#901



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December 15, 2019

Dennis M. Keefe, PhD,
Director, Office of Food Additive Safety
HFS-200
Food and Drug Administration
5100 Paint Branch Pkwy
College Park, MD 20740-3835

Re: GRAS Notice for Glucosyl Hesperidin

Dear Dr. Keefe:

The attached GRAS Notification is submitted on behalf of the Notifier, Hayashibara Co., Ltd. of Okayama, Japan, for Glucosyl Hesperidin (GH). GH is a hesperidin molecule modified by enzymatic addition of a glucose molecule. It is intended for use as a general food ingredient, in food. The document provides a review of the information related to the intended uses, manufacturing and safety of GH.

Hayashibara Co., Ltd. (Hayashibara) has concluded that GH is generally recognized as safe (GRAS) based on scientific procedures under 21 CFR 170.30(b) and conforms to the proposed rule published in the *Federal Register* at Vol. 62, No. 74 on April 17, 1997. The publically available data and information upon which a conclusion of GRAS was made has been evaluated by a panel of experts who are qualified by scientific training and experience to assess the safety of GH under the conditions of its intended use in food. A copy of the Expert Panel's letter is attached to this GRAS Notice. Hayashibara therefore would like to respectfully submit notice to the Agency that GH, manufactured by Hayashibara, is exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act, because such use is GRAS.

GH is manufactured by Hayashibara in Japan using food grade hesperidin, dextrin and processing aids that are removed during the production process, including two enzymes commonly used in food grade starch processing. From absorption and metabolic data provided in the GRAS Notice it is believed that GH is absorbed into the body and metabolized in essentially the identical manner as natural hesperidin.

It is Hayashibara's contention that GH is GRAS by its *substantial equivalence* to hesperidin, and therefore safety related data of hesperidin is provided in this Notice. Additionally, a recent paper has been published that includes two genotoxicity studies, a 4-week oral feeding study, a 13-week sub-chronic toxicity study, and a teratogenicity study using GH from commercial Hayashibara production lots.

It has been listed and commercialized as an existing food additive in Japan since 1998. GH is also allowed to be used as a food ingredient and a food additive in Taiwan and Korea, respectively.

Hayashibara Co., Ltd. greatly appreciates the Agency's review of the submitted materials, and would be pleased to provide any additional information that the Agency needs to complete the review of this GRAS Notice.

Sincerely,

Alan B. Richards, PhD
President, VRSI

#901

# Generally Recognized As Safe Notice for Glucosyl Hesperidin (GH)

## Manufactured by Hayashibara Co., Ltd.

Prepared for

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

Prepared by

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


December 13, 2019

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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 Glucosyl Hesperidin in accordance with 21 CFR §170.225 Part 1.

## 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
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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

Glucosyl Hesperidin. For brevity, the substance will be abbreviated in many parts of the document as GH.

## 1.5 Intended conditions of use of the notified substance

Glucosyl Hesperidin (GH) is proposed for use in a variety of food categories provided below (Table 1-1). The intended uses or physical or technical effect are as a food ingredient to provide a source of antioxidants, coloring adjuncts, flavor enhancer, and flavoring agents and adjuvants. It should be noted that GH is not a "color" agent per

se, but rather a color adjunct. It is expected that GH will be used in foods, and beverages for consumption by adults and children 1-year old and older.

Table 1-1 provides the food categories (21 CFR §170.3(n) and concentrations of the maximum amount of GH to be used in each food category. The actual intended uses below may only consist of a subset of the food categories listed (see Part 3). An example is Food Category (31) below. Only yogurt will be included as a potential for Milk products.

**Table 1-1 Food categories, estimated maximum use of Glucosyl Hesperidin**

| FDA 21 CFR §170.3(n) Food Category | Maximum Amount of GH Per Serving of Food |
|---|---|
| (3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks. | 500 mg |
| (7) Coffee and tea, including regular, decaffeinated, and instant types. | 500 mg |
| (16) Fresh fruits and fruit juices, including only raw fruits, citrus, melons, and berries, and home-prepared "ades" and punches made therefrom. | 500 mg |
| (25) Hard candy and cough drops, including all hard type candies. | 500 mg |
| (31) Milk products, including flavored milks and milk drinks, dry milk, toppings, snack dips, spreads, weight control milk beverages, and other milk origin products. | 500 mg |
| (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. | 500 mg |
| (36) Processed vegetables and vegetable juices, including all commercially processed vegetables, vegetable dishes, frozen multicourse vegetable meals, and vegetable juices and blends. | 500 mg |
| (38) Soft candy, including candy bars, chocolates, fudge, mints, and other chewy or nougat candies. | 500 mg |

### 1.6 Basis for GRAS determination

Hayashibara Co., Ltd. has determined and is therefore notifying the Agency that the intended use of Glucosyl Hesperidin 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

Hayashibara Co., Ltd. has concluded that the notified substance, Glucosyl Hesperidin, 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 data and Information included in this GRAS Notice, Parts 2-7 are not exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

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

As stated above there are no trade secrets or confidential information contained in this GRAS Notice, and can be shared with the USDA FIS if it is thought that there would be a product that could contain USDA products.

## 1.9 Certification

Hayashibara Co., Ltd. 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 Hayashibara Co., Ltd., which is pertinent to the evaluation of the safety and GRAS status of the use of Glucosyl Hesperidin for general food consumption by humans.

December 13, 2019

Alan B. Richards, Ph.D.
Authorized Agent

# 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR 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 that is the topic of this GRAS Notice is called Glucosyl Hesperidin. It is produced by enzymatic linkage of hesperidin, and dextrin. Hesperidin is a major flavonoid naturally found in sweet oranges, lemons and tangerines, and can also be identified in lower concentrations in many other plants (Barthe, et al., 1988; Garg, et al., 2001). Hesperidin and other flavonoids are highly insoluble, which limits their use as a food ingredient. Hayashibara Co., Ltd. of Okayama, Japan, the Notifier of this GRAS Notice, developed a derivative of hesperidin by enzymatically combining natural hesperidin with a glucose molecule (Hijiya, Miyake, 1991; Miyake, Yumoto, 2000). This product, Glucosyl Hesperidin (GH), has very high water solubility and retains the antioxidative activity of native hesperidin. The main component of GH is monoglucosyl hesperidin (MGH).

### 2.1.1 Common or Usual Names and Identity

Glucosyl Hesperidin
Enzymatically Modified Hesperidin
α-Glucosyl hesperidin

### 2.1.2 IUPAC Nomenclature of the Main Constituent

(2S)-7-({6-O-[6-deoxy-α-L-mannopyranosyl-(1→]-4-O-[α-D-glucopyranosyl-(1→]-β-D-glucopyranosyl-(1→}oxy)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4H-chromen-4-one

### 2.1.3 CAS Registry Number of the Main Constituent

Monoglucosyl hesperidin 161713-86-6

### 2.1.4 CAS Name of the Main Constituent

4H-1-Benzopyran-4-one, 7-[(O-6-deoxy-α-L-mannopyranosyl-(1→6)-O-[α-D-glucopyranosyl-(1→4)]-β-D-glucopyranosyl)oxy]-2,3-dihydro-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-, (2S)-

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

4G-α-D-glucopyranosyl-hesperidin
Alpha-glucosyl-hesperidin
Hayashibara Hesperidin S (Trade name in Japan and Korea)

## 2.2 Chemical Formula, Structure and Molecular Weight

### 2.2.1 Empirical Formula of the Main Constituent

Monoglucosyl Hesperidin $C_{34}H_{44}O_{20}$

### 2.2.2 Structural Formula of the Main Constituent

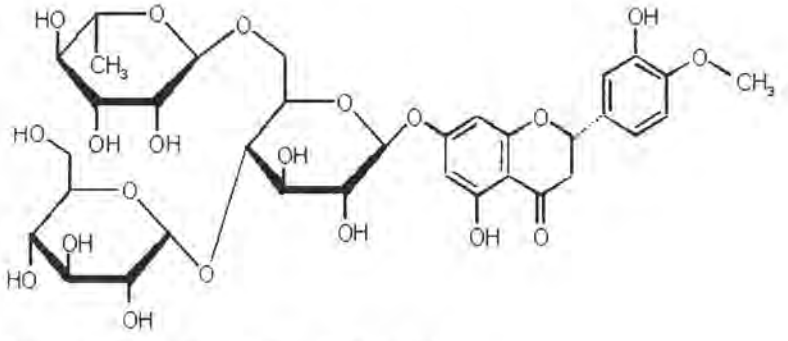


**Figure 2-1 Structure of MGH**

### 2.2.3 Molecular Weight of the Main Constituent

Monoglucosyl hesperidin 772.70 daltons

## 2.3 Raw Materials

Hayashibara Co., Ltd. manufactures one grade of GH for food. There is a second grade of glucosyl hesperidin that is used for cosmetic applications (GH-C). It differs in composition because it is subjected to an additional enzymatic step. The reason this second cosmetic grade is mentioned in this document is because i) the main component of both products is monoglucosyl hesperidin (MGH), and ii) there are some safety-related pre-clinical data in which GH-C was used that will be presented as supporting information.

To manufacturer GH, the following substances, listed in Tabe 2-1, are suitable for food

production (food or food additive grade) and used in accordance with Food Sanitation Act of Japan (FSAJ, 1947). It is possible that the raw materials used for the manufacturing of GH might be changed, deleted and/or added due to technological benefits, but only after validating that there is no untoward effect to the safety and quality of GH.

**Table 2-1 Raw materials and Processing Aids Used in the Production of GH**

| | | |
|---|---|---|
| Hesperidin | Dextrin | Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) *Geobacillus stearothermophilus* |
| Glucoamylase (EC 3.2.1.3) *Rhizopus oryzae* | Ascorbic acid | Sodium hydroxide solution |
| Magnesium chloride | Hydrocholoric acid | [1]Sodium pyrosulfite |
| Sulfuric acid | Sodium chloride | Ethanol |
| Activated carbon | Diatomaceous earth | Powdered cellulose |
| Perlite | Ion exchange resins | Adsorption separation resin |

[1]Sodium pyrosulfite was assayed in the finished product and found to be less than 0.003g/kg, which is below the limit of detection.

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

### 2.4.1 Specifications

Hayashibara Co., Ltd. developed the specifications for GH, which are shown in Table 2-2 below.

**Table 2-2 Final Product Specifications for GH**

| Variables | Specifications |
|---|---|
| Description | A pale yellow to yellow-brown powder having a slight, characteristic odor |
| Identification (1) | A brown color develops. |
| Identification (2) | Exhibits a peak at the position corresponding to monoglucosyl hesperidin, having an absorption maximum at a wavelength of 280 – 286 nm. |
| Monoglucosyl hesperidin | 75.0 – 85.0% (on the dried base) |
| Total hesperidin | Not less than 70.0% (on the dried base) |
| Loss on drying | Not more than 6.0% |
| Residue on ignition | Not more than 2.0% |
| pH | 5.0 – 7.0 |
| Lead (as Pb) | Not more than 0.1 μg/g |
| Arsenic (as As) | Not more than 1.5 μg/g |
| Total aerobic microbial count | Not more than 300 CFU/g |
| Coliform organisms | Negative/0.01 g |

### 2.4.2 Methods of Analysis

Table 2-3 provides the methods of analysis for the specifications.

**Table 2-3 Analytical Methods for Each of the Specifications**

| Variables | Analytical Methods |
|---|---|
| Description | Japan's Specifications and Standards for Food Additives[1] "General Notices" |
| Identification (1) | Japan's Specifications and Standards for Food Additives "Enzymatically Modified Hesperidin" |
| Identification (2) | Japan's Specifications and Standards for Food Additives "Enzymatically Modified Hesperidin" |
| Monoglucosyl hepseridin | In-house Method (HPLC) |
| Total hesperidin | In-house Method (HPLC) |
| Loss on drying | Japan's Specifications and Standards for Food Additives "Enzymatically Modified Hesperidin" |
| Residue on ignition | Japan's Specifications and Standards for Food Additives "General Tests" |
| pH | Japan Industrial Standard (JIS Z8802) |
| Lead | Method I of Atomic Absorption Spectrophotometric Graphite Furnace Method of Appendix III: Chemical Tests and Determinations in the FCC |
| Arsenic | Japan's Specifications and Standards for Food Additives "Enzymatically Modified Hesperidin" |
| Total aerobic microbial count | Japanese Pharmacopoeia "Microbiological Examination of Non-sterile Products" |
| Coliform organisms | Standard Methods of Analysis for Hygenic Chemists |

### 2.4.3 Analysis of Multiple Lots

To demonstrate that Hayashibara Co., Ltd., is able to consistently manufacture GH to meet proposed specifications above, the company analyzed 5 lots of the commercial product (Hayashibara Hesperidin S). The results are provided in Table 2-4. Lots 8G051, 8G221, 8H071 and 8H221 are non-consecutive; while 8H301 is consecutive with 8H221, and therefore non-consecutive with the other 3. The content of MGH and of hesperidin ranged from 75.1 to 78.4%, and from 73.9 to 76.6%, respectively. Water loss using a standardized drying method was not more than 2.7%. Residue on ignition and pH of the product ranged from 0.04 to 0.08%, and 5.6 to 5.8, respectively. Lead and arsenic content were less than the proposed specification, and below the

sensitivity of the analytical test. Therefore the term "qualified" was used. Total aerobic microbial count was within the set limit and all lots were negative for coliform organisms.

