 5. -continued Hematology

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | WBC ($\times 10^3/mm^3$) | Differential leukocyte ratios (%) NEUT | LYMPH | MONO | EOSN | BASO | LUC |
|---|---|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 7.34 ± 1.89 | 18.8 ± 4.5 | 76.2 ± 5.8 | 2.8 ± 1.0 | 1.6 ± 0.7 | 0.1 ± 0.0N | 0.6 ± 0.4N |
| | 1000 | 10 | 7.39 ± 1.90 | 15.1 ± 7.7 | 80.8 ± 7.8 | 2.4 ± 0.5 | 1.3 ± 0.5 | 0.1 ± 0.0 | 0.4 ± 0.1 |
| | 3000 | 10 | 8.34 ± 2.98 | 17.1 ± 6.3 | 78.7 ± 6.6 | 2.1 ± 0.8 | 1.5 ± 0.5 | 0.1 ± 0.1 | 0.5 ± 0.3 |
| | 5000 | 10 | 7.13 ± 2.98 | 16.9 ± 6.5 | 78.3 ± 7.5 | 2.7 ± 0.9 | 1.5 ± 0.6 | 0.1 ± 0.1 | 0.5 ± 0.3 |
| Female | 0 | 10 | 4.60 ± 1.04N | 13.8 ± 3.7 | 79.8 ± 5.8 | 3.5 ± 2.0N | 2.4 ± 0.9N | 0.1 ± 0.1 | 0.4 ± 0.1N |
| | 1000 | 10 | 4.18 ± 0.79 | 14.7 ± 4.0 | 78.9 ± 4.3 | 3.3 ± 1.0 | 2.4 ± 0.6 | 0.1 ± 0.1 | 0.6 ± 0.3 |
| | 3000 | 10 | 3.88 ± 1.15 | 14.0 ± 6.0 | 79.8 ± 6.2 | 2.8 ± 0.7 | 2.9 ± 0.7 | 0.1 ± 0.1 | 0.5 ± 0.3 |
| | 5000 | 10 | 5.21 ± 1.95 | 12.9 ± 4.9 | 81.3 ± 5.8 | 2.5 ± 0.6 | 2.8 ± 1.4 | 0.1 ± 0.1 | 0.5 ± 0.1 |

NEUT: Neutrophil LYMPH: Lymphocyte MONO: Monocyte EOSN: Eosinophil BASO: Basophil LUC: Large unstained cells
Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 5. -continued Hematology

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Reticulocyte (%) | Reticulocyte ($\times 10^9$/L) | PLT ($\times 10^3$/mm$^3$) |
|---|---|---|---|---|---|
| Male | 0 | 10 | 1.8 ± 0.2 | 159.2 ± 13.1 | 968 ± 107 |
| | 1000 | 10 | 1.9 ± 0.3 | 162.4 ± 27.1 | 925 ± 91 |
| | 3000 | 10 | 1.9 ± 0.3 | 162.0 ± 25.3 | 1042 ± 97 |
| | 5000 | 10 | 1.8 ± 0.3 | 156.3 ± 19.4 | 939 ± 111 |
| Female | 0 | 10 | 1.6 ± 0.2 | 135.9 ± 16.0 | 1027 ± 105N |
| | 1000 | 10 | 1.9 ± 0.4 | 150.3 ± 26.0 | 1044 ± 83 |
| | 3000 | 10 | 1.7 ± 0.4 | 140.6 ± 27.2 | 1055 ± 108 |
| | 5000 | 10 | 1.7 ± 0.3 | 135.3 ± 29.5 | 971 ± 208 |

Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 5. Hematology

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | HCT (%) | HGB (g/dL) | RBC ($\times 10^6/mm^3$) | MCV ($\mu m^3$) | MCH (pg) | MCHC (%) |
|---|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 44.5 ± 1.0N | 15.5 ± 0.3N | 8.78 ± 0.29 | 50.7 ± 0.9 | 17.6 ± 0.4 | 34.8 ± 0.2N |
| | 1000 | 10 | 44.7 ± 1.0 | 15.6 ± 0.4 | 8.65 ± 0.23 | 51.8 ± 1.7 | 18.1 ± 0.7 | 34.9 ± 0.3 |
| | 3000 | 10 | 44.4 ± 1.6 | 15.5 ± 0.5 | 8.54 ± 0.29 | 52.0 ± 1.2 | 18.2 ± 0.4 | 35.0 ± 0.4 |
| | 5000 | 10 | 45.5 ± 2.4 | 15.8 ± 0.9 | 8.73 ± 0.50 | 52.2 ± 1.2* | 18.1 ± 0.4 | 34.7 ± 0.7 |
| Female | 0 | 10 | 43.6 ± 1.8 | 15.5 ± 0.5 | 8.24 ± 0.38 | 52.9 ± 1.0 | 18.8 ± 0.5 | 35.6 ± 0.4 |
| | 1000 | 10 | 43.1 ± 1.7 | 15.3 ± 0.5 | 8.11 ± 0.35 | 53.1 ± 1.1 | 18.8 ± 0.4 | 35.5 ± 0.4 |
| | 3000 | 10 | 43.2 ± 1.1 | 15.3 ± 0.4 | 8.18 ± 0.24 | 52.8 ± 1.4 | 18.7 ± 0.5 | 35.4 ± 0.2 |
| | 5000 | 10 | 42.6 ± 1.6 | 15.2 ± 0.4 | 8.15 ± 0.31 | 52.3 ± 1.1 | 18.6 ± 0.4 | 35.6 ± 0.5 |

Mean ± S.D.
Significant difference from the control; *: $P \leq 0.05$ (Dunnett's multiple test)
N: Non parametric analysis

Table 4 -continued Food consumption Exp. No. G768 (499-047)

Sex: Female

| Group No. / Dose mg/kg/day | | Day of treatment 78 => 85 | 85 => 90 | Unit:g/animal/day |
|---|---|---|---|---|
| 01 Control 0 | N | 10 | 10 | |
| | Mean | 17 | 15 | |
| | S.D. | 1 | 2 | |
| 02 MT-Syr. 1000 | N | 10 | 10 | |
| | Mean | 18 | 16 | |
| | S.D. | 2 | 2 | |
| 03 MT-Syr. 3000 | N | 10 | 10 | |
| | Mean | 17 | 15 | |
| | S.D. | 2 | 2 | |
| 04 MT-Syr. 5000 | N | 10 | 10 | |
| | Mean | 16 | 14 | |
| | S.D. | 1 | 1 | |

No significant difference from the control
MT-Syr.: Maltosyl Trehalose Syrup

Table 5. -continued Hematology

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | NEUT ($\times10^3/mm^3$) | LYMPH ($\times10^3/mm^3$) | MONO ($\times10^3/mm^3$) | EOSN ($\times10^3/mm^3$) | BASO ($\times10^3/mm^3$) | LUC ($\times10^3/mm^3$) |
|---|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 1.34 ± 0.35 | 5.64 ± 1.69 | 0.21 ± 0.10 | 0.11 ± 0.04 | 0.01 ± 0.00 | 0.04 ± 0.04N |
| | 1000 | 10 | 1.07 ± 0.48 | 6.01 ± 1.84 | 0.18 ± 0.05 | 0.10 ± 0.05 | 0.01 ± 0.01 | 0.03 ± 0.01 |
| | 3000 | 10 | 1.33 ± 0.44 | 6.66 ± 2.64 | 0.17 ± 0.10 | 0.12 ± 0.05 | 0.01 ± 0.01 | 0.05 ± 0.03 |
| | 5000 | 10 | 1.08 ± 0.35 | 5.71 ± 2.72 | 0.19 ± 0.11 | 0.10 ± 0.04 | 0.01 ± 0.01 | 0.04 ± 0.03 |
| Female | 0 | 10 | 0.63 ± 0.22 | 3.68 ± 0.93N | 0.16 ± 0.10N | 0.11 ± 0.04N | 0.00 ± 0.00 | 0.02 ± 0.01 |
| | 1000 | 10 | 0.62 ± 0.20 | 3.30 ± 0.68 | 0.13 ± 0.04 | 0.10 ± 0.03 | 0.00 ± 0.00 | 0.03 ± 0.01 |
| | 3000 | 10 | 0.59 ± 0.45 | 3.05 ± 0.72 | 0.11 ± 0.03 | 0.11 ± 0.05 | 0.00 ± 0.00 | 0.02 ± 0.02 |
| | 5000 | 10 | 0.67 ± 0.35 | 4.24 ± 1.67 | 0.13 ± 0.06 | 0.14 ± 0.09 | 0.00 ± 0.00 | 0.03 ± 0.01 |