**Table 2-4 Five (5) Lot Analyses of GH**

| Variables | Specifications | Lot No. | | | | |
|---|---|---|---|---|---|---|
| | | 8G051 | 8G221 | 8H071 | 8H221 | 8H301 |
| Description | A pale yellow to yellow-brown powder having a slight, characteristic odor | Qualified | Qualified | Qualified | Qualified | Qualified |
| Identification (1) | A brown color develops. | Qualified | Qualified | Qualified | Qualified | Qualified |
| Identification (2) | Exhibits a peak at the position corresponding to monoglucosyl hesperidin, having an absorption maximum at a wavelength of 280 – 286 nm. | Qualified | Qualified | Qualified | Qualified | Qualified |
| Monoglucosyl hesperidin | 75.0 – 85.0% (d.b.) | 77.4 | 76.6 | 78.4 | 75.5 | 75.1 |
| Total hesperidin | ≥ 70.0% (d.b.) | 75.8 | 74.1 | 76.6 | 74.3 | 73.9 |
| Loss on drying | ≤ 6.0% | 2.7 | 2.6 | 2.5 | 2.7 | 2.7 |
| Residue on ignition | ≤ 2.0% | 0.04 | 0.08 | 0.07 | 0.05 | 0.06 |
| pH | 5.0 – 7.0 | 5.7 | 5.6 | 5.8 | 5.7 | 5.7 |
| Lead (as Pb) | ≤ 0.1 μg/g | Qualified | Qualified | Qualified | Qualified | Qualified |
| Arsenic (as As) | ≤ 1.5 μg/g | Qualified | Qualified | Qualified | Qualified | Qualified |
| Total aerobic microbial count | ≤ 300 CFU/g | 0 | 2 | 0 | 0 | 0 |
| Coliform organisms | Negative/0.1 g | Negative | Negative | Negative | Negative | Negative |
| Protein (not a Specification) | -- | < 50 μg/g | < 50 μg/g | < 50 μg/g | < 50 μg/g | < 50 μg/g |

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

The protein concentration, which is not a lot specification, was under the limits of detection.

### 2.4.4 Microbial and Toxicant assays

Microbiological and other toxicant analyses of GH are summarized in Table 2-5. The results showed that *Escherichia coli*, heat-resistant acidophilic bacteria, *Staphylococcus aureus*, *Salmonella* sp. and *Bacillus cereus* were all negative. Total combined yeast and mold counts were < 10 CFU/g. Sulfur dioxide, total aflatoxins (aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$) and enterotoxin were not detected.

**Table 2-5 Microbial and Toxicant Analyses of GH**

| Variables | Lot No. | |
|---|---|---|
| | 5H311 | 6L08 |
| *Escherichia coli* | Negative/ 2.22 g | Negative/ 2.22 g |
| Heat-resistant acidphilic bacteria | Negative/ 10 g | Negative/ 10 g |
| *Staphylococcus aureus* | Negative/ 0.01 g | Negative/ 0.01 g |
| *Salmonella* sp. | Negative/ 25 g | Negative/ 25 g |
| *Bacillus cereus* | Negative/ 0.01 g | Negative/ 0.01 g |
| Total combined yeasts and molds count | <10 CFU/g | <10 CFU/g |
| Sulfur dioxide | Not detected | Not detected |
| Total aflatoxins | Not detected | Not detected |
| Enterotoxin | Not detected | Not detected |

### 2.4.5 Nutritional Analysis of Glucosyl Hesperidin

Table 2-6 includes the nutritional analysis of 2 lots of GH. The mean values for the total carbohydrates and energy were 97.5 g/100 g and 390 kcal/100 g, respectively. The water content was 2.5 g/100 g, and protein, lipid, ash, dietary fiber and sodium were undetectable in GH. Essentially the carbohydrate and water content account for 100% of the composition of GH.

**Table 2-6 Nutritional Content of GH**

| Variables | Lot No. | | Average |
|---|---|---|---|
| | 5H311 | 6L08 | |
| Water (g/100 g) | 2.3 | 2.7 | 2.5 |
| Protein (g/100 g) | < 0.1 | < 0.1 | < 0.1 |
| Lipid (g/100 g) | < 0.1 | < 0.1 | < 0.1 |
| Ash (g/100 g) | < 0.1 | < 0.1 | < 0.1 |
| Carbohydrate (g/100 g) | 97.7 | 97.3 | 97.5 |
| Dietary fiber (g/100 g) | < 0.5 | < 0.5 | < 0.5 |
| Energy (kcal/100 g) | 391 | 389 | 390 |
| Sodium (mg/100 g)[1] | Not detected | Not detected | Not detected |

1) Detection limit: 1 mg/100 g

### 2.4.6 Quantitative Composition

The composition of GH (Batch HS-009-1) was analyzed using both HPLC and LC/MS methods. The constituents of the GH product were determined and are presented in Table 2-7. Main components were monoglucosyl hesperidin and hesperidin which accounts for 92.8% of GH (dwb). The sum of the di- and maltooligosyl hesperidin molecules is 2.8%, and monoglycosyl flavonoids contribute 3.1%. The naturally occurring flavonoids, which are glycosylated, are normally found in preparations of food grade hesperidin. β- and γ-Cyclodextrins, and other small amounts of free saccharides are produced from the raw material, dextrin, by reactions with cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) and glucoamylase (EC 3.2.1.3), respectively.

The unknown fraction is believed to likely be carbohydrates, as the non-water composition is essentially 100% carbohydrate as shown in the nutritional composition of GH in Table 2-6, and there is less than 0.1% protein, 0.1% lipid, 0.1% ash, and 0.5% dietary fiber, the latter being a carbohydrate.

**Table 2-7 Composition Analysis of GH**

| Composition | | Content (%) |
|---|---|---|
| Monoglucosyl hesperidin | Hesperidin derivatives (glycosylated hesperidin) | 78.7 |
| Hesperidin | | 14.1 |
| Diglucosyl hesperidin | | 0.9 |
| Maltooligosyl (n ≥ 3) hesperidin | | 1.9 |
| Monoglucosyl narirutin | Glycosylated impurity flavonoides derived from the raw material "Hesperidin" | 1.2 |
| Monoglucosyl diosmin | | 1.1 |
| Monoglucosyl neoponcirin | | 0.8 |
| β-Cyclodextrin | Free saccharides derived from the raw material "Dextrin" | 0.1 |
| γ- Cyclodextrin | | 0.2 |
| Other free saccharides | | 0.5 |
| Unknown | | 0.6 |

## 2.5 Manufacturing Process

### 2.5.1 Introduction

Flavonoids are a large group of natural compounds, which are one of the most commonly found categories in the plant kingdom. In the mid-1970's, it was reported that there were about 800 different known flavonoids, with more being continually isolated (Kuhnau, 1976). Today that number is thought to be about 6,000 (Panche, et al., 2016). The basic structure underlying all the different compounds is a 1,4-benzopyrane (4-chromenone) molecule that has a phenyl group attached at the 2-carbon position. While most plants contain several bioflavonoids, different bioflavonoids are found in specific species or groups of plants. This fact aids in the isolation and manufacture of a particular bioflavonoid(s), such as hesperidin. Hesperidin is the basic structure of GH, the subject of this Notification. One dramatic feature of hesperidin and many other bioflavonoids is their low water solubility (0.002 g/100 g water).

A common method for the manufacture of hesperidin for food use is by multiple extractions of orange peel tissue. The pulp material is first soaked in alkaline solution, and then acidified. The hesperidin precipitates as crystals, but contains other bioflavonoids. The precipitate is washed and then the crystals are re-dissolved in alkaline solution and acidified. This process is repeated.

The rationale for the development and manufacture of GH was to create a hesperidin-based bioflavonoid with high water solubility. To do this, Hayashibara Co., Ltd.,

developed a patented process, which enzymatically attaches a starch fragment (oligosaccharides) to the existing disaccharide (rutinose) (Hijiya, Miyake, 1991; Miyake, Yumoto, 2000). This rutinose is naturally bound to the aglycone molecule, hesperetin, creating hesperidin (see Part 2.2.2, Figure 2-1). The enzyme used to attach the oligosaccharides is cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19), which is a common enzyme used in many food production processes. To increase hesperidin content per unit weight, excess glucose molecules of the attached oligosaccharides are cleaved using glucoamylase (EC 3.2.1.3). This enzyme removes essentially all the glucose molecules except the terminal one because steric hindrance prevents the α-glucose from being cleaved. The addition of this single glucose molecule to hesperidin increases the solubility by approximately 100,000 fold. The product is sent through a purification system common to many food ingredients, which results in GH. The main component of GH being monoglucosyl hesperidin (MGH).

The following is a more detailed description of the manufacturing process of GH. If the Agency would like to know the various values, Hayashibara would be willing to supply this information under terms of confidentiality. It should also be noted that food-grade hesperidin and dextrin are used as the starting material for the GH production process. All enzymes, chemicals and purification resins used as processing aids have a purity that makes them suitable for use in the present process.

### 2.5.2 Hayashibara Production Process

Hayashibara developed and patented the novel method for producing GH.

**Dissolution of raw materials**
Sodium hydroxide solution is added to water to make a solution of relatively high pH and the water is heated. Hesperidin and sodium pyrosulfite are dissolved in this mixture, and ascorbic acid and magnesium chloride are added. Next, dextrin is added to the mixture and stirred until all the substances have dissolved.

**Reaction**
The pH is lowered with sulfuric acid. CGTase is added and allowed to react for hours. This enzyme attaches the oligosaccharides to the hesperidin molecule. To stop the first reaction and adjust the pH to the optimal value for the second reaction, the pH is lowered. The second enzyme, glucoamylase, is added to cleave off all but the α-glucose. The reaction continues with the second enzyme at an optimum pH and temperature for a number of hours. At this point, the solution is heated to stop the reaction, degrade the enzymes, and the solution is allowed to cool.

**Purification**

The GH solution undergoes a multi-step purification process. The first step is filtration through diatomaceous earth. After this, the solution is sent through an adsorption-separation resin. The MGH, natural hesperidin and other minor molecules (Table 2-7) adhere to the resin, while free glucose and oligosaccharides, and other substances pass through. To elute the MGH and hesperidin, ethanol is used. The ethanol is distilled from the eluate, and further purification is accomplished using ion exchange resins for decolorization. The final purification step uses a combination of powdered cellulose and activated carbon.