NEUT: Neutrophil LYMPH: Lymphocyte MONO: Monocyte EOSN: Eosinophil BASO: Basophil LUC: Large unstained cells
Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 5. -continued Hematology

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | PT (sec.) | APTT (sec.) | Fibrinogen (mg/dL) |
|---|---|---|---|---|---|
| Male | 0 | 10 | 13.5 ± 2.3 | 25.3 ± 2.5 | 274 ± 25 |
| | 1000 | 10 | 12.7 ± 2.0 | 23.7 ± 2.3 | 283 ± 26 |
| | 3000 | 10 | 11.8 ± 2.2 | 23.5 ± 1.7 | 282 ± 17 |
| | 5000 | 10 | 15.1 ± 4.3 | 25.2 ± 3.9 | 284 ± 32 |
| Female | 0 | 10 | 8.7 ± 0.2 | 16.2 ± 0.5N | 216 ± 32 |
| | 1000 | 10 | 8.5 ± 0.2 | 17.2 ± 2.0 | 206 ± 20 |
| | 3000 | 10 | 8.6 ± 0.2 | 16.8 ± 2.1 | 209 ± 19 |
| | 5000 | 10 | 8.8 ± 0.2 | 16.2 ± 0.9 | 215 ± 20 |

Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 7. Urinalysis

Exp. No. G768 (499-047)

Day: 86

| Sex | Dose level (mg/kg/day) | No. of animals | Volume (mL) | Osmotic pressure (mOsm/kg) | Sodium (mmol/L) | Potassium (mmol/L) | Chloride (mmol/L) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 14.5 ± 4.9N | 1330 ± 431 | 63.8 ± 32.4 | 163.6 ± 60.8 | 96.1 ± 48.7 |
| | 1000 | 10 | 18.4 ± 8.0 | 1014 ± 384 | 47.7 ± 26.2 | 120.7 ± 48.2 | 72.4 ± 34.9 |
| | 3000 | 10 | 11.2 ± 3.3 | 1398 ± 359 | 69.8 ± 25.7 | 174.8 ± 51.3 | 96.9 ± 36.5 |
| | 5000 | 10 | 15.9 ± 8.0 | 1181 ± 637 | 74.1 ± 53.4 | 144.4 ± 80.9 | 98.2 ± 74.9 |
| Female | 0 | 10 | 14.1 ± 5.0 | 1196 ± 374 | 85.1 ± 41.5 | 149.4 ± 51.7 | 108.1 ± 52.5 |
| | 1000 | 10 | 11.3 ± 2.8 | 1427 ± 346 | 92.4 ± 28.4 | 175.2 ± 44.3 | 122.9 ± 40.6 |
| | 3000 | 10 | 12.1 ± 5.1 | 1417 ± 381 | 96.9 ± 27.6 | 184.0 ± 49.7 | 135.2 ± 37.1 |
| | 5000 | 10 | 9.7 ± 4.2 | 1425 ± 410 | 87.6 ± 22.7 | 173.6 ± 55.1 | 117.2 ± 41.8 |

Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 6. Blood chemistry

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Glucose (mg/dL) | Triglyceride (mg/dL) | T.cholesterol (mg/dL) | BUN (mg/dL) | Creatinine (mg/dL) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 153 ± 6N | 63 ± 21N | 57 ± 12 | 13.9 ± 1.6 | 0.31 ± 0.02N |
| | 1000 | 10 | 155 ± 13 | 51 ± 20 | 59 ± 12 | 14.3 ± 2.0 | 0.31 ± 0.05 |
| | 3000 | 10 | 153 ± 18 | 79 ± 48 | 67 ± 9 | 14.5 ± 2.6 | 0.34 ± 0.04 |
| | 5000 | 10 | 170 ± 17 | 97 ± 36 | 60 ± 7 | 14.7 ± 3.2 | 0.34 ± 0.06 |
| Female | 0 | 10 | 139 ± 18 | 31 ± 13 | 77 ± 13 | 15.1 ± 2.3N | 0.39 ± 0.04N |
| | 1000 | 10 | 142 ± 15 | 48 ± 22 | 79 ± 18 | 15.0 ± 1.2 | 0.37 ± 0.04 |
| | 3000 | 10 | 138 ± 10 | 43 ± 19 | 70 ± 11 | 15.4 ± 2.5 | 0.39 ± 0.10 |
| | 5000 | 10 | 139 ± 14 | 29 ± 10 | 73 ± 21 | 15.2 ± 4.4 | 0.42 ± 0.09 |

Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 6. -continued Blood chemistry

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | T.bilirubin (mg/dL) | AST (U/L) | ALT (U/L) | ALP (U/L) | Gamma-GTP (U/L) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 0.06 ± 0.01 | 73 ± 16N | 27 ± 5 | 332 ± 83 | 0.3 ± 0.3 |
| | 1000 | 10 | 0.07 ± 0.01 | 60 ± 5 | 22 ± 2 | 305 ± 63 | 0.4 ± 0.2 |
| | 3000 | 10 | 0.07 ± 0.01 | 75 ± 19 | 28 ± 6 | 292 ± 41 | 0.4 ± 0.2 |
| | 5000 | 10 | 0.07 ± 0.01 | 60 ± 8 | 24 ± 5 | 298 ± 59 | 0.3 ± 0.2 |
| Female | 0 | 10 | 0.09 ± 0.02 | 107 ± 66N | 59 ± 74N | 144 ± 35 | 0.8 ± 0.7N |
| | 1000 | 10 | 0.09 ± 0.03 | 80 ± 24 | 30 ± 17 | 110 ± 36 | 0.4 ± 0.2 |
| | 3000 | 10 | 0.09 ± 0.01 | 64 ± 9# | 21 ± 3# | 150 ± 44 | 0.5 ± 0.3 |
| | 5000 | 10 | 0.09 ± 0.02 | 65 ± 7# | 17 ± 3## | 144 ± 38 | 0.6 ± 0.3 |

Mean ± S.D.
Significant difference from the control; #: $P \leq 0.05$ ##: $P \leq 0.01$ (Steel's test)
N: Non parametric analysis

Table 6. -continued Blood chemistry

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Calcium (mg/dL) | I.phosphorus (mg/dL) | Sodium (mmol/L) | Potassium (mmol/L) | Chloride (mmol/L) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 9.65 ± 0.19 | 6.09 ± 0.67 | 143.6 ± 1.0 | 4.72 ± 0.20 | 106.5 ± 1.2 |
| | 1000 | 10 | 9.71 ± 0.21 | 5.87 ± 0.44 | 143.9 ± 0.8 | 4.67 ± 0.27 | 107.4 ± 1.2 |
| | 3000 | 10 | 9.69 ± 0.19 | 6.03 ± 0.50 | 143.4 ± 1.1 | 4.67 ± 0.30 | 106.7 ± 1.2 |
| | 5000 | 10 | 9.72 ± 0.34 | 5.72 ± 0.68 | 143.4 ± 0.9 | 4.79 ± 0.22 | 107.2 ± 1.0 |
| Female | 0 | 10 | 9.92 ± 0.33 | 5.18 ± 0.87 | 142.5 ± 1.0 | 4.40 ± 0.25 | 107.4 ± 1.2 |
| | 1000 | 10 | 10.02 ± 0.41 | 4.68 ± 0.60 | 142.1 ± 1.0 | 4.35 ± 0.23 | 107.2 ± 1.6 |
| | 3000 | 10 | 9.88 ± 0.37 | 4.85 ± 0.74 | 142.5 ± 0.8 | 4.35 ± 0.19 | 107.0 ± 1.3 |
| | 5000 | 10 | 9.82 ± 0.38 | 4.99 ± 0.45 | 142.3 ± 0.9 | 4.39 ± 0.26 | 107.8 ± 1.4 |

Mean ± S.D.
No significant difference from the control.