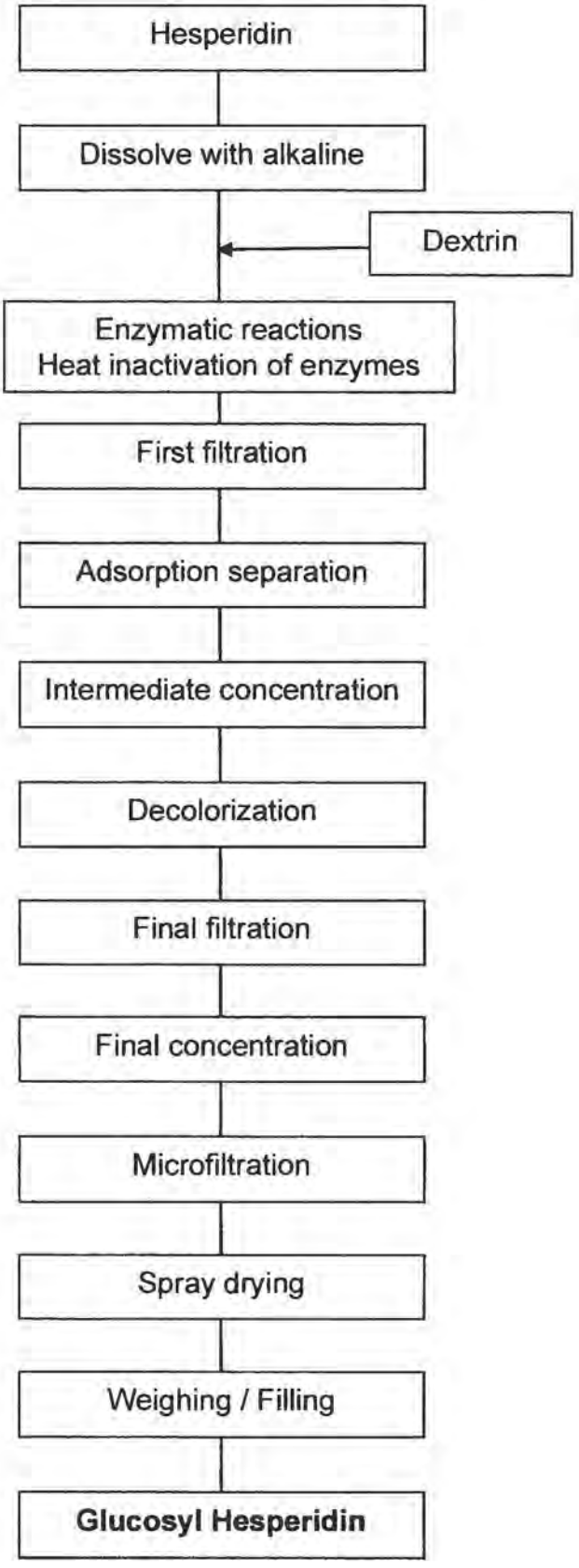
**Drying and packaging**

The purified solution is concentrated by heat under vacuum to the appropriate concentration. The liquid is spray-dried to a low moisture content. The powdering procedure is conducted under clean air, and the powder is packaged.

Aluminum laminated bags are used as the packaging material. One (1) kg or 10 kg of GH is accurately weighed into aluminum-laminated bags. Stability tests (below) demonstrate their applicability for this product.

The schematic diagram below (Figure 2-2) shows the production steps of GH.

**Figure 2-2 Production Flow of GH**



### 2.5.3 Control Points in the Manufacturing Process

Hayashibara Co., Ltd. maintains quality control by determining specified control points within the manufacturing process of GH. The critical control points and control points are listed in Table 2-8.

**Table 2-8 Critical Control Point (CCP) and Control Points (CP) in the Manufacturing Process of GH**

| Processing Step | Critical Control Point (CCP) and Control Point (CP) |
|---|---|
| Enzymatic reaction | CP: Heating temperature |
| Spray drying | CCP: Pressure decrease of liquid drying filter<br>CP: Foreign substances |

### 2.5.4 Tracking Program

Hayashibara's manufacturing process is organized into units consisting of a series of enzymatic reactions and purification steps. One (1) unit of enzymatic reaction and purification is completed within 2 weeks. The production date for the materials produced within that week is assigned a lot number. The reference numbers and quantities of all materials and processing aids used in each unit of enzymatic reaction and purification are recorded for each lot number and stored. Thus, it is possible for Hayashibara to track not only the finished product, but also each raw material component that was used in the processing of that finished product. All raw materials and finished products can be traced to a particular week of production.

## 2.6 Product Stability

To test the stability of GH, 3 different lots of product were packed in aluminum-laminated bags. The samples were stored at room temperature in a storage room for a total of 42 or 44 months. Lot No. 3E091 was tested for description, loss on drying, pH, total aerobic microbial count, coliform organisms, content of MGH and content of hesperidin at 0, 6, 12, 24, 36 and 42 months. Identification (1), identification (2), lead, arsenic and residue on ignition were tested at 0 and 42 months. As for Lots 3E311 and 3F131, all the specifications were tested at 0, 36 and 44 months (Table 2-9).

Accelerated stability tests were also conducted. The samples for the accelerated storage test were stored at 40°C: RH 75% for 7 months. Loss on drying, pH, content of MGH and total hesperidin were tested at 0, 1, 3, 6, and 7 months. Total aerobic microbial count and coliform organisms were tested at 0 and 7 months (Table 2-10).

The stability study showed that virtually no change had occurred, and that the product remained within the prescribed specifications throughout the test periods. Considering the test results, it was concluded that GH was stable for at least 36 months of storage under the standard test conditions, and 7 months under the accelerated temperature and humidity.

**Table 2-9 Stability of GH**

| Variables | Specifications | Lot No. | Storage period (months) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Start | 6 | 12 | 24 | 36 | 42 (or 44) |
| Description | A pale yellow to yellow-brown powder having a slight, characteristic odor | 3E091 | Qualified | Qualified | Qualified | Qualified | Qualified | Qualified |
| | | 3E311 | Qualified | | | | Qualified | Qualified |
| | | 3F131 | Qualified | | | | Qualified | Qualified |
| Identification (1) | A brown color develops. | 3E091 | Qualified | | | | | Qualified |
| | | 3E311 | Qualified | | | | Qualified | Qualified |
| | | 3F131 | Qualified | | | | Qualified | Qualified |
| Identification (2) | Exhibits a peak at the position corresponding to monoglucosyl hesperidin, having an absorption maximum at a wavelength of 280 – 286 nm. | 3E091 | Qualified | | | | | Qualified |
| | | 3E311 | Qualified | | | | Qualified | Qualified |
| | | 3F131 | Qualified | | | | Qualified | Qualified |
| Lead (as Pb) | ≤ 10 μg/g | 3E091 | Qualified | | | | | Qualified |
| | | 3E311 | Qualified | | | | Qualified | Qualified |
| | | 3F131 | Qualified | | | | Qualified | Qualified |
| Arsenic (as As) | ≤ 1.5 μg/g | 3E091 | Qualified | | | | | Qualified |
| | | 3E311 | Qualified | | | | Qualified | Qualified |
| | | 3F131 | Qualified | | | | Qualified | Qualified |
| Loss on drying | ≤ 6.0% | 3E091 | 3.1 | 2.7 | 2.7 | 3.0 | 3.1 | 3.0 |
| | | 3E311 | 2.8 | | | | 3.6 | 3.6 |
| | | 3F131 | 2.7 | | | | 3.4 | 3.3 |

**Table 2-9 Stability of GH (Cont.)**

| Variables | Specifications | Lot No. | Storage period (months) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Start | 6 | 12 | 24 | 36 | 42 (or 44) |
| Residue on ignition | ≤ 2.0% | 3E091 | 0.06 | | | | | 0.03 |
| | | 3E311 | 0.07 | | | | 0.06 | 0.01 |
| | | 3F131 | 0.06 | | | | 0.11 | 0.03 |
| pH | 5.0 – 7.0 | 3E091 | 5.4 | 5.9 | 6.0 | 5.6 | 5.9 | 5.6 |
| | | 3E311 | 5.8 | | | | 5.7 | 5.7 |
| | | 3F131 | 5.5 | | | | 5.6 | 5.6 |
| Total aerobic microbial count | ≤ 300 CFU/g | 3E091 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 3E311 | 0 | | | | 1 | 0 |
| | | 3F131 | 0 | | | | 0 | 0 |
| Coliform organisms | Negative/0.1 g | 3E091 | Negative | Negative | Negative | Negative | Negative | Negative |
| | | 3E311 | Negative | | | | Negative | Negative |
| | | 3F131 | Negative | | | | Negative | Negative |
| Mono-glucosyl hesperidin | 75.0 – 85.0% (d.b.) | 3E091 | 77.0 | 76.3 | 76.7 | 76.7 | 77.2 | 77.2 |
| | | 3E311 | 77.1 | | | | 77.8 | 76.6 |
| | | 3F131 | 77.2 | | | | 77.3 | 76.6 |
| Total hesperidin | ≥ 70.0% (d.b.) | 3E091 | 75.2 | 74.5 | 74.8 | 74.7 | 75.3 | 75.3 |
| | | 3E311 | 76.1 | | | | 76.1 | 74.9 |
| | | 3F131 | 75.7 | | | | 75.5 | 74.8 |

**Table 2-10 Stability of GH (Accelerated Test)**

| Variables | Specifications | Lot No. | Storage period (months) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Start | 1 | 3 | 6 | 7 |
| Loss on drying | ≤ 6.0% | 7B011 | 2.5 | 2.5 | 2.4 | 2.4 | 2.5 |
| | | 7B281 | 2.7 | 2.5 | 2.9 | 2.7 | 2.7 |
| pH | 5.0 – 7.0 | 7B011 | 5.6 | 5.7 | 5.6 | 5.6 | 5.6 |
| | | 7B281 | 5.6 | 5.7 | 5.6 | 5.7 | 5.7 |
| Total aerobic microbial count | ≤ 300 CFU/g | 7B011 | 0 | | | | 0 |
| | | 7B281 | 0 | | | | 0 |
| Coliform organisms | Negative/0.1 g | 7B011 | Negative | | | | Negative |
| | | 7B281 | Negative | | | | Negative |
| Monoglucosyl hesperidin | 75.0 – 85.0% (d.b.) | 7B011 | 75.2 | 76.3 | 75.6 | 76.2 | 75.5 |
| | | 7B281 | 75.4 | 76.5 | 76.4 | 76.5 | 76.3 |
| Total hesperidin | ≥ 70.0% (d.b.) | 7B011 | 71.6 | 73.0 | 72.5 | 73.0 | 72.4 |
| | | 7B281 | 71.5 | 72.4 | 72.3 | 72.4 | 72.3 |

## 2.7 Physico-Chemical Properties, and Physical and Technical Effects

The following section includes various analytical assays, which have been used by Hayashibara Co., Ltd. to characterize the physico-chemical properties, and physical and technical effects of GH for which it can be used in food and beverages. The last section provides a listing for the physical or technical functional effects for which GH can be used as listed in both 21CFR §170.3 (o) (1–32) (FDA. 2009).

### 2.7.1 Physico-Chemical Properties

The following section includes studies on the physico-chemical properties of GH. These properties are the basis for the physical and technical effects of the substance discussed in the following section, and the technical functional effects from listed in 21CFR §170.3 (o) (1–32) (FDA. 2009).

#### 2.7.1.1 Solubility in various solvents

All solubility studies used GH Lot No. 5B121. The Notifier would be pleased to provide the FDA with any information related to the methodologies and equipment used for these studies.

**Water**

The solid content of GH-saturated solution was determined at 3 different temperatures by the heat-drying method using reduced-pressure (drying aid method). The solubility measurements at 5, 10 and 25°C were not less than 123, not less than 153 and not less than 197 g/100 g-water, respectively. The reason that each reading was reported as "not less than" was because In each case the solution could not be stirred to saturation because of the high viscosity of the solution (Hayashibara Biochemical Laboratories, Inc; now Hayashibara Co., Ltd., by merger).

**5, 10 and 20 Volume % Ethanol**

The solubility of GH in 5, 10 and 20 volume % ethanol was measured with the heat-drying method using reduced-pressure (drying aid method). The solubility in 5, 10 and 20 volume % ethanol at 25°C were not less than 195, not less than 195, and not less than 190 g/100 g-solvent. As with the water assay the solutions could not be stirred to complete saturation because of the high viscosity.

**99.5% Ethanol**

The solid content of GH-saturated solution was measured using the heat-drying method under reduced-pressure. The solubility was 1.99 g/100 g-99.5% ethanol.

**Soybean Oil**

GH was not soluble in soybean oil (< 0.3 mg/100 g-soybean oil at 25°C.

### 2.7.1.2 Melting and Boiling Point

GH Lot No. 5B121 were assayed for melting point/melting range and boiling point/boiling range. A differential scanning calorimeter (DSC), and thermo gavimetric-differential thermal analyzer (TG-DTA) were used. The DSC data showed that there was an endothermic peak at approximately 50°C, but TG-DTA data revealed that there was a total 4.7% gradual weight loss at the point where the endothermic peaks were observed on the DSC curve. It is thought likely that the endothermic peak may be caused by sublimation of water. TG-DTA data also showed that there was rapid weight loss at approximately 250°C, which is likely caused by decomposition of the GH. Therefore, it was concluded that the test sample did not melt under the test condition but decomposed at about 250°C.