Table 6. -continued Blood chemistry

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Albumin (%) | Alpha-1 (%) | Alpha-2 (%) | Beta (%) | Gamma (%) | A/G |
|---|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 51.7 ± 2.8 | 20.3 ± 2.4 | 8.2 ± 1.0 | 14.4 ± 1.1 | 5.4 ± 0.6N | 1.08 ± 0.12 |
| | 1000 | 10 | 50.5 ± 1.2 | 21.7 ± 1.9 | 7.7 ± 0.9 | 14.4 ± 1.1 | 5.7 ± 1.7 | 1.02 ± 0.05 |
| | 3000 | 10 | 52.2 ± 1.8 | 20.4 ± 2.0 | 8.1 ± 1.2 | 14.4 ± 1.3 | 4.9 ± 1.2 | 1.09 ± 0.08 |
| | 5000 | 10 | 51.9 ± 1.9 | 20.2 ± 2.4 | 8.4 ± 1.0 | 14.2 ± 0.9 | 5.4 ± 0.9 | 1.08 ± 0.08 |
| Female | 0 | 10 | 58.7 ± 2.3 | 15.2 ± 1.9 | 6.4 ± 0.9 | 14.2 ± 1.5 | 5.5 ± 1.5 | 1.43 ± 0.13 |
| | 1000 | 10 | 58.1 ± 2.1 | 17.2 ± 2.1 | 6.3 ± 1.0 | 13.1 ± 1.2 | 5.3 ± 1.2 | 1.39 ± 0.12 |
| | 3000 | 10 | 57.8 ± 2.1 | 15.8 ± 3.3 | 6.4 ± 0.9 | 13.5 ± 0.9 | 6.5 ± 1.8 | 1.38 ± 0.12 |
| | 5000 | 10 | 56.6 ± 2.4 | 15.5 ± 2.7 | 7.2 ± 1.0 | 14.0 ± 1.3 | 6.7 ± 1.4 | 1.31 ± 0.12 |

Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 7. -continued Urinalysis

Exp. No. G768 (499-047)

Day: 86

| Sex | Dose level (mg/kg/day) | No. of animals | Sodium (mmol/day) | Potassium (mmol/day) | Chloride (mmol/day) |
|---|---|---|---|---|---|
| Male | 0 | 10 | 0.85 ± 0.30 | 2.20 ± 0.52N | 1.28 ± 0.45 |
| | 1000 | 10 | 0.75 ± 0.24 | 1.90 ± 0.21 | 1.13 ± 0.25 |
| | 3000 | 10 | 0.75 ± 0.25 | 1.86 ± 0.43 | 1.05 ± 0.39 |
| | 5000 | 10 | 0.85 ± 0.38 | 1.78 ± 0.56 | 1.10 ± 0.51 |
| Female | 0 | 10 | 1.09 ± 0.36 | 1.97 ± 0.56 | 1.39 ± 0.49 |
| | 1000 | 10 | 1.03 ± 0.32 | 1.94 ± 0.45 | 1.37 ± 0.43 |
| | 3000 | 10 | 1.08 ± 0.23 | 2.02 ± 0.28 | 1.49 ± 0.20 |
| | 5000 | 10 | 0.82 ± 0.37 | 1.60 ± 0.65 | 1.10 ± 0.54 |

Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 7. -continued Urinalysis

Exp. No. G768 (499-047)

Day: 85,86

| Sex | Dose level (mg/kg/day) | No. of animals | Color 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | pH 5 | 5.5 | 6 | 6.5 | 7 | 7.5 | 8 | 8.5 | ≥9 | Occult blood - | +/- | 1+ | 2+ | 3+ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 10 | | | | | | | | | | | | | | | | | 4 | 5 | 1 | 5 | 5 | | | |
| | 1000 | 10 | 10 | | | | | | | | | | | | | | | | | 2 | 5 | 3 | 4 | 5 | 1 | | |
| | 3000 | 10 | 10 | | | | | | | | | | | | | | | | 1 | | 4 | 5 | 7 | 3 | | | |
| | 5000 | 10 | 10 | | | | | | | | | | | | | | | | 1 | | 7 | 2 | 1 | 9 | | | |
| Female | 0 | 10 | 10 | | | | | | | | | | | | | 1 | | | | 1 | 2 | 6 | 9 | 1 | | | |
| | 1000 | 10 | 10 | | | | | | | | | | | | | 1 | | | | 2 | 2 | 5 | 9 | 1 | | | |
| | 3000 | 10 | 10 | | | | | | | | | | | | | | 1 | | | | 3 | 5 | 1 | 9 | 1 | | |
| | 5000 | 10 | 10 | | | | | | | | | | | | | | | 2 | | | 1 | 7 | 10 | | | | |

Color : 1= Colorless, 2= Slight yellow, 3= Yellow-brown, 4= Red, 5= Red-brown, 6= Dark red, 7= Dark brown, 8= Brown-black, 9= Milky white, 10= Fluorescent green, 11= Blue

Table 7, -continued Urinalysis

Exp. No. G768 (499-047)

Day: 85

| Sex | Dose level (mg/kg/day) | No. of animals | Ketone bodies - | +/- | 1+ | 2+ | 3+ | 4+ | Glucose(g/dL) - | 0.1 | 0.25 | 0.5 | ≥1.0 | Protein(mg/dL) - | +/- | 30 | 100 | ≥300 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 8 | 1 | 1 | | | | 10 | | | | | 7 | 2 | 1 | | |
| | 1000 | 10 | 9 | 1 | | | | | 10 | | | | | 5 | 4 | 1 | | |
| | 3000 | 10 | | | 2 | 7 | 1 | | 10 | | | | | | 1 | 6 | 3 | |
| | 5000 | 10 | 4 | 3 | 3 | | | | 10 | | | | | | 6 | 3 | 1 | |
| Female | 0 | 10 | 9 | | 1 | | | | 10 | | | | | 9 | | | 1 | |
| | 1000 | 10 | 9 | 1 | | | | | 10 | | | | | 8 | 1 | 1 | | |
| | 3000 | 10 | 8 | 2 | | | | | 10 | | | | | 6 | 3 | 1 | | |
| | 5000 | 10 | 6 | 4 | | | | | 10 | | | | | 1 | 5 | 4 | | |

Table 7. -continued Urinalysis

Exp. No. G768 (499-047)

Day: 85

| Sex | Dose level (mg/kg/day) | No. of animals | Bilirubin - | 1+ | 2+ | 3+ | Urobilinogen (E.U./dL) 0.1 | 1.0 | 2.0 | 4.0 | 8.0 | ≥ 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 10 | | | | 10 | | | | | |
| | 1000 | 10 | 10 | | | | 10 | | | | | |
| | 3000 | 10 | 10 | | | | 6 | 4 | | | | |
| | 5000 | 10 | 10 | | | | 10 | | | | | |
| Female | 0 | 10 | 10 | | | | 9 | 1 | | | | |
| | 1000 | 10 | 10 | | | | 9 | 1 | | | | |
| | 3000 | 10 | 10 | | | | 9 | 1 | | | | |
| | 5000 | 10 | 10 | | | | 6 | 4 | | | | |

Table 7. -continued Urinalysis : Microscopic examination of sediment Exp. No. G768 (499-047)

Day: 86

| Sex | Dose level (mg/kg/day) | No. of animals | Erythrocytes - 1+ 2+ 3+ | Leukocytes - 1+ 2+ 3+ | Squamous cells - 1+ 2+ 3+ | Transitional epi. - 1+ 2+ 3+ | Renal tubular epi. - 1+ 2+ 3+ |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 10 | 10 | 10 | 10 | 10 |
| | 1000 | 10 | 10 | 10 | 10 | 10 | 10 |
| | 3000 | 10 | 10 | 10 | 10 | 10 | 10 |
| | 5000 | 10 | 10 | 10 | 10 | 10 | 10 |
| Female | 0 | 10 | 10 | 9 1 | 10 | 10 | 10 |
| | 1000 | 10 | 10 | 10 | 10 | 10 | 10 |
| | 3000 | 10 | 10 | 9 1 | 10 | 10 | 10 |
| | 5000 | 10 | 10 | 9 1 | 10 | 10 | 10 |

epi.: epithelial cells

Table 7. -continued Urinalysis : Microscopic examination of sediment Exp. No. G768 (499-047)