### 2.7.1.3 Hygroscopicity

The hygroscopicity of GH (Lot No. 0411192) was measured over time. Samples were held at relative humidities (RH) of 33.0, 52.8, 60.0 75.2, and 90.1% in sealed

chambers for 14 days. All samples approached a maximum hydration value at Day 1 and remained relatively constant during the experiment (day 14). The water content of samples was increased as the RH of the chamber increased. Caking was not observed at RH of 33.0, 52.8, 60.0 and 75.2% for the period of storage. However, the sample at 90.1% started to deliquesce at Day 2.

**Table 2-11 Change of Water Content at Various Relative Humidities**

| Relative humidity | Water content (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Initial | Day 1 | Day 2 | Day 3 | Day 4 | Day 7 | Day 10 | Day 14 |
| 33.0% | 1.50 | 3.39 | 3.51 | 3.59 | 3.61 | 3.73 | 3.64 | 3.74 |
| 52.8% | 1.50 | 6.45 | 6.61 | 6.67 | 6.68 | 6.79 | 6.68 | 6.79 |
| 60.0% | 1.50 | 8.14 | 8.22 | 8.28 | 8.26 | 8.33 | 8.20 | 8.28 |
| 75.2% | 1.50 | 10.67 | 10.64 | 10.67 | 10.63 | 10.62 | 10.42 | 10.53 |
| 90.1% | 1.50 | 16.07 | 16.81 | 16.99 | 17.01 | 17.19 | 17.34 | 17.01 |

### 2.7.2 Physical and Technical Effects in Food Applications

Glucosyl Hesperidiin (commercial name HAYASHIBARA HESPERIDIN S) has been commercially sold and used in a wide variety of foods in Japan since 1998, for a number of technical effects in accordance with the Food Sanitation Law of Japan. GH can function in foods in a similar manner to other antioxidants, like native hesperidin. The one major advantage is that GH is over 100,000 times more soluble than hesperidin in water (Mitsuzumi, 2011).

During the processing and subsequent storage of orange juice, flavonoids, mainly consisting of hesperidin, can crystallize causing a white precipitate at the bottom of the container or add turbidity to the product (Cameron, et al., 1997; Baker, Cameron, 1999). Some tests showed that fresh orange juice (240 mL) provides 9.7 mg of soluble hesperidin, whereas pasteurized commercial juices contain only 3.7 mg of soluble hesperidin. This indicates that about 40% of the hesperidin in the natural products is available to the consumer of commercial juices because of removal during processing (Gil-Izquierdo, et al., 2001). The addition of GH to orange juice and canned mandarin oranges prevents such precipitation. This effect lasted for at least 6 months for canned mandarin oranges and for orange juice, which increases the quality and nutritional value of the product (Terada, et al., 1995; Kometani, et al., 1997; Nishimura, et al., 1998).

Flavor compounds and color pigments commonly oxidize once they have been incorporated into food products. GH can help stabilize these substances. A study compared the stability of natural pigments with and without GH. GH solutions (0.01 – 0.1%) were mixed with natural pigments (0.05 – 0.1%) and the solutions placed under high intensity lights. The absorbance of each solution was measured at various incubation times. All pigments that have been investigated have shown increased stability when GH was used. The absorption spectra of GH showed absorbance in the ultraviolet light region, but not in the range of visible light (Kometani, et al., 1994; Kometani, et al., 1995). This allows the addition of GH to pigments and foods without changing the color of the products, while protecting natural and added pigments.

Sensory experiments showed that GH might reduce "off" notes in some foods by covering unwanted flavors without altering the active materials. Among the off flavors, GH is reported to mask bitterness, sourness and a "grassy" flavor that is termed "greenness" in Japan. A sensory panel found the addition of

0.1% GH reduced bitterness, and correspondingly increased the preference of green tea, coffee, vegetable juice, and grapefruit juice. Seafood products such as surimi can have an overly "fishy" flavor, whereas the addition of GH reduced this flavor and was judged to enhance palatability. GH can modify other strong flavors (Kometani, et al., 2000).

A list of the technical effects of GH in various foods is provided in Table 2-12. Because these are commercial products, the specific function for all the products may not be known.

### 2.7.3 Technical Effects of GH as Listed in US 21 CFR §170.3 (o)

In order to classify the various technical 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 GH are covered under several of the following terms. The equivalent functional classes to 21 CFR §170.3 (o) (1-32) are also provided below.

**Table 2-12 Technical Effects of GH in US 21CFR §170.3 (o)**

| | |
|---|---|
| (3) Antioxidants | Substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation. |
| (4) * Colors and coloring adjuncts | Substances used to preserve, or enhance the color or shading of a food, including color stabilizers, color fixatives, color-retention agents, etc. |
| (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. |
| (12) Flavoring agents and adjuvants | Substances added to impart or help impart a taste or aroma in food. |

* GH is not a color.

# Part 3. Dietary Exposure

This part of the GRAS Notice is to fulfill the requirements of 21 CFR §170.235 regarding the dietary exposure of Glucosyl Hesperidin (GH) as a result of its intended uses and use levels in the identified food categories.

As provided in Part 1.5 of this GRAS Notice, GH can be used to provide the following physical and technical effects as an antioxidants; coloring adjunct; flavor enhancer; flavoring agents and adjuvants. It should be noted that GH is not a "color" agent per se, but rather a coloring adjunct.

GH is intended to be used in the following food categories: yogurts, chocolates, gummi and hard candies, instant and bottled/canned coffees, instant and bottled/canned teas, carbonated waters, soft drinks, fruit and fruit flavored drinks, beverage powders, fortified water, nutritional drinks and powders, energy and sports drinks and powders, fluid replacement drinks, and other functional beverages. The maximum amount of GH in each of these foods and beverages is 500 mg per serving.

## 3.1 Source of food consumption data for the estimated daily intakes (EDI)

Using food intake data reported in the 2013-2016 NHANES, the exposure levels to GH, **as hesperidin**, that will result from the intended uses were estimated. GH is not found naturally in foods; however, hesperidin the chemical basis of GH and is a chemically and metabolically closely related substance, which is commonly found in the human diet, especially in citrus containing foods. The most recent NHANES (2013-2016) compiled by the National Center for Health Statistics and the Nutrition Coordinating Center was used to calculated exposure estimates. The NHANES was conducted between 2013-2016 with non-institutionalized individuals in the U.S. The NHANES provides the most current food consumption data available for the American population. The food and dietary supplement record for each individual includes the gram weight and nutrient data for all foods consumed during the day of the recall. All estimates were generated with USDA sampling weights to adjust for differences in representation of subpopulations. For this study 1 g is considered equivalent to 1 mL for soft drinks and formula diets for meal replacement. NHANES 2013-2016 dietary data age 1+, after exclusions for breast-fed children, pregnant or lactating females and unreliable data, was used to estimate intake of hesperidin. SAS 9.4 along with Strata, and day 2 dietary weights were used for analyses of mean, median, 90$^{th}$ percentile, and standard errors (SE) for GH exposure (based on hesperidin consumption). Intake was calculated as the average of day 1 and day 2 intake (Appendix 1). The sample population was limited to subjects with both day 1 and day 2 dietary data.

### 3.2 Estimated daily intake (EDI) of naturally occurring Hesperidin from diet

GH is not a natural substance found in the diet. However, when GH is consumed by humans the added glucose is enzymatically cleaved, primarily in the small intestine, to form hesperidin (see Part 6.5). Hesperidin is one of many similar dietary nutritional substances known as "flavonoids" or "bioflavonoids" because of their perceived nutritional benefit. Hesperidin is principally found in oranges and other citrus fruit where it is the principle bioflavonoid from these sources (Fisher, 1982; Garg, et al., 2001; Peterson, et al., 2006).

One of the bases upon which the Notifier is claiming that the consumption of GH is GRAS is its "substantial equivalence" to the consumption of hesperidin from dietary sources. Hesperidin has also recently been reviewed by the Agency and the Notifier given a "no questions" letter (GRN 000796; Part 6.6.4).

Therefore the Notifier of this GRAS Notice has calculated the EDI for the consumption of hesperidin from all sources in the diet, exclusive of intended use, from the NHANES flavonoid database (Sebastian, et al., 2016). However, because of the limitations of analytical methods, the database of hesperidin content in foods is provided as hesperetin. Hesperetin is the direct metabolite of hesperidin during digestion and absorption (see Part 6.4). For the calculation of dietary consumption of hesperidin, the concentrations of hesperetin in foods were converted to hesperidin, and values consequently provide the amount of hesperidin consumed in the diet.

The mean and 90$^{th}$ percentiles of "total" and "consumers only" consumption of hesperidin for the total population is 16.16 ± 0.52 and 56.25 ± 1.46 mg/person/day, and 28.98 ± 0.79 and 79.40 ± 2.49 mg/person/day, respectively. The 90$^{th}$ percentile dietary exposure to hesperidin in the total population (79.40 mg/person/day) is similar to the 72.1 mg/person/day reported in GRN 000796 for the same population group. The total population's values for mg/kg-bw/day (consumers only; mean and 90$^{th}$ percentile) were 0.52 ± 0.02 and 1.36 ± 0.05, respectively. The highest EDI by age group is male teenagers 13-18 years. Looking at these 'consumers only' for mean and 90$^{th}$ percentile in this group provides values of 45.46 ± 3.63 and 104.72 ± 7.73 mg/person/day, respectively. Values for all groups in mg/person/day and mg/kg-bw/day are provided in Appendix 1 (Appendix 1, Tables 6 and 7).

### 3.3 Estimated daily intake (EDI) of GH, as Hesperidin, from intended use

The EDI of GH was calculated from the food categories and NHANES 2013-2016 average food intake (Appendix 1, Tables 2 and 3). However, because the EDI of the

intake from the diet is for hesperidin, the values for GH were converted into the amount of hesperidin that is provided in the maximum consumption of 500 mg GH per serving (i.e. 364.9 mg hesperidin per serving).

GH is a product that contains a high concentration of monoglucosyl hesperidin (MGH) as the main constituent, hesperidin, di- and maltooligosyl hesperidin, and low concentrations of other monoglucosyl bioflavonoids (derived from impurities in food grade natural hesperidin and dextrin sources from which it is made; Table 2-7). During consumption of GH the glucose molecules that were enzymatically attached to hesperidin during manufacture, are enzymatically hydrolyzed, primarily in the small intestine, resulting in the release of hesperidin and glucose (Part 6.4). Therefore it is hesperidin that is further metabolized and taken into the body as hesperetin.

The mean and 90$^{th}$ percentile consumption of GH, as hesperidin, added to the diet for "consumers only" for the total population is 602.71 ± 12.81 and 1,259.15 ± 34.64 mg/person/day, respectively. Further, the values for this group in mg/kg-bw/day are 9.35 ± 0.15 and 19.33 ± 0.37, respectively (Appendix 1, Tables 4 and 5). The values for the actual consumption of GH for the mean and 90$^{th}$ percentile would be 825.86 ± 17.56 and 1,725.33 ± 47.47 mg GH/person/day, respectively and 12.82 ± 0.21 and 26.49 ± 0.51 mg GH/kg bw/day, respectively (Appendix 1, Tables 2 and 3). In Part 6.7.4 a published 13-week sub-chronic rat toxicity study reports female (n = 10) and male (n = 10) HanRcc:WIST (SPF) rats were fed a diet of 50,000 ppm of GH for 13 weeks provided a NOEL of 3,083.99 mg/kg-bw for males and 3,427.84 mg/kg-bw for females. Using the mean of the female/male NOEL, this is equal to a safety factor of an average female/male NOEL of 3,256 mg of GH/kg-bw/day (Matsumoto et al., 2019). Suggesting a safety margin of greater than 100 fold.

The highest mean and 90$^{th}$ percentile consumption (consumers only) is for male adults (19-99 years). The amounts of GH, as hesperidin, are 733.90 ± 23.64 and 1,541.07 ± 49.77 mg/person/day, respectively (8.46 ± 0.27 and 17.64 ± 0.67 mg/kg-bw/day, respectively). The same values for GH are 1,005.61 ± 32.39 and 2,111.64 ± 68.07 mg/person/day, respectively (11.59 ± 0.37 and 24.16 ± 0.91 mg GH/kg bw/day, respectively; Appendix 1, Tables 2, 3, 4 and 5).