Day: 86

| Sex | Dose level (mg/kg/day) | No. of animals | Casts - | Casts + | Fat globules - | Fat globules + | Mucous threads - | Mucous threads + | Crystals - | Crystals + |
|---|---|---|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 10 | | 10 | | 10 | | 10 | |
| | 1000 | 10 | 10 | | 10 | | 10 | | 10 | |
| | 3000 | 10 | 10 | | 10 | | 9 | 1 | 10 | |
| | 5000 | 10 | 10 | | 10 | | 9 | 1 | 10 | |
| Female | 0 | 10 | 10 | | 10 | | 10 | | 10 | |
| | 1000 | 10 | 10 | | 10 | | 10 | | 10 | |
| | 3000 | 10 | 10 | | 10 | | 10 | | 10 | |
| | 5000 | 10 | 10 | | 10 | | 10 | | 10 | |

Table 8. -continued Ophthalmology Exp. No. G768(499-047)

Day86 Sex: Male

| Dose level (mg/kg/day) | 0 | 5000 |
|---|---|---|
| Number of animals | 10 | 10 |
| Anterior part (Conjunctiva,Sclera,Cornea,Iris) | | |
| Normal | 2 | 7 |
| Corneal opacity | 8 | 3 |
| Optic media (Posterior chamber,Lens,Corpus vitreum) | | |
| Normal | 7 | 6 |
| Particulate opacity in lens | 3 | 4 |
| Fundus oculi | | |
| Normal | 9 | 10 |
| Chorioretinal atrophy | 1 | 0 |

Table 8. -continued Ophthalmology Exp. No. G768(499-047)

Day86 Sex: Female

| Dose level (mg/kg/day) | 0 | 5000 |
|---|---|---|
| Number of animals | 10 | 10 |
| Anterior part (Conjunctiva,Sclera,Cornea,Iris) | | |
| Normal | 4 | 5 |
| Corneal opacity | 6 | 5 |
| Optic media (Posterior chamber,Lens,Corpus vitreum) | | |
| Normal | 8 | 8 |
| Particulate opacity in lens | 2 | 2 |
| Fundus oculi | | |
| Normal | 10 | 10 |

Table 9. Organ weight

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Body weight (g) | Brain (g) | Heart (g) | Lungs (g) | Liver (g) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 446 ± 45 | 2.22 ± 0.09 | 1.35 ± 0.14 | 1.40 ± 0.14 | 11.02 ± 0.94 |
| | 1000 | 10 | 452 ± 52 | 2.19 ± 0.09 | 1.32 ± 0.15 | 1.38 ± 0.14 | 11.22 ± 1.99 |
| | 3000 | 10 | 459 ± 57 | 2.17 ± 0.10 | 1.34 ± 0.12 | 1.37 ± 0.09 | 11.59 ± 1.59 |
| | 5000 | 10 | 465 ± 33 | 2.19 ± 0.06 | 1.33 ± 0.12 | 1.38 ± 0.11 | 11.46 ± 1.30 |
| Female | 0 | 10 | 286 ± 23 | 2.08 ± 0.07 | 0.87 ± 0.05 | 1.10 ± 0.06 | 6.54 ± 0.65 |
| | 1000 | 10 | 300 ± 26 | 2.06 ± 0.04 | 0.95 ± 0.09 | 1.12 ± 0.06 | 7.32 ± 0.84 |
| | 3000 | 10 | 292 ± 25 | 2.08 ± 0.08 | 0.93 ± 0.12 | 1.13 ± 0.08 | 6.88 ± 0.88 |
| | 5000 | 10 | 291 ± 18 | 2.08 ± 0.07 | 0.92 ± 0.10 | 1.15 ± 0.07 | 6.80 ± 0.50 |

Mean ± S.D.
No significant difference from the control.

Table 9. -continued Organ weight

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Kidneys (g) | Spleen (g) | Adrenal glands (mg) | Testes (g) | Ovaries (mg) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 2.90 ± 0.28 | 0.65 ± 0.09 | 55 ± 9 | 3.33 ± 0.39 | |
| | 1000 | 10 | 2.93 ± 0.27 | 0.72 ± 0.12 | 54 ± 7 | 3.16 ± 0.34 | |
| | 3000 | 10 | 2.87 ± 0.32 | 0.67 ± 0.11 | 56 ± 7 | 3.20 ± 0.28 | |
| | 5000 | 10 | 2.75 ± 0.25 | 0.66 ± 0.11 | 53 ± 7 | 3.33 ± 0.22 | |
| Female | 0 | 10 | 1.86 ± 0.19 | 0.47 ± 0.05 | 63 ± 8 | | 74 ± 11 |
| | 1000 | 10 | 1.98 ± 0.16 | 0.51 ± 0.03 | 67 ± 6 | | 90 ± 9* |
| | 3000 | 10 | 1.93 ± 0.23 | 0.45 ± 0.08 | 68 ± 14 | | 93 ± 15** |
| | 5000 | 10 | 1.90 ± 0.11 | 0.51 ± 0.05 | 67 ± 9 | | 88 ± 15 |

Mean ± S.D.
Significant difference from the control; *: $P \leq 0.05$ **: $P \leq 0.01$ (Dunnett's multiple test)

Table 9. -continued Organ weight

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Thyroid glands (mg) | Pituitary (mg) | Thymus (mg) | Prostate (mg) | Uterus (mg) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 24 ± 5 | 12 ± 3 | 250 ± 45 | 1549 ± 229 | |
| | 1000 | 10 | 27 ± 8 | 12 ± 2 | 347 ± 94** | 1474 ± 185 | |
| | 3000 | 10 | 24 ± 6 | 13 ± 2 | 292 ± 65 | 1532 ± 245 | |
| | 5000 | 10 | 24 ± 5 | 12 ± 1 | 290 ± 65 | 1613 ± 260 | |
| Female | 0 | 10 | 20 ± 3 | 16 ± 2 | 257 ± 54 | | 628 ± 225N |
| | 1000 | 10 | 23 ± 5 | 19 ± 3 | 238 ± 61 | | 707 ± 304 |
| | 3000 | 10 | 22 ± 3 | 16 ± 4 | 275 ± 105 | | 640 ± 239 |
| | 5000 | 10 | 22 ± 4 | 16 ± 3 | 249 ± 52 | | 575 ± 91 |

Mean ± S.D.
Significant difference from the control; **: P ≤ 0.01 (Dunnett's multiple test)
N: Non parametric analysis

Table 9. -continued Organ weight

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Epididymides (mg) | Salivary glands (g) | Seminal vesicle (mg) |
|---|---|---|---|---|---|
| Male | 0 | 10 | 1279 ± 104N | 0.68 ± 0.08N | 1408 ± 250 |
| | 1000 | 10 | 1278 ± 125 | 0.75 ± 0.14 | 1468 ± 193 |
| | 3000 | 10 | 1201 ± 88 | 0.68 ± 0.06 | 1543 ± 188 |
| | 5000 | 10 | 1256 ± 230 | 0.69 ± 0.07 | 1579 ± 254 |
| Female | 0 | 10 | | 0.44 ± 0.03 | |
| | 1000 | 10 | | 0.47 ± 0.04 | |
| | 3000 | 10 | | 0.47 ± 0.06 | |
| | 5000 | 10 | | 0.47 ± 0.04 | |

Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 10. Organ weight per body weight

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Body weight (g) | Brain (%) | Heart (%) | Lungs (%) | Liver (%) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 446 ± 45 | 0.502 ± 0.053 | 0.304 ± 0.027 | 0.316 ± 0.028N | 2.477 ± 0.147 |
| | 1000 | 10 | 452 ± 52 | 0.490 ± 0.048 | 0.292 ± 0.012 | 0.307 ± 0.018 | 2.469 ± 0.218 |
| | 3000 | 10 | 459 ± 57 | 0.479 ± 0.051 | 0.293 ± 0.021 | 0.302 ± 0.032 | 2.529 ± 0.172 |
| | 5000 | 10 | 465 ± 33 | 0.474 ± 0.036 | 0.285 ± 0.017 | 0.296 ± 0.012 | 2.460 ± 0.131 |
| Female | 0 | 10 | 286 ± 23 | 0.733 ± 0.065 | 0.306 ± 0.020 | 0.386 ± 0.036 | 2.291 ± 0.170 |
| | 1000 | 10 | 300 ± 26 | 0.691 ± 0.064 | 0.319 ± 0.021 | 0.376 ± 0.027 | 2.440 ± 0.166 |
| | 3000 | 10 | 292 ± 25 | 0.714 ± 0.055 | 0.318 ± 0.022 | 0.389 ± 0.027 | 2.345 ± 0.120 |
| | 5000 | 10 | 291 ± 18 | 0.717 ± 0.029 | 0.315 ± 0.029 | 0.396 ± 0.031 | 2.338 ± 0.117 |