### 3.4 Estimated daily intake (EDI) of Hesperidin based on naturally occurring Hesperidin from the diet and intended use of Glucosyl Hesperidin, as Hesperidin from the total diet

The highest mean and 90$^{th}$ percentile consumption of GH, as hesperidin, consumers only, from the intended use and the diet is in male teenagers (13-18 years) with intake estimates of 677.34 ± 54.25, and in adult males (19-99 years) of 1,492.83 ± 56.10

mg/person/day, respectively (Table 3-1). This is equal to 9.63 ± 0.70, and 16.86 ± 0.64 mg/kg-bw/day, respectively (consumers only; Table 3-2). The values for the total population of consumers only are 544.98 ± 12.96 and 1,202.26 ± 36.56 mg/person/day, respectively. The mean and 90$^{th}$ percentile amount of GH (as GH) consumed by the total population (consumers only) would be 825.86 and 1,725 mg/person/day, plus 28.98 and 79.40 mg/person/day of hesperidin from the diet, respectively.

However, the highest per user mean and 90$^{th}$ percentile intake estimates of hesperidin (consumers only), on a body weight basis, are for young children 1-6 years being 18.45 ± 0.55 and 39.84 ± 1.52 mg/kg-bw/day, respectively (Table 3-2). The second highest per kg intake estimates (consumers only) are in children 7-12 years. The amounts for the mean and 90$^{th}$ percentile are 12.31 ± 0.39 and 26.06 ± 1.23 mg/kg-bw/day. The mg/kg-bw/day values for the remaining groups are similar to the total population all consumers 90$^{th}$ percentile (8.51 ± 0.16 and 18.38 ± 0.38, respectively). The two younger groups are approximately 2.2 and 1.4 fold greater than mean and 90$^{th}$ percentile of the total population. As mentioned in Part 3.3 above, the published data from a 13-week sub-chronic toxicity study in rats (Part 6.7.4) approximately 82 for children 1-6 years, 125 for 7-12 years, and 177 for the total population of the consumers only in 90$^{th}$ percentile groups.

As mentioned in Part 3.3 above, the published data from the 13-week sub-chronic toxicity study in rats (Part 6.7.4) showed a mean female/male NOEL of 3,256 mg GH/kg-bw/day (Matsumoto et al., 2019). Using this mean it provides a safety factor of approximately 82 for children 1-6 years, 125 for 7-12 years, and 177 for the total population of the consumers only in the 90$^{th}$ percentile groups.

As an additional measure of safety GRN 000719 and 000796 used a 96-week sub-chronic carcinogenicity study of methyl hesperidin in B6C3F$_1$ mice as a pivotal safety study, and to compare the NOAEL of 7,500 mg/kg-bw/day for male mice to the EDI of hesperidin (Kurata et al., 1990). In GRN 000796 the estimated 90$^{th}$ percentile dietary exposure to hesperidin from the diet and intended uses in the total population (not consumers only) was reported in the Agency Response letter as 39.5 mg/kg-bw/day, which is similar to the highest intake sub-group of consumers only in this Notice. This would provide a safety factor of approximately 188 and 288 fold less than the NOAEL in this 96-week sub-chronic carcinogenicity study of methyl hesperidin for the two younger consumption groups and over 400 times for the total population, consumers only, 90$^{th}$ percentile (18.38 mg/kg-bw/day). The Notifier believes that using this comparison is valid because of the chemical and metabolic substantial equivalence of GH to hesperidin.

These data suggests that the consumption of GH as hesperidin and also the consumption of GH appears to have a large safety margin when compared with the pivotal safety data provided in two safety studies (Kurata et al., 1990; Matsumoto et al., 2019).

It should be noted that the calculations of estimated daily intake of GH, as hesperidin, is a slight overestimation of GH intake because MGH, which consists of 75-85% of GH has a molecular weight of about 20% greater than hesperidin. Therefore the amount of hesperidin consumed in GH is less than the same weight of hesperidin.

### 3.5 Estimated daily intake calculation by food code, gender and age groups

The EDI of GH, as hesperidin, was calculated by multiplying each NHANES respondents' 2-day average food intake by the maximum use level (500 mg GH; 364.9 mg hesperidin) for each food code. The mean amounts provide the consumption of hesperidin per person per day. The daily intake of GH (hesperidin) was also divided by the respondent's bodyweight to give the daily consumption on a per kg bodyweight basis. The daily consumption of hesperidin that is naturally found in various foods was also calculated alone and in combination with the intended intake of GH (hesperidin).

**Table 3-1 Summary of the EDI of Hesperidin Under the Intended Use and Diet by Population Group, mg/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/d) | | All-users Intake (or consumers only, mg/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90th Pctl | % | n | Mean | 90th Pctl |
| Young children | 1-6 | 267.69 ± 10.01 | 642.60 ± 33.23 | 86.77 | 1,476 | 308.52 ± 9.04 | 693.36 ± 29.93 |
| Children | 7-12 | 394.38 ± 14.12 | 852.59 ± 32.13 | 90.17 | 1,565 | 437.39 ± 12.55 | 885.43 ± 41.36 |
| Total teenagers | 13-18 | 519.69 ± 28.43 | 1080.58 ± 58.91 | 91.15 | 1,488 | 570.18 ± 31.73 | 1,108.96 ± 49.29 |
| Male teenagers | 13-18 | 627.21 ± 49.60 | 1315.53 ± 104.12 | 92.60 | 739 | 677.34 ± 54.25 | 1,330.92 ± 117.63 |
| Female teenagers | 13-18 | 412.55 ± 30.96 | 869.43 ± 65.44 | 89.70 | 749 | 459.94 ± 32.81 | 906.12 ± 64.33 |
| Total adults | 19-99 | 520.75 ± 12.68 | 1,224.45 ± 36.81 | 90.36 | 7,933 | 576.28 ± 14.48 | 1,293.18 ± 38.81 |
| Male adults | 19-99 | 593.63 ± 20.33 | 1,445.61 ± 53.15 | 89.58 | 3,780 | 662.66 ± 23.67 | 1,492.83 ± 56.10 |
| Female adults | 19-99 | 449.78 ± 12.88 | 1,060.28 ± 33.43 | 91.12 | 4,153 | 493.59 ± 13.70 | 1,095.49 ± 33.33 |
| Total population | 1-99 | 491.25 ± 11.39 | 1,146.54 ± 30.28 | 90.14 | 12,462 | 544.98 ± 12.96 | 1,202.26 ± 36.56 |

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; y = years.

**Table 3-2 Summary of the EDI of Hesperidin Under the Intended Use and Diet by Population Group, mg/kg-bw/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/kg-bw/d) | | All-users Intake (or consumers only, mg/kg-bw/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90th Pctl | % | n | Mean | 90th Pctl |
| Young children | 1-6 | 15.87 ± 0.59 | 37.98 ± 1.40 | 86.04 | 1,463 | 18.45 ± 0.55 | 39.84 ± 1.52 |
| Children | 7-11 | 11.01 ± 0.41 | 25.15 ± 0.99 | 89.42 | 1,557 | 12.31 ± 0.39 | 26.06 ± 1.23 |
| Total teenagers | 13-18 | 7.80 ± 0.40 | 16.49 ± 0.77 | 90.68 | 1,478 | 8.60 ± 0.44 | 17.14 ± 0.70 |
| Male teenagers | 13-18 | 8.91 ± 0.64 | 17.31 ± 1.16 | 92.58 | 738 | 9.63 ± 0.70 | 18.08 ± 1.22 |
| Female teenagers | 13-18 | 6.69 ± 0.48 | 15.28 ± 1.11 | 88.78 | 740 | 7.54 ± 0.51 | 15.85 ± 1.21 |
| Total adults | 19-99 | 6.40 ± 0.15 | 14.84 ± 0.27 | 89.65 | 7,871 | 7.14 ± 0.18 | 15.43 ± 0.36 |
| Male adults | 19-99 | 6.77 ± 0.22 | 15.75 ± 0.55 | 88.54 | 3,747 | 7.65 ± 0.26 | 16.86 ± 0.64 |
| Female adults | 19-99 | 6.04 ± 0.19 | 14.02 ± 0.34 | 90.73 | 4,124 | 6.66 ± 0.20 | 14.41 ± 0.34 |
| Total population | 1-99 | 7.61 ± 0.15 | 17.56 ± 0.32 | 89.44 | 12,369 | 8.51 ± 0.16 | 18.38 ± 0.38 |

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; y = years.

## Part 4. Self-limiting Levels of Use

Glucosyl Hesperidin (GH) is a modified natural bioflavonoid that consists of a glucose molecule attached to the glucose molecule of hesperidin (Figure 1-1). The intended uses are to provide one of 4 technical effects listed in 21 CFR §170.3 (o). These include use as antioxidants, coloring adjuncts, flavor enhancer, and flavoring agents and adjuvants. The Estimated Daily Intake (EDI) provided in Part 3 was calculated using very conservative values. The use and use levels of GH are based on its functional properties and intended technical effects. Further the cost is always a fundamental consideration.

There are two factors that suggest that the EDI of consumption of GH will be much less than the values calculated. As shown in Part 3 the total population mean and 90$^{th}$ percentile consumption of consumers only of GH, as hesperidin at 544.98 ± 12.96 and 1,202.26 ± 36.56 mg/person/day, respectively (Table 3-2).

a. Hayashibara has marketed GH in Japan since 1998. Sales of GH in Japan from 2012 through 2017 averaged approximately 11,000 kg. If the Japanese population (127 million) were to consume this entire quantity (6 years) of GH in a 1-year period, the mean **yearly** intake would only equal 0.52 g. The daily mean intake would be 0.0014 g and a 90th percentile of approximately 0.0028 g. The actual average amounts of GH that have been consumed yearly, and daily and for the 90$^{th}$ percentile in Japan have been 0.087, 0.00024, and approximately 0.00048 g, respectively.

However, there is another GH-like product sold in Japan for which Hayashibara does not know the amount of sales. This would obviously increase the daily mean intake in Japan of hesperidin derivatives. However, even if the consumption of GH or a GH-like product were multiplied many fold it would provide consumptions of much less than what is calculated in Part 3.

b. Additionally, GH is a relatively expensive ingredient, which may set a self-limiting amount in most applications. Therefore, GH will not likely be formulated into all foods within a given category, and the level of its use may vary depending upon the desired effect in the final food product.

Given these factors, Hayashibara feels it is reasonable to assume that the daily exposure amount for GH estimated in Table 3-2 is conservatively high and the real amount would be substantially less than the estimate. Further the Notifier submits

that there is a sufficient margin of safety to support the consumption levels presented in Part 3.5.

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

The statutory basis for the conclusion of GRAS status is through scientific procedures.

While glucosyl hesperidin (GH) has not been consumed in the US before 1958, it should be noted that the base substance from which is it produced, hesperidin, has been in the human diet since ancient times, primarily being consumed from citrus fruits (Garg et al., 2001). Hesperidin is one of thousands of flavonoids that are found in most plant-based foods. Consumption of hesperidin previous to 1958 is pertinent to this GRAS Notice because the main constituent of GH is monoglucosyl hesperidin (MGH; $\geq$ 75.0%). MGH is digested in the gut and assimilated into the body as hesperidin (see Part 6). Additionally, GH contains approximately 15% native hesperidin (see Part 2.4.6).

# Part 6: Narrative

## 6.1 Introduction

This section on the safety of Glucosyl Hesperidin (GH) includes company information on a history of human use of GH, a review of *in vitro*, animal and human information and data related to the safety of natural hesperidin, hesperidin-containing complexes, and GH. Data on natural and hesperidin-containing complexes are included because GH is hydrolyzed to hesperidin under normal conditions in the small intestine before it is absorbed. Following absorption, it is metabolized in an identical manner to natural hesperidin (see Part 6.3 below). Therefore, the safety of GH is directly related to the safety of hesperidin. The main component of GH is monoglucosyl hesperidin (MGH; Part 2.4.6). The Notifier claims and provides data and information that GH is "substantially equivalent" to natural hesperidin and has a similar absorption mechanism and the same metabolism and nutritional benefits as consumption of hesperidin. The following will provide evidence of these claims.

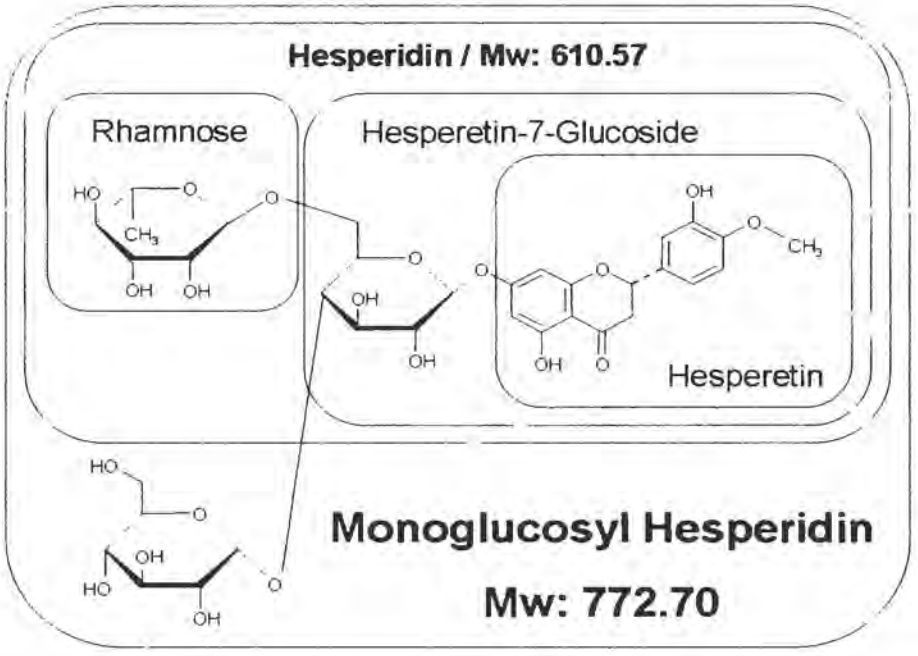
Hesperidin is a major flavonoid in the human diet; however, it is almost exclusively found in citrus species and is purified as a by-product of citrus fruit processing (Garg, et al., 2001; Manach, et al., 2003). It is a glycosylated flavanone (a class of flavonoid), which is composed of hesperetin (aglycone) and rutinose. Rutinose is the name for a disaccharide consisting of glucose, which is attached to the hesperetin molecule and rhamnose, which is attached to the glucose (Figure 6-1; Evans, 1996; Matsumoto, et al., 2019). The basic structure consists of a 1,4-benzopyrone having a phenyl group at the C-2 position (Whalley, 1962). Hesperidin is one of the most highly concentrated flavonoids in oranges (especially sweet organges), tangors, tangerines (mandarin), tangelos, and lemons (Fisher, 1982; Garg, et al., 2001; Peterson, et al., 2006). The concentration of hesperidin is greatest in the albedo, membranes and pith of oranges and lower in juice vesicles (Garg, et al., 2001). In addition, hesperidin is also reported to be found in aromatic herbs and mint; however, in a USDA document of selected foods containing hesperidin, including herbs, only peppermint is listed as containing hesperidin (Manach, et al., 2003; Bhagwat, et al, 2013). In the US millions of tons of oranges and other citrus fruits are consumed each year (USDA ERA, 2018). There appears to be no information in the literature that suggests that consumption of hesperidin in the diet results in any safety-related issues in humans. Part 6 of this GRAS contains published and unpublished studies demonstrating the safety of hesperidin and GH.

Hesperidin has been reviewed for safety by the FDA on various occasions. In a 1982 SCOGS report on Hesperidin and another flavanone preparations the Select

Committee noted that hesperidin had previously been given GRAS status as an antioxidant to preserve the flavor of milk-based beverages (6.6.1 below; Fisher, 1982). The report noted it had been given prior sanctioned status in special dietary foods up to 1 g daily, and also for over-the-counter distribution with a recommended dosage of no more than 1 g per day. Later the SCOGS was given GRAS status for enhancing and preserving flavor at a level of 30 ppm in flavored milk (Fisher, 1982). Very recently the Agency provided a "no objection" letter for GRN 000796, which is a GRAS Notice for an orange extract (85% hesperidin). The ingredient is for use in a number of food categories, many similar to what is included in this document, and at the same maximum amount per serving, 500 mg. Additonaly, an earlier GRN (000719) on a orange pumice product, containing a relatively high concentration of hesperidin also received a "no objection" letter.

Despite the recent increased interest in antioxidants in the diet, and a relatively long history of use of hesperidin as a dietary supplement, the actual applications of isolated hesperidin in food systems are limited because of the extremely low aqueous solubility of hesperidin (only about 1 g in 50 liters of water or about 0.002% w/v at ambient temperature). Therefore, the development of a more water-soluble hesperidin derivative has been sought.

**Figure 6-1 The Structure of Monoglucosyl Hesperidin**




Matsumoto et al. 2019, Fundam.Toxicolog Sci. 6(8) 299-317.

## 6.2 Human Consumption and Exposure to GH

In Japan GH has been listed since 1998 in the Existing Food Additives List, and monographed in the 9th edition of Japan's Specifications and Standards of Food Additives (JMHLW, 2018) under the name of Enzymatically Modified Hesperidin. In Japan GH can be legally labeled as hesperidin, as an abbreviated name, on foods containing GH (Cabinet Office Ordinance, No. 10, 2015). Under the Health Promotion Act in Japan two products of powdered soft drink containing GH as nutritional ingredient were approved in December 2011 to be "Food for Specified Health Uses (FOSHU)" products for consumers, and currently 12 products containing GH as a nutritional ingredient have been approved as of September 2019 (JMHLW, 2002). Under Food Labeling Act in Japan (CAA, 2013), a tablet product containing GH as a nutritive ingredient was notified in April 2015 by systematic review to be a "Foods with Functional Claims" product, and 43 products containing GH as an ingredient have been notified as of August 2019. GH has been sold mainly in Japan since 1998; however, in Taiwan, GH is listed in their Food Ingredients List under the name of alpha-glycosyl hesperidin and categorized as "草、木本植物類來源製取之原料 (Raw materials derived from grass and woody plants)" (Taiwan FDA, 1975). In Korea, GH is monographed in the Korea Food Additives Code under the name of Enzymatically Modified Hesperidin (MFDS, 2019). The ingredient is used in a wide variety of foods. The sales and related consumption of GH in Japan from 2007 through 2017 were approximately 75 metric tons.

The Notifier, Hayashibara Co., Ltd., is not aware of any consumer complaints associated with GH or products in which GH is used. Further, the Notifier is not aware of any adverse effects to company personnel who work with the raw materials, participate in the manufacturing process, or handle GH on a continuous basis, that appear to have any relation to this exposure.

## 6.3 Safety of the GH manufacturing process

Scientists at Hayashibara Co., Ltd. (the Notifier), worked on the development of water-soluble bioflavonoids, including hesperidin. In several industries, cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) is currently used to catalyze the conversion of oligosaccharides to cyclodextrins by intermolecular transglycosylation (Szejtli, 1988). The Notifier developed a process whereby a suitable glycosyl residue (1 to 15 glucose units) is transferred to the C4 position of the glucose unit of the hesperidin molecule by CGTase. The CGTase is isolated from a Non-GMO strain of *Bacillus stearothermophilus* (now *Geobacilius stearothermohilus*; Kometani, et al., 1994). Glucoamylase (EC 3.2.1.3) is then used, if needed, to cleave off terminal glucose units from the formed glycosyl molecule resulting in a single glucose molecule

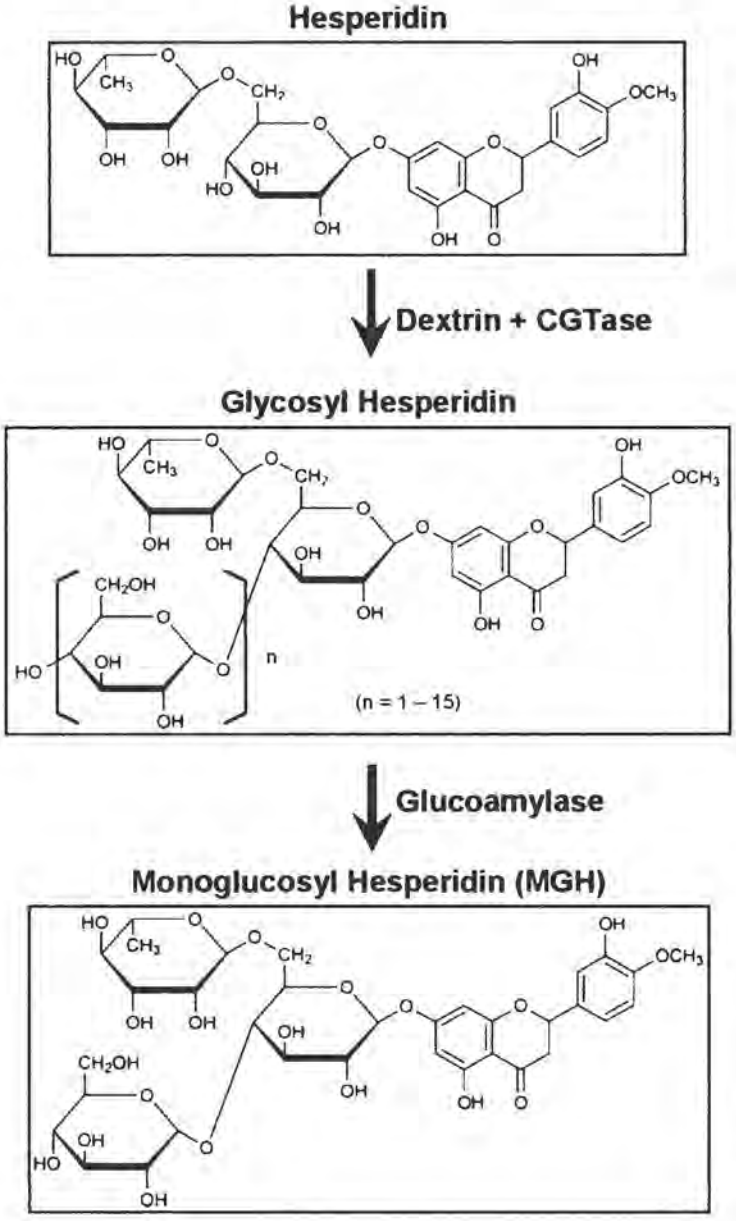
attached to the rhamnosyl glucoside moiety (C4 position of the glucose) of hesperidin (Figure 6-2 below; Hijiya, Miyake, 1991; Miyake, Yumoto, 2000). The end products of this process are monoglucosyl hesperidin (MGH; 75 - 85%), hesperidin (approximately 15%), other glycosylated flavonoids, and small amounts of cyclodextrins and free saccharides, which make up GH (see Tables 2-2 and 2-7). All raw materials, enzymes, equipment and processes used are food or food additive grade and are very common to the food and dietary supplement industry (see Table 2-1). The production of GH by the Notifier is consistent with the "Specifications and Standards for Food, Food Additives, etc. Under the Food Sanitation Law in Japan".

As mentioned above, GH is produced via an enzymatic process that includes two enzymes that have been common in food manufacture and processing for many years. These enzymes are Cyclodextrin Glucanotransferase (CGTase; E.C. 2.4.1.19) and Glucoamylase (E.C. 3.2.1.3).

CGTase is produced from the non-GM source organism *Geobacillus stearothermophilus* (basonyme, *Bacillus stearothermophilus*). *G. stearothermophilus* is a well-known rod-shaped, spore-forming, non-pathogenic, non-toxigenic, ubiquitous, gram-positive soil organism in the order of Firmicutes. It is known for being responsible for food spoilage, but has not been observed to be pathogenic to any host. The Notifier was not able to identify any information associating *G. stearothermophilus* to human infection. The organism is commonly used commercially as a challenge microorganism for sterilization processes. *G. stearothermophilus* is listed as BioSafety Level 1 by the American Type Culture Collection (ATCC), is not listed in the FDA Bad Bug Book (2nd ed.), and is classified by EFSA as having a Qualified Presumption of Safety (QPS).

The production of CGTase via *G. stearothermophilus* by the Notifier operates under a Quality Management System, which has been assessed and found to comply with the requirements of ISO 9001:2008/JIS Q 9001:2008. The enzyme is filtered to remove all the source microorganisms. CGTase, produced by *G. stearothermophilus*, is monographed in Japan's Specifications and Standards for Food Additives for use in the processing of foods (JMHLW, 2018). CGTase is commonly used cyclodextrins from amylaceous polysaccharides, such as starch. However, the enzyme can also attach oligoglucose chains to many other substrates. During the process to manufacture GH, this results in glycosyl heperidin, which consists of hesperidin attached to a chain with multiple glucose units at the C-4 position of the hesperidin glucose moiety.