Mean ± S.D.
No significant difference from the control.
N: Non parametric analysis

Table 10. -continued Organ weight per body weight

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Kidneys (%) | Spleen (%) | Adrenal glands (%) | Testes (%) | Ovaries (%) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 0.654 ± 0.058 | 0.146 ± 0.020 | 0.012 ± 0.002 | 0.753 ± 0.107 | |
| | 1000 | 10 | 0.651 ± 0.053 | 0.160 ± 0.015 | 0.012 ± 0.002 | 0.703 ± 0.080 | |
| | 3000 | 10 | 0.627 ± 0.039 | 0.145 ± 0.021 | 0.012 ± 0.002 | 0.704 ± 0.086 | |
| | 5000 | 10 | 0.592 ± 0.043* | 0.141 ± 0.019 | 0.011 ± 0.002 | 0.718 ± 0.056 | |
| Female | 0 | 10 | 0.653 ± 0.064 | 0.165 ± 0.024 | 0.022 ± 0.003 | | 0.026 ± 0.005 |
| | 1000 | 10 | 0.660 ± 0.036 | 0.169 ± 0.013 | 0.023 ± 0.003 | | 0.030 ± 0.004 |
| | 3000 | 10 | 0.658 ± 0.042 | 0.152 ± 0.023 | 0.023 ± 0.004 | | 0.032 ± 0.003* |
| | 5000 | 10 | 0.653 ± 0.040 | 0.175 ± 0.017 | 0.023 ± 0.003 | | 0.030 ± 0.006 |

Mean ± S.D.
Significant difference from the control; *: $P \leq 0.05$ (Dunnett's multiple test)

Table 10. -continued Organ weight per body weight Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Thyroid glands (%) | Pituitary (%) | Thymus (%) | Prostate (%) | Uterus (%) |
|---|---|---|---|---|---|---|---|
| Male | 0 | 10 | 0.005 ± 0.001 | 0.003 ± 0.001 | 0.056 ± 0.011 | 0.350 ± 0.059 | |
| | 1000 | 10 | 0.006 ± 0.001 | 0.003 ± 0.000 | 0.076 ± 0.016** | 0.326 ± 0.027 | |
| | 3000 | 10 | 0.005 ± 0.001 | 0.003 ± 0.000 | 0.064 ± 0.015 | 0.337 ± 0.052 | |
| | 5000 | 10 | 0.005 ± 0.001 | 0.003 ± 0.000 | 0.062 ± 0.012 | 0.347 ± 0.049 | |
| Female | 0 | 10 | 0.007 ± 0.001 | 0.005 ± 0.001 | 0.090 ± 0.020 | | 0.217 ± 0.063N |
| | 1000 | 10 | 0.008 ± 0.002 | 0.006 ± 0.001 | 0.079 ± 0.018 | | 0.238 ± 0.107 |
| | 3000 | 10 | 0.008 ± 0.001 | 0.006 ± 0.001 | 0.095 ± 0.036 | | 0.219 ± 0.079 |
| | 5000 | 10 | 0.008 ± 0.001 | 0.006 ± 0.001 | 0.085 ± 0.017 | | 0.198 ± 0.031 |

Mean ± S.D.
Significant difference from the control; **: $P \leq 0.01$ (Dunnett's multiple test)
N: Non parametric analysis

Table 10. -continued Organ weight per body weight

Exp. No. G768 (499-047)

Day: 91

| Sex | Dose level (mg/kg/day) | No. of animals | Epididymides (%) | Salivary glands (%) | Seminal vesicle (%) |
|---|---|---|---|---|---|
| Male | 0 | 10 | 0.290 ± 0.038 | 0.154 ± 0.014 | 0.318 ± 0.060 |
| | 1000 | 10 | 0.284 ± 0.027 | 0.165 ± 0.027 | 0.325 ± 0.027 |
| | 3000 | 10 | 0.266 ± 0.039 | 0.151 ± 0.017 | 0.340 ± 0.050 |
| | 5000 | 10 | 0.272 ± 0.051 | 0.149 ± 0.012 | 0.340 ± 0.049 |
| Female | 0 | 10 | | 0.153 ± 0.013 | |
| | 1000 | 10 | | 0.158 ± 0.021 | |
| | 3000 | 10 | | 0.161 ± 0.016 | |
| | 5000 | 10 | | 0.162 ± 0.019 | |

Mean ± S.D.
No significant difference from the control.

Table 11. Gross necropsy findings Exp. No. G768 (499-047)

| Dose level ( mg/kg/day ) | | Male animals | | | | Female animals | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1000 | 3000 | 5000 | 0 | 1000 | 3000 | 5000 |
| No. of animals necropsied | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Organ | Findings | | | | | | | | |
| CARDIOVASCULAR SYSTEM | | | | | | | | | |
| heart | white patch | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HEMATOPOIETIC SYSTEM | | | | | | | | | |
| lymph nodes | enlarged | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| thymus | reddish | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| DIGESTIVE SYSTEM | | | | | | | | | |
| stomach | nodule | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | white patch | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| liver | hepatodiaphragmatic nodule | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| URINARY SYSTEM | | | | | | | | | |
| kidneys | depression, focal | 1 | 1 | 2 | 0 | 0 | 2 | 1 | 1 |
| REPRODUCTIVE SYSTEM | | | | | | | | | |
| testes | soft | 0 | 0 | 0 | 1 | - | - | - | - |
| uterus | dilated lumen | - | - | - | - | 1 | 3 | 1 | 0 |
| vagina | nodule | - | - | - | - | 1 | 0 | 0 | 0 |

-: Not applicable

Table 12. Histopathological findings Exp. No. G768 (499-047)

| Dose level ( mg/kg/day ) | Male animals 0 | Male animals 5000 | Female animals 0 | Female animals 5000 |
|---|---|---|---|---|
| No. of animals necropsied | 10 | 10 | 10 | 10 |
| Organ / Findings | | | | |
| CARDIOVASCULAR SYSTEM | | | | |
| heart | (10) | (10) | (10) | (10) |
| cardiomyopathy | 2 | 3 | 0 | 0 |
| HEMATOPOIETIC SYSTEM | | | | |
| thymus | (10) | (10) | (10) | (10) |
| hemorrhage | 1 | 1 | 0 | 0 |
| RESPIRATORY SYSTEM | | | | |
| lungs | (10) | (10) | (10) | (10) |
| aggregation, macrophage | 0 | 1 | 0 | 2 |
| DIGESTIVE SYSTEM | | | | |
| glandular stomach | (10) | (10) | (10) | (10) |
| cyst | 0 | 0 | 1 | 2 |
| pancreas (exocrine) | (10) | (10) | (10) | (10) |
| inflammatory change | 0 | 1 | 0 | 0 |
| liver | (10) | (10) | (10) | (10) |
| fatty change, hepatocyte | 4 | 4 | 3 | 4 |
| microgranuloma | 6 | 5 | 5 | 4 |
| hepatodiaphragmatic nodule | 0 | 0 | 1 | 0 |
| URINARY SYSTEM | | | | |
| kidneys | (10) | (10) | (10) | (10) |
| cast, hyaline | 1 | 1 | 0 | 0 |
| cyst | 1 | 0 | 0 | 0 |
| mineralization | 3 | 1 | 4 | 4 |
| regeneration, tubule | 3 | 1 | 0 | 1 |
| inflammatory change | 2 | 0 | 0 | 1 |
| REPRODUCTIVE SYSTEM | | | | |
| testes | (10) | (10) | ( -) | ( -) |
| atrophy, seminiferous tubule | 1 | 1 | - | - |
| epididymides | (10) | (10) | ( -) | ( -) |
| decrease, sperm | 0 | 1 | - | - |
| prostate | (10) | (10) | ( -) | ( -) |
| inflammatory change | 7 | 5 | - | - |

(): No. of animals examined microscopically at this site.
-: Not applicable

Table 12. -continued Histopathological findings

Exp. No. G768 (499-047)

| Dose level ( mg/kg/day ) | Male animals | | Female animals | |
|---|---|---|---|---|
| | 0 | 5000 | 0 | 5000 |
| No. of animals necropsied | 10 | 10 | 10 | 10 |
| Organ / Findings | | | | |
| REPRODUCTIVE SYSTEM | | | | |
| uterus | ( -) | ( -) | (10) | (10) |
| dilatation, lumen | - | - | 1 | 0 |
| vagina | ( -) | ( -) | (10) | (10) |
| cyst | - | - | 1 | 0 |
| SPECIAL SENSE SYSTEM | | | | |
| Harderian glands | (10) | (10) | (10) | (10) |
| inflammatory change | 0 | 1 | 0 | 0 |

(): No. of animals examined microscopically at this site.
-: Not applicable

**Responses to FDA Questions of October 6, 2021 regarding TG4 Syrup GRN 001004**

## CHEMISTRY

### Question
1. In Section 2.1 for identity (page 14), you describe that there is approximately 7.7% of other saccharides that are in TG4 syrup. According to the saccharide composition of TG4 Syrup by HPLC analysis (Section 2.6.1), the average content of other saccharides varies from 2.1% (Table 2-11) to 18.3% (Table 2-12). Please describe what the "other saccharides" impurities are in the TG4 syrup and clarify the levels of "other saccharides" in the final TG4 syrup.