**Figure 6-2 Schematic of the Manufacturing Process of Glucosyl Hesperidin**




CGTase from *G. stearothermophilus* has been characterized and safely used in Japan for many years in the food industry (Kitahata, Okada, 1982). It has been used for the commercial production of GH by the Notifier since 1998, and to the Notifier's knowledge has not been associated with any untoward affects on humans or animals.

Concerning the safety of *G. stearothermophilus*, a preparation of α-amylase from *B. stearothermophilus* is recognized as a direct food substance in 21 CFR 184.1012, and

the 1,4-α-glucan branching enzyme preparation from non-genetically-modified *G. stearothermophilus* strain TRBE14 was issued a "no question letter" to GRN 000405. Conversely, many naturally occurring organisms have been reported to produce CGTase, including many strains of *G. stearothermophilus* (BRENDA: The Comprehensive Enzyme Information System; http://www.brenda-enzymes.org/enzyme.php?ecno=2.4.1.19). In the US, the production and safety of α-, β- and γ-cyclodextrins produced using CGTase from a recombinant strain of *Escherichia coli* KI2, harbouring the CGTase gene of *Klebsiella oxytoca,* was reviewed in separate GRAS Notices. "No questions" letters for α-, β- and γ-cyclodextrins (GRN 000155, 000074, and 000046, respectively) were issued through scientific procedures. No safety concerns for the CGTase enzymes were described in the Agency response letters.

The second enzyme, Glucoamylase (EC 3.2.1.3) is one of many common enzyme carbohydrases that are used to decompose starch into glucose, and is one of the principal enzymes used to produce food grade glucose.

Glucoamylase is commercially produced in Japan from fermentation of *Rhizopus oryzae*, which is a non-GM, non-pathogenic, non-toxigenic, non-afflatoxin producing filamentous fungi. This organism is classified as Biosafety Level 1 by ATCC and not listed in the FDA Bad Bug Book (2nd ed.). Glucoamylase produced by *Rhizopus oryzae* is monographed in Japan's Specifications and Standards for Food Additives for use in the processing of foods (JSFA, 9th Edition). While the principle use of glucoamylase is to hydrolyze starch into glucose, in the production of GH it can be used to hydrolyze the terminal end glucose molecules from the chains of glucose that were attached to hesperidin at the C-4 position of the hesperidin glucose molecule by the CGTase enzyme (Figure 6-1 and 6-2). The purpose is to have a high concentration of monoglucosyl hesperidin (75 to 85%) in GH.

In the US, carbohydrase from *Rhizopus oryzae* are recognized as secondary direct food additives permitted in food for human consumption in 21 CFR 173.130. The glucoamylase from *Rhizopus oryzae* used for GH production qualifies under 21 CFR 173.130 as a carbohydrase, except that it is a powder and does not need to be refrigerated or labeled as needing refrigeration.

Several naturally occurring organisms have been reported to produce glucoamylase, including *Rhizopus oryzae*, *Rhizopus niveus*, *Bacillus* sp., *Saccharomyces cerevisiae*, *Lactobacillus amylovorus*, *Aspergillus tubingensis*, etc, (BRENDA: The Comprehensive Enzyme Information System; https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.3).

In the US, under 21 CFR 172.892, glucoamylase (EC 3.2.1.3) is permitted to be used to make food starch-modified which is a food additive permitted in food for human consumption. Additionally amyloglucosidase (a.k.a. glucoamylase) derived from *Rhizopus niveus* is recognized as a secondary direct food additive permitted in food for human consumption in 21 CFR 173.110.

The two enzymes, CGTase and Glucoamylase, are used in this order with other raw materials (processing aids) to first produce glycosyl hesperidin from hesperidin and dextrin, and then to cleave off the extra glucose units from the glycosyl hesperidin. This results in a high concentration of monoglucosyl hesperidin (75 – 85%) in the GH product. After the process is completed, the mixture is heated to inactivate the enzymes. Further processing steps remove much of the non-essential substances, including protein. While not a specification, protein from the commercial production lots has been assayed and were found to be < 50 µg/g.

## 6.4 Metabolism of Hesperidin

The following detailed information and data presented below demonstrates that the metabolism of GH is substantially equivalent to that of natural hesperidin from which it is produced. The only difference is that the glucose molecule that is enzymatically added to hesperidin to make MGH is removed primarily in the small intestine, resulting in a free glucose and the original hesperidin. The hesperidin from MGH is metabolized by the identical pathway as when native hesperidin is consumed.

### 6.4.1 Absorption of Hesperidin

Most flavonoids, including hesperidin (hesperetin-7-rutinoside; Figure 6-1) are found in foods as β-glycosides (Kuhnau, 1976). Data from several different flavonoid systems have demonstrated that the glycosylated forms do not enter into the body, but first undergo deglycosylation. Studies have demonstrated that the production of the "aglycone" form for many flavonoids occurs in the brush border of the small intestine by lactase phloridzin hydrolase (LPH). Another identified membrane receptor system (SGLT1) binds to certain flavonoid glycosides, transports them into the enterocyte, where they are hydrolyzed to the aglycone by cytosolic β-glucosidase (Nemeth, et al., 2003). However, Griffiths and Barrow demonstrated that the enzymes capable of hydrolyzing hesperidin to its absorbable aglycone, hesperetin (Fig. 6-1), are not found in the upper small intestinal tract (Griffiths, Barrow, 1972). It was also reported that when hesperidin was incubated with cecal contents from rats that hesperetin was formed, as well as *m*-hydroxyphenylpropionic acid (*m*-HPPA; Scheline, 1968). This substance is a further metabolite of hesperidin. Using both germ-free animals and animals whose digestive tracts were sterilized by antibiotics, it was demonstrated that

neither hesperetin nor m-HPPA are formed (Griffiths, Barrow, 1972). This suggests that hesperidin is actually hydrolyzed to hesperetin in the lower intestine where bacterially produced factors hydrolyze it to the aglycone form for enterocyte transit.

Additional evidence that the initial fate of hesperidin occurs in the lower and not the upper small intestine, as with some other flavonoids, is the time to maximum concentration in the plasma ($T_{max}$; Manach, Donovan, 2004). Two studies have shown that the $T_{max}$ for hesperidin provided in orange juice was 5.6 and 5.4 hours, respectively (Manach, et al., 2003; Erlund, et al., 2001). The type of sugar moiety attached to the flavonoid base is a major determinant of the absorption of dietary flavonoid glycosides in man (Hollman, et al., 1999; Manach, Donovan, 2004). In a study by Nielsen et al. the authors enzymatically cleaved the rhamnose moiety (rutinoside consists of glucose and rhamnose) to create hesperetin-7-glucoside (Fig. 6-1; Nielsen et al., 2006 ). The remaining glucose unit on this molecule can then be hydrolyzed by an enzyme (LPH) located in the small intestine. The data bore out this conclusion in that the $T_{max}$ for hesperetin-7-glucoside was 30 minutes, while the $T_{max}$ for hesperidin was about 5.5 hours. Taken together, these data strongly suggest that the bulk of hesperidin must be enzymatically converted to hesperetin by enzymes in the lower digestive tract.

Further, all available evidence suggests that, in general, flavonoids must be in the aglycone or more highly metabolized forms to be absorbed into the body (Aherne, O'Brien, 2002). More recent data has demonstrated that enterocytes absorb and metabolize several polyphenols. In some cases the metabolites in the plasma may exceed the parent complexes (see below; Williamson, 2017). Other than the aforementioned SGLT1 system, it is thought that passive diffusion is responsible for much of the absorption in both the small and large intestine (Aherne, O'Brien, 2002; Manach, Donovan, 2004).

In a series of publications over several years Caco-2 cell monolayers (Caco-2 cells) have been used to elucidate the absorption pathway of hesperidin. Caco-2 cells are a common model system for investigating small intestinal epithelial transport (Kim, et. al., 1999). In this first experiment the authors reported that hesperidin did not permeate across the cell monolayer, which hesperidin glycosides did in a time- and dose-dependent manner. It was thought that this occurred because of hesperidin's low solubility. Further, the transport was energy-independent and inversely correlated to the transepithelial electrical resistance (TER). This suggests that hesperidin glucosides pass via a paracellular pathway. Using the same Caco-2, two publications in 2008 reported on the cellular permeability of hesperidin and hesperetin (Kobayashi, Konishi, 2008; Kobayashi, et al, 2008). In the presence of a proton gradient, hesperetin permeated the cell in the apical-to-basolateral (ap-to-bl) direction more than

400 fold greater than hesperidin. The transepithelial movement of hesperidin was the same with or without the proton gradient and inversely proportional to the TER, which was again suggestive of paracellular diffusion (Kobayashi, et al, 2008). Without the proton gradient the flux of hesperetin in either direction (ap-to-bl, or bl-to-ap) was essentially the same. The results suggest that hesperetin is efficiently absorbed in the intestine by transcellular proton gradient active-transport; whereas, hesperidin is poorly transported via a paracellular path, and most transport is performed via conversion to hesperetin (Kobayashi, et al, 2008). The second study looked a group of flavanones, including hesperidin, and concluded that the proton gradient active transport appears common to flavanones (Kobayashi, Konishi, 2008). The final paper used Caco-2 cells and an artificial membrane assay with pH gradients and iso-pH conditions (Kobayashi, et al., 2012). The authors concluded that both passive diffusion and active transport of hesperetin contribute to absorption through the human intestinal epithelium. In total, it appears that using the Caco-2 cell model for human intestinal absorption of hesperidin shows that, most all hesperidin is hydrolyzed to hesperetin (aglycone) and then enters the system via a transcellular active transport mechanism, and this pathway is common to other flavanones aglycones (Kobayashi, et al., 2012).

### 6.4.2 Metabolites of Hesperidin

Once flavonoids, including hesperidin, are hydrolyzed, the aglycones appear to enter the epithelial cells of the gastrointestinal tract, and are conjugated with glucuronic acid or sulfate either in the enterocyte or in the liver (Kuhnau, 1976; Manach, Donovan, 2004). A number of animal and human studies suggest that this is also the metabolic process for hesperidin (Ameer, et al., 1996; Erlund, et al., 2001; Manach, et al., 2003; Del Rio, 2013). Matsumoto, et al. (2004) identified and quantitated the major glucuronide metabolites in rats after consumption of hesperidin. Hesperetin-glucuronides were present as the major metabolite, while hesperetin-sulfates were not present. Hesperetin-sulfoglucuronides were not directly detected in this study, but the difference between total conjugates and glucouronides suggested their presence. Further, this study in rats showed the presence of conjugated forms of metabolites of hesperetin (homoeriodictyol-conjugates), showing that metabolic products other than hesperetin are formed (Matsumoto, et al., 2004). As noted previously, rat cecal contents (microorganisms) primarily produce hesperetin from hesperidin, which is then metabolized to *m*-HPPA (Scheline, 1968). Further, *in vivo* studies in rats and rabbits also demonstrated *m*-HPPA as a primary metabolite; however, in a study with one human subject, *m*-HPPA was not observed (Booth, et al., 1958, Fisher 1982). This suggests that there are likely species differences in metabolic pathways and metabolite production between humans and other animals (Booth, et al., 1958). Regardless, of the specific metabolites, it is almost certain that the aglycone of

hesperidin (hesperetin) and perhaps its further metabolites are in a conjugated form in the plasma. This mechanism is similar for essentially all known flavonoids. Studies in humans support this. In the previously cited report by Manach, et al. (2003), human volunteers consumed orange juice. No aglycones or sulfated conjugates were observed in the plasma, while all the detectable hesperetin was present as glucuronidated conjugates, 13% of which were sulpho-glucuronidated conjugates (Manach, et al., 2003). The analysis also showed that no other metabolites were identified, suggesting that no further metabolic transformations occurred, at least during the experimental period. In a more recent study reviewed by by Del Rio et al. human subjects were given orange juice that contained 168 umol of hesperetin-*7-O*-rutinoside Del Rio, et al. 2013). Plasma contained hesperetin-*7-O*-glucuronide and a non-specified hesperetin-*O*-glucuronide.

A more recent study with humans showed that absorption of orange flavonoids may be affected by factors such as processing and individual variabilities, and the bioactivity of the absorbed phytochemicals depends on how they are metabolized during absorption. 20 subjects consumed orange fruit (79.7 $\pm$ 17.7 mg hesperetin/portion), and also juice (71.8 $\pm$ 8.1 mg hesperetin/portion) in a randomized cross-over study. An additional group of 109 subjects were recruited and only consumed the orange juice. The 109 subjects data were combined with the juice consumption data of the 20 subjects. As mentioned above in other studies hesperidin was found in plasma and urine as hesperetin conjugates, but not aglycones or rutinosides. Low concentrations of hesperetin were detected in plasma within 15 min of ingestion, and the pharmacokinetic profile for hesperetin in plasma was similar for both the fruit and juice. There was a small peak at 1– 1.5 h followed by the major peak at about 6 h with a subsequent decline to near baseline over the next 48 h. The data on excretion is given below in the next section (Brett, et al., 2009).

### 6.4.3 Excretion of Hesperidin

As mentioned previously, the metabolism and excretion of many different flavonoids are reported in the literature (Manach, Donovan, 2004). There are a few studies in which hesperidin was administered to humans to determine plasma kinetics and excretion. One (1) male was given different dosing regimens of substances containing hesperidin and naringin, which is another flavonoid also found in citrus fruits, but primarily in grapefruit (Ameer, et al., 1996). Subsequent treatments were given 8 weeks after the proceeding treatment. In the first experiment, purified preparations of 500 mg of hesperidin and 500 mg of naringin were mixed in water and immediately swallowed. In a second study grapefruit juice and orange juice were mixed and given in 5 uneven doses over 48 hours. The first two of the 5 doses of the juices were to simulate a "loading dose". Plasma and urine samples were hydrolyzed with β-

glucuronidase to release the aglycone form. The treatment of all hesperidin-related substances with β-glucuronidase is necessary because the glucuronide moiety interferes with HPLC detection.

Hesperetin was detected in the urine within 3 hours of consumption, and for 38 hours after; however, only about 3% of the administered dose was identified in the urine. When hesperidin was ingested in the mixed juice, hesperetin was present in the urine by 6 hours after the first dose and up to 24 hours after the last of the five doses. In this second experiment about 24% of the hesperidin dose appeared in the urine, most likely as glucuronide conjugates. Other related metabolites of hesperetin were observed in urine samples, but these were thought by the authors to be absorbed from the juice, and not formed after absorption (Ameer, et al., 1996). The authors did not explain the large percentage difference between the two studies in recovery of hesperetin in the urine. It may have been caused by the large dose of hesperidin in the first experiment, a solubility issue, the influence of a cofactor in juice, other unknown factors or a combination of these. No mention was made of any untoward effects reported by the subject.

In the review of the following studies absorption, metabolism and excretion will all be discussed together. In a similar study to that reported by Ameer and co-workers, 8 subjects consumed 8 mL/kg orange juice (Erlund, et al., 2001). The concentration of the hesperidin in the orange juice was 218 mg/L. The authors reported that all subjects had hesperetin in their plasma and urine. The time to maximum plasma concentration ($T_{max}$), was 5.4 ± 1.6 hours, which is in agreement with that of other studies; however, the relative urinary excretion was only 5.3%, which is much lower than the previous report (24%) when juice was given. There was a highly significant correlation between the plasma time curver (AUC) and urinary excretion. The data suggest that urinary excretion may be only a small factor in the elimination of hesperidin. Biliary excretion has been identified as one pathway for excretion, which suggests that the substances can enter the entero-hepatic cycle. However, this has only been demonstrated in rats and may not occur in humans (Hackett, et al., 1979; Aherne, O'Brien, 2002). Further metabolism of the absorbed hesperetin is also a likely mechanism (Kim, et al., 1999).

Manach, et al. (2003) provided 5 healthy individuals with 0.5 or 1 liter of commercial orange. The juice provided 444 mg/L of hesperidin. No hesperetin was observed in the plasma before the study or by 24 hours after ingestion. Conjugated hesperetin could be detected at about 3 hours after consumption and the $T_{max}$ for the 0.5 and 1.0 liter doses were 5.4 and 5.8 hours, respectively. The $AUC_{0-24}$ hours were approximately 4.2 (0.5-L orange juice) and 9.3 μmol/L·hour (1.0-L orange juice), respectively. These values are consistent with those reported by Ameer, et al. (1996)

using purified hesperidin, and appears to correlate with those also reported by Erlund, et al., (2001).

In another study, 16 healthy human volunteers consumed three samples of "orange juice" (Nielsen, et al., 2006). All orange juice samples were given as 5 mL/kg bw. One dose was orange juice (low dose), the second was orange juice fortified with 3 times the normal concentration of hesperidin (high dose), and the last dose was orange juice treated with a rhamnosidase, which hydrolyzes the rhamnose moiety. This hydrolytic process results in the hesperidin derivative, heperetin-7-glucoside, (H7G; Figure 6-1 above), which is much more water soluble than the native hesperidin, and as mentioned earlier, does not require the rutinose to be cleaved by lower gut bacterial enzymes. Rather, it can be hydrolyzed into the aglycone form by enzymes in the small intestine. The doses given to each group were 0.93 ± 0.06 (mg/kg-bw; low dose), 2.92 ± 0.18 (mg/kg-bw; high dose) and 1.21 ± 0.08 (mg/kg-bw; H7G dose). Analysis of subject blood demonstrated that the $T_{max}$ of hesperetin for the low and high hesperidin doses were 7.0 and 7.4 hours, respectively, while for the H7G dose the $T_{max}$ was only 0.6 hours. While these times are a little slower than that reported for the hesperidin in other juice products, they are within the general time range. The $T_{max}$ for the H7G dose appears to be close to that of other flavonoids that are absorbed in the small intestine (Manach, et al., 2004). The $AUC_{0\text{-}10}$ hours of hesperetin for the low, high and H7G doses were 1.16 ± 0.52, 4.16 ± 1.50, and 3.45 ± 1.27 mmol/L·hour, respectively. The plasma concentration of hesperetin for the high dose was still elevated after 10 hours and therefore the AUC would have been even greater after 24 hours. The plasma concentration for the H7G dose essentially returned to baseline by 4 hours after ingestion. Statistical evaluation of the AUC showed that both the high and H7G doses were significantly greater than the low dose. This suggests that the absorption of flavonoids in the small intestine appears to be more rapid than that which occurs in the large intestine. Urine was collected in three fractions (0 - 5, 5 - 10 and 10 - 24 hours after consumption). The low dose showed a relative urinary excretion (RUE %) of 4.06 ± 1.77, the high dose 8.90 ± 3.83, and the H7G dose 14.40 ± 6.75%. The reason for the RUE of the H7G group being significantly greater is not known. One possible explanation is that the $C_{max}$ of the H7G dose was significantly greater and absorption occurred over a much shorter period of time. Therefore the higher blood concentration may have resulted a more rapid clearance into the urine.

In the study described by Del Rio, et al. (2013) in which human subjects were given 250 mL of orange juice containing 168 µmol of hesperetin-*7-O*-rutinoside, their combined $C_{max}$ was 922 n*M* at a $T_{max}$ of 4.4 h, which is similar to other studies. Additionally when the 0-24 hour urine was examined, not only were hesperetin-*7-O*-glucuronide and the non-specified hesperetin-*O*-glucuronide observed, but also a third

unassigned hesperetin-*O*-glucuronide, two hesperetin-*O*-glucuronides-*O*-sulfonates, and a hesperetin-*O*-diglucuronides (Del Rio, et al., 2013). The quantity of all metabolites in the urine equate to 6.5% of the hesperetin-7-*O*-rutinoside consumed. The presence of these additional metabolites suggest active metabolic processing after absorption.

Finally, in the aforementioned study of Brett, et al. the excretion of hesperetin over 48 hours was 3.9 ± 3.6% (Brett, et al., 2009).

### 6.5 Metabolism of GH (MGH) in the Rat and Human

The following section includes 4 metabolic studies on rats (1 published & 3 unpublished) and one human unpublished study performed by the Notifier (Hayashibara Co., Ltd.) to investigate the similarities in the metabolism of the main component of GH, monoglucosyl hesperidin (MGH), and hesperidin after ingestion. The first two studies used water as the solvent/diluent for the substances. Because of the large disparity in the water solubility of these two substances sodium carboxymethyl cellulose (CMC-Na) was employed for the latter two animal studies. The results of these studies demonstrate that when the solubility is equalized between the two substances, the consumption of MGH results in a metabolic process that is essentially identical to natural hesperidin. The human bioavailability study consisted of two experiments. The first includes a single dose of MGH, while the second test includes the same dose given daily for 14 consecutive days. As with all the data and information in this GRAS Notice the unpublished study reports are available for review by the Agency upon request.

The data demonstrate that GH is metabolized in a similar fashion as hesperidin. The main difference is that the GH with high concentrations of MGH, has greater solubility and can be absorbed to a greater extent.

#### 6.5.1 Rat Study 1 on Monoglucosyl Hesperidin

The information discussed in the preceding sections is necessary to understand the basic mechanism of the metabolism of MGH. Yamada, et al. compared the bioavailability of MGH with that of hesperidin in rats (Yamada, et al., 2006a, published). The animals received equal molar amounts (1 mmol/kg bw) of MGH and hesperidin. Additionally tissue homogenates were prepared from the small intestine and cecum contents of rats. These were used to measure the amounts of enzymatic activity of α- and β-glucosidase.

Results from the small intestinal/cecal contents of α- and β-glucosidase activity study demonstrated that the α-glucosidase of the small intestine and cecal contents were equal; whereas the β-glucosidase activity of the cecal contents was a little more than 5 times greater than that of the small intestine. Tissue homogenates of the small intestine or cecal contents were incubated with MGH, and the concentrations of MGH, hesperidin, and hesperetin were assayed at 0, 1 and 4 hours. In both experiments there was some conversion of MGH to hesperidin at time 0, suggesting a rapid hydrolysis. Further, these data showed that the small intestine hydrolyzed all the MGH to hesperidin in 1 hour; however, no hesperetin was produced. When the cecal contents and MGH were incubated for 1 hour, all the MGH was converted to hesperidin and a small fraction of hesperetin was formed. After 4 hours of incubation, the concentration of hesperidin was about the same, but the amount of hesperetin increased several fold. Indicating that the glucose unit can be cleaved (α-glucosidase) to hesperidin in the upper digestive tract, but for hydrolysis of the rutinose moiety and conversion to hesperetin it requires the presence of β-glucosidase, which occurs principally in the cecum. At time 0 there was about 35% more MGH in the small intestine samples than in the samples containing cecal contents. This suggests that a portion of the added MGH (and possibly hesperidin) may be bound to the cecal contents and unavailable for assay. However, this would not necessarily mean that it could not be eventually hydrolyzed by β-glucosidase to hesperetin and subsequently absorbed. The information in this *in vitro* experiment supports the hypothesis of Manach and Donovan (2004) and others (Nielson, et al., 2006; Garg, et al., 2001) that flavonoid molecules with a rutinose moiety require hydrolysis by β-glucosidase, which is found primarily in the lower digestive tract. It also demonstrates that the added glucose in MGH is hydrolyzed primarily in the small intestine (Yamada, et al., 2006a).

Evaluation of the serum hesperetin-glucuronide concentration in the rats appears to support the *in vitro* data. The serum profile after consumption of hesperidin showed no hesperetin in the serum until 6 hours after administration. The concentration increased until 9 hours, remained the same at 12 hours, and was almost back to baseline 27 hours after consumption. It is likely that the actual peak occurred between 9 and 12 hours.

Serum concentrations of hesperetin-glucuronide after MGH administration were elevated at 15 minutes and remained significantly higher and constant for 3 hours. The peak serum concentration of the MGH group was reached at 6 hours and steadily declined to just above baseline at 27 hours after administration. The mean serum concentrations of hesperetin-glucuronide of the MGH group were consistently and significantly higher than the hesperidin group at all sampling times from 15 minutes until 9 hours after administration. The kinetic profile of the hesperidin administration group showed no hesperetin-glucuronide in the serum through the 3-hour sample. At

the 6-hour sample the mean concentration was about 0.5 µmol/L, and the values essentially were the same at sample times 9 and 12 hours, and the mean value returned to 0 at 27 hours after administration. The AUC for the MGH group was significantly greater than for the hesperidin group. No free hesperetin, hesperidin