### Response
1. The confusion lies in the results of the specific assays that were performed related to "other saccharides". In Part 2.1 (page 14) it presents the percentages of molecules having only α-1-4 linkages (except glucose), namely G1, G2, G3 and G4. The total is about 36.3%. The amount of molecules containing terminal α-1,1 linkages, namely TG3 and TG4, is about 56%. This leaves 7.7% of "Other saccharides", all of which are also exclusively made of glucose monomers. These consist of polyglucose of G5 or larger, and TG molecules of TG5 or greater. The term "Other saccharides" is specific to each analytical method reported in the Tables.

Tables 2-11 and 2-12 provided data using different analytical methods to further demonstrate the composition of the TG4 Syrup. This process is complex because, as noted, some of the molecules co-resolve and therefore multiple methods need to be used to tease apart these types of molecules. Table 2-11 included molecules of G5 and G6, and TG5 – TG7, which were not mentioned in the above paragraph (Part 2.1). If you add the percentage of these to "Other saccharides" (2.1%) in this Table the value is 7.6%. Table 2-12 only included the analysis of G3, G4, G5, TG3, TG4 and TG5. It did not include G1, G2, G6 or TG6 and TG7. When these are added with any larger molecules, it averages 18.3% of "Other saccharides".

Table 2-13, using cation-exchange HPLC, is the closest to the values found in Part 2.1. The only difference is that G4 and TG3 co-resolve (18.4%); however, using other analyses it was shown that TG3 is about 3.5%, and G4 is 15%, with non-listed (Other saccharides) being 7.7%.

### Question
2. In Section 1.5 (page 8), you describe that "TG4 Syrup will be used as a carbohydrate source that can be substituted for standard starch-based syrups." Therefore, if the notified use would be a complement to the current standard starch-based syrups in the food industry, please address whether there would be an impact on overall dietary starch-based syrup intake for U.S. consumers.

### Response
2. As mentioned in part 3.3, page 49 based on the years of use in Japan TG4 Syrup is only likely to replace a portion of the starch syrup that would normally be used in a particular product, but could theoretically be used to replace the total amount. This is

unlikely because of the cost. In Parts 3 and 4 it uses the word “replace” several times. Because of the functional properties of TG4 Syrup it is Hayashibara’s experience from over 10 years of sales in Japan that TG4 will not be additive, and has not impacted the overall dietary starch syrup intake by Japanese consumers. As related in the Notice, TG4 Syrup amounts to less than 1.6% of the starch syrup consumed in Japan after years of marketing because of the self-limiting cost and/or technical functions that are not suitable for use in most products using starch-based syrups. Therefore the Notifier does not believe that TG4 Syrup would impact dietary consumption in the US.

**Question**

3. In Section 2.1 (page 14), you describe that G4 is one of the 2 main constituents of the TG4 syrup, but G4 is not included in the specifications. Please provide a specification or a narrative as to why G4 was not included in the proposed specifications for TG4 syrup.

**Response**

3. While G4 is the second of the two main components of TG4 syrup, it is the trehalose moiety (TGn), primarily TG4, that provides the product with its unique technical functions, and hence it is used in the specifications. G4 is found in most all starch-based syrups and simply offers the functionality of many of the other glucose (Gn) molecules. A higher or lower percentage of G4 would not appreciable change the technical functionality of TG4 Syrup. Therefore it is not believed that a G4 specification would offer any benefit.

**Question**

4. You provided a parameter for “Color in solution” by Absorption spectrophotometer, 30% (Japanese Agricultural Standards) in the specifications table (Table 2-1, page 20). However, please clarify what the color value is for in the specification, and if you are measuring the browning intensity of the sugar solution from Maillard reaction, please also provide maximum absorption (λmax) for the browning index using a UV-Vis spectrophotometer.

**Response**

4. Hayashibara uses the “Color of solution” in many of their starch-based products. This is also a standard assay used by essentially all producers of these types of products in Japan. The purpose is not specifically because of the Maillard reaction, but rather to detect possible color development during the later stages of production and storage. The Maillard reaction is more likely to occur during liquefaction and saccharification when the protein content is higher. Much of the protein is removed during the purification steps (Part 2.5.2)

The analytical method for “Color of solution” is as follows, which is almost the same as the “Trehalose” monograph in USP-NF and FCC:

| | |
|---|---|
| Sample solution: | Dissolve 30 g of the sample in recently boiled water to make 100 mL. |
| Analysis: | Determine the absorbance of the Sample solution at 420 nm and 720 nm using a 10-cm cuvette. Calculate Color in Solution: |

$$\text{Result} = A_{420} - A_{720}$$

$A_{420}$ = absorbance at 420 nm

$A_{720}$ = absorbance at 720 nm

Acceptance criteria: NMT 0.100

Yellow color may develop because of the Maillard reaction, caramelization, etc. during production. The $A_{420}$ determines the yellow color, which is the complementary color of violet. As also described in the "Trehalose" monograph in USP-NF and FCC, $A_{720}$ determines turbidity of solution (insoluble floating matter such as active carbon, diatomaceous earth, etc., if any), which does not transmit light including at $A_{420}$. Therefore, $A_{720}$ is subtracted from $A_{420}$ to obtain the real "Color of solution".

**Question**

5. You provide results of arsenic (< 2 ppm) and lead (< 0.1 ppm) in Table 2.3 (page 22). However, please include the heavy metals in the proposed specifications table (Table 2.1) along with analytical method.

**Response**

5. Hayashibara has added "Arsenic ($As_2O_3$) not more than 2 ppm" and "Lead not more than 0.1 ppm", and the analytical methods to the proposed product specifications (see attachment).

**Question**

6. You provide the analytical results of $SO_2$ in the nutritional components table (Table 2-4). However**,** if $SO_2$ was used to reduce and prevent color formation in the browning reaction in the refined syrup, please include residual $SO_2$ in the proposed Specifications, as proposed for trehalose in the previous notification (GRN 000045).

**Response**

6. In 21CFR 168.120 for Glucose Syrups, it gives a specification for $SO_2$ of not more than 40 mg/kg. In four commercial lots the $SO_2$ concentration was below the 3 mg/kg limit of detection, which is greater than 10 fold under the concentration that is considered safe. It is felt that the recovery/purification process of TG4 Syrup has demonstrated a consistent record of removal and therefore should not be needed as a specification.

Trehalose GRN 000045 does not have a specification for $SO_2$.

**Question**

7. In Section 3.5 (EDI based on data from the US surveys), you provide the mean estimated daily intake of TG4 syrup by age-gender groups using the mean number of calories consumed from added sugars (NCHS 2012-13), and 95th intake using the percentiles of usual intake of total sugars from food and beverages (Dietary Reference Intake in the US, 2007-2010), respectively. However, we consider that estimates of chronic (long-term) intake should generally be derived for the general population instead of by age-gender group. We also note that the Dietary Reference Intake database provides total sugar intake at the mean (directly from the day 1 dietary recall) and distribution of usual intake estimate for "All individuals 1 and over." Therefore, please revise and provide an estimate for the mean as well for the high percentile ($90^{th}$ percentile) consumers to reflect chronic average daily intake for the general U.S.

population. Additionally, an adult body weight of 60 kg is typically used for the dietary intake assessments.

**Response**
7. The Notifier was providing a worst-case scenario for consumption. The average EDI for the general population is "2.55 g/person/day", calculated from EDI of TG4 Syrup (g/person/day) in Table 3-2 (p. 58) and the population pyramid of the U.S. in 2020 (https://www.populationpyramid.net/ja/%E3%82%A2%E3%83%A1%E3%83%AA%E3%82%AB%E5%90%88%E8%A1%86%E5%9B%BD/2020/) as follows:

(2.55 g/person/day × 3.0%) + (4.04 × 3.1%) + (5.17 × (3.3 + 3.3)%) + (4.65 × (3.4 + 3.7 + 3.5 + 3.3)%) + (3.96 × (3.1 + 3.0 + 3.1 + 3.2)%) + (2.62 × (3.0 + 2.6 + 2.0+ 1.3 + 0.8 + 0.5 + 0.2 + 0.1 + 0.0)%) + (2.29 × 2.9%) + (3.43 × 3.0%) + (3.68 × (3.1 + 3.1)%) + (3.22 × (3.3 + 3.5 + 3.4 + 3.2)%) + (2.76 × (3.1 + 3.0 + 3.1 + 3.3)%) + (2.13 × (3.2 + 2.8 + 2.3 + 1.6 + 1.1 + 0.7 + 0.4 + 0.1 + 0.0)%)

= 77.4 + 78.9 + 166.5 + 354.6 + 316.7 + 267.6 + 74.1 + 75.5 + 159.6 + 344.6 + 318.6 + 315.7 mg/person/day

= 2.55 g/person/day

The EDI for the general population is "0.0425 g/kg-bw/day" in case of using the body weight of 60 kg for the dietary intake assessments according to the comment from the FDA as follows:

2.55 g/person/day ÷ 60 kg-bw /person = **0.0425 g/kg-bw/day**

**Question**
8. In Part 4 (page 60), you describe that "the trehalose moiety does not participate in Maillard reactions (Figure 2-15), so it would not be used in the many applications..." However, we note that Figure 2-15 was not provided in the submission. Please provide Figure 2-15 for the completion of the submission.

**Response**
8. Please find attached Figure 2-15, which was not provided in the submission.

**TOXICOLOGY**

**Question**
1. In the published 90-day oral feeding study (Matsumoto et al. 2020), it was stated that several gross findings were observed at necropsy.

a. Notably, there were animals in the TG4 syrup group with kidney nodules and herniated livers, which were not observed in any control animals (see Table 7 in Appendix B on page 120). Please explain why these are not considered to be treatment-associated observations, and why they are not a safety concern.

b. The notifier also presents data from the 90-day repeated oral dose toxicity study showing an increase in ketone bodies and protein in the urine of animals given TG4 syrup (see page 89 and Table 7 in Appendix B on page 149). The notifier states that these results are not significant because no dose relationship is observed. However, there are clearly trends towards an increase in both parameters at higher doses, and there is in fact a dose-relationship seen in females. Please explain why these results are not a safety concern.

**Response**

1.a. A general comment that should be made is that TG4 Syrup is composed of essentially only glucose which is completely digested in the small intestine in a manner identical to trehalose and starch-based syrups that have been GRASed and consumed for many years without any reported adverse effects, other than those associated with saccharidase deficiencies (see Response 3. below).

The study in question was performed by a U.S laboratory that meets Good Laboratory Practice requirements of the EPA, FDA and OECD. It was a 90-day Preliminary study with only control and 10% TG4 Syrup consumption groups. The conclusion of the laboratory was that the observations at necropsy were “sporadic, not seen in both sexes and not treatment associated”. The clinical chemistry values were reviewed and showed neither untoward kidney-associated values for the male group, nor abnormal hepatic values for the female group. The only exception was a significantly lower Na+ value in the TG4 Syrup treatment group than in the control group; however, both values were within the standard range for this age and strain of animal. No organ weight or relative weight differences were observed. Additionally there was no histopathology that would suggest any treatment associated kidney or liver issues.

Further, subsequent to this preliminary study a full 90-day gavage repeated oral dose toxicity study was performed using a control and three treatment groups. Gross inspection in this second study did not reveal similar gross lesions in the liver, except a single hepatodiaphramatic nodule of 2x4 mm in a control female rat. The kidneys showed focal depressions that were observed in both female and male animals, and in a male control and not in a dose dependent manner. Histopathology also provided no suggestion of toxicity in the kidneys or liver. The preliminary 90-day study had a mean high dose (10%) of 7,141 mg/kg/day, while in the second study the highest dose was 5,000 mg/kg/day by gavage. Because there were no lesions consistent between the two studies, lesions in both male and female, even at the highest doses, and no supporting clinical chemistry or other physical variations in the preliminary 90-day study; therefore it was concluded that the original determination of the study laboratory of “sporadic, not seen in both sexes and not treatment associated” was consistent with all the associated available data.

1.b. This study was performed by a laboratory that works under OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17, and OECD Guideline for Testing of Chemicals 408 (21st September 1998: Repeated Dose 90-Day Oral Toxicity Study in Rodents).

In males, both ketones and proteins were more positive in the 3000 mg/kg/day group than in the 5000 mg/kg/day group, it was judged that there was no dose response. Regarding protein, in females, there was an example showing 100 mg/dL even in the control group, therefore the toxicologist concluded that this trend in protein urea was artifactual.

To support these conclusions the laboratory examined the control data from 35 trials from 2013 to 2018, which bracket that date of the study (attached). According to the data 1+ ketone body reactions were observed in 19.6% of the male control groups, and +/- reactions in the 10.6%. In addition, in regard to protein, reactions at the level of 100 mg/dL are frequently observed in both males and females (attached). Considering this fact, it was concluded that the results of male with 5000 mg/kg/day in both ketone body and protein are not uncommonly high. Similarly females receiving the 5000 mg/kg, both ketone body and protein scores are not uncommon. Further, although a slightly higher score is observed, this tendency is often seen in repeated oral toxicity tests of food materials rich in enzymes and amino acids. Finally, it was concluded that these urine values were of low or no clinical significance because there were no blood biochemical test results indicating decreased renal function, abnormal glucose metabolism, or starvation, and no pathological findings of impaired changes in the liver, pancreas, or kidneys.

**Question**

2. Using the NOAEL of 5000 mg/kg bw/d from the Matsumoto et al. 2020 study, it is not clear that your exposure estimate provides sufficient margin of exposure. Please provide your rationale as to how you came to your GRAS conclusion based on this study and the exposure estimate based on your proposed uses for TG4 syrup.

**Response**

2. The Notifier used calculations that were extremely conservative, which resulted in the low Margin of Exposure (MOE). One of those variables was the use of the highest EDI consumption groups by age and sex. **Chemistry** Question 7. above requested that the EDI for estimates of chronic (long-term) intake be recalculated for the general population instead of by age-gender groups. Using this recalculation from the response to item 7., provides the EDI of 0.0425 g/kg-bw/day. Therefore, the Margin of Exposure (MOE) is calculated to be 3.65/0.0425 = **85.9**

It is concluded that for a macroingredient composed of approximately 99.9% glucose this MOE should be sufficient.

This is also calculated with a market share of 5% of the starch-based syrups. As was noted in Part 4 about 73% of these syrups consist of HFCS, for which it would be highly unlikely for TG4 Syrup to substitute. Further, if the market share potential of TG4 Syrup in the U.S. is 1.6%, which is the market share in Japan after over 10 years of sales, instead of the worst case 5% used for the EDI calculation in Table 3-2, the MOE would be “268”.

**Question**

3. The notifier has discussed safety of TG4 syrup in the context of patients with trehalase enzyme deficiencies (see page 69 of the notice). Please also address safety of TG4 consumption in patients with primary carbohydrate malabsorption and digestion disorders, for example, α-glucosidase enzyme deficiencies, and secondary diseases, such as pancreatic insufficiency.

**Response**

3. As shown in Figure 6-1 TG4 Syrup demonstrates the same glycemic index and profile as glucose showing that the entire dose of TG4 Syrup is digested into glucose. Figure 6-2 shows the similar insulin profile as glucose. The reason only trehalase deficiency was discussed is that the addition of trehalose to the diet is a fairly recent occurrence and unknown to many. However, the consumption of dietary starch-based syrups has been known for many decades, and deficiencies of disaccharidases well studied.

U.S starch syrup production started in 1831, and now produces starch-based syrup from corn, wheat, potato and rice (Hobbs, 2009). As stated in Part 4., the per capita consumption of such starch-based sweeteners in 2016 was 25.6 kg, approximately 8.27 x $10^9$ kg in total (USDA, 2019). About 73% of the total consists of HFCS, which required very little pancreatic or small intestinal enzymes to digest (USDA, 2019). Rather it is the other 27% of starch-based syrup sold in the US that requires the enzymes that were referred to in the Agency's question about individuals with primary carbohydrate malabsorption and digestive disorders. The enzymes involved in carbohydrate breakdown and absorption as glucose would include pancreatic alpha-amylase, the two enzyme complexes called maltose-glucoamylase (MGAM) and sucrose-isomaltase (SI), lactase, and trehalase.

TG4 Syrup does not contain starch, which is the primary substrate of pancreatic α-amylase. A primary or secondary deficiency in this enzyme would not cause the malabsorption of glucose. Next, TG4 Syrup does not contain lactose, so a lactase deficiency would not be an issue in the consumption of TG4 Syrup. According to the NIH, lactose intolerance, because of lactase deficiency, is the most common carbohydrate deficiency in the US and world (NIH, 2020).

As with lactase and trehalase deficiencies (absent or low concentrations), the consumption of the respective enzyme substrates results in transient self-limiting osmotic laxation with accompanying symptoms of abdominal pain/distention, tenesmus, borborygmus, flatus, and nausea/vomiting.

If an individual has a deficiency of either of the other two small intestinal enzyme complexes (MGMA, SI) consumption of TG4 Syrup would result in similar symptoms resulting from osmotic laxation as described above. However, this would also be the case for any other starch-based syrup that the person would consume. The FDA has already considered all of these starch-based substances that are similar to TG4 Syrup as GRAS (21CFR 168.120; 184.1865; 184.1444; 184:1277).

Therefore consumption of TG4 Syrup would present no more of a safety concern than the approximately 4.4 x $10^9$ kg of starch-based syrups that are consumed each year in the US (2016 data; USDA, 2019).

**Question**
4. On page 77, the notifier states: “Carcinogenicity of fructose appears to be similar to glucose” in the section related to cariogenic activity of sugars, starches, and foods. Please clarify that this statement should be cariogenicity and not carcinogenicity.

**Response**
4. As was noted the use of “Carcinogenicity of fructose .....”, was a mistake and should have read “Cariogenicity of fructose .....”.

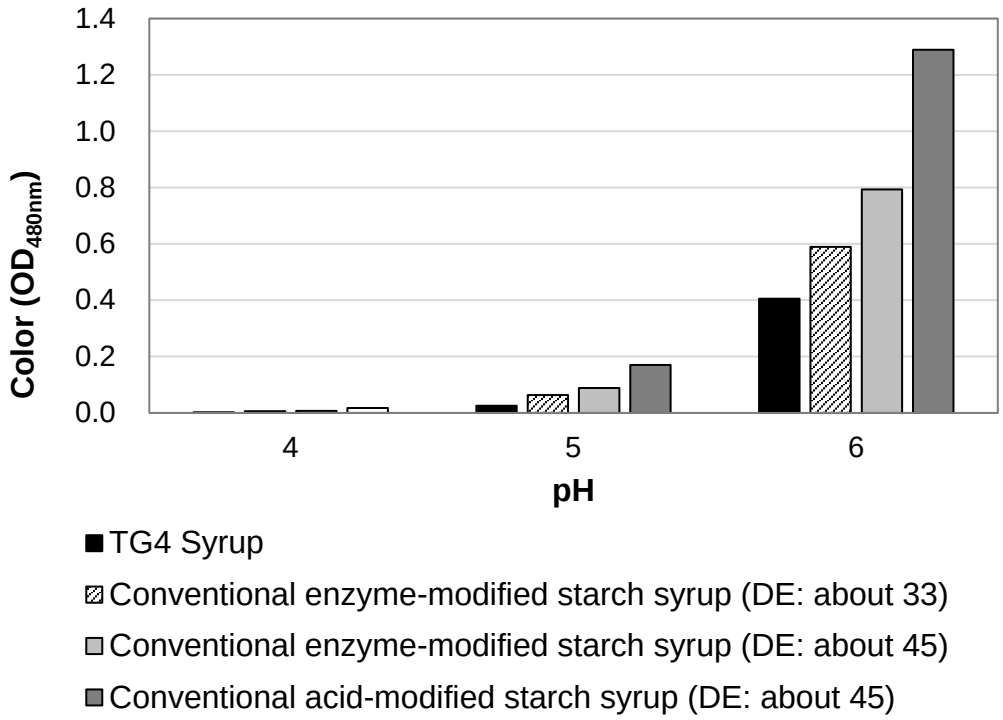
**Question**
5. On page 86, the notifier details the search parameters and databases used for the literature search performed for the safety assessment of this GRAS notice. Please specify the timeframe this literature search encompasses.

**Response**
5. The date of the last review of the parameters used for the literature search performed for the safety assessment of the GRAS submission was in early December of 2020.

**Table 2-1 Final Product Specifications for TG4 Syrup**

| Variables | Specifications | Analytical Methods |
|---|---|---|
| Dry solid | Not less than 72.0% | Refractive index method (Industrial Analytical Methods for Starch-derived Saccharides) |
| Total ash | Not more than 0.05% | Electric conductivity method (Industrial Analytical Methods for Starch-derived Saccharides) |
| pH | 3.5 – 6.5 | pH determination, 30% (Japanese Industrial Standards) |
| Color in solution | Not more than 0.100 | Absorption spectrophotometer, 30% (Japanese Agricultural Standards) |
| Turbidity | Not more than 0.050 | Absorption spectrophotometer, 30% (Japanese Agricultural Standards) |
| Sugar composition (on the dry basis) | | HPLC method (Industrial Analytical Methods for Starch-derived Saccharides) |
| Glucose | Not more than 6.0% | |
| Maltosyltrehalose | Not less than 50.0% | |
| Arsenic ($As_2O_3$) | Not more than 2 ppm | Arsenic Limit Test, Method 1, Apparatus B (Japan's Specifications and Standards for Food Additives) |
| Lead | Not more than 0.1 ppm | Modified Atomic Absorption Technique (FCC) |
| Total aerobic microbial count | Not more than 300 CFU/g | Pour plate method, Standard agar (Standard Methods of Analysis for Hygiene Chemists) |
| Coliform organisms | Negative | BGLB method (Standard Methods of Analysis for Hygiene Chemists) |

**Figure 2-15 Malliard Reaction of TG4 Syrup Compared to Other Common Starch Syrups.**



# Toxicology

## 1.b.

Backgroud data

Surveyed test:****
●Animal species/Strain: Rat/CrlCD(SD)
●Search period: August 13, 2013～September 5, 2018
●Number of tests: 35 tests

Aggregated tests: E530,E819,F076,F448,F494,F531,F657,F709,F958,G086,G117,G219,G402, G533,G764,G768,G772,G779,G803,G841,G884,H025,H033,H045,H046,H07

Test tem:
**Ketone bodies**

| Data | Male | | | Female | | |
|---|---|---|---|---|---|---|
| − | 156 | ( | 47.9 )* | 283 | ( | 86.5 ) |
| +/− | 104 | ( | 31.9 ) | 34 | ( | 10.4 ) |
| 1+ | 64 | ( | 19.6 ) | 10 | ( | 3.1 ) |
| 2+ | 2 | ( | 0.6 ) | 0 | ( | 0.0 ) |
| 3+ | 0 | ( | 0.0 ) | 0 | ( | 0.0 ) |
| 4+ | 0 | ( | 0.0 ) | 0 | ( | 0.0 ) |
| Total | 326 | ( | 100 ) | 327 | ( | 100 ) |

**Protein**

| Data | Male | | | Female | | |
|---|---|---|---|---|---|---|
| − | 134 | ( | 41.1 ) | 241 | ( | 73.7 ) |
| +/− | 104 | ( | 31.9 ) | 38 | ( | 11.6 ) |
| 30 | 59 | ( | 18.1 ) | 25 | ( | 7.6 ) |
| 100 | 27 | ( | 8.3 ) | 22 | ( | 6.7 ) |
| >=300 | 2 | ( | 0.6 ) | 1 | ( | 0.3 ) |
| 計 | 326 | ( | 100 ) | 327 | ( | 100 ) |

*(Occurrence rate)(%)

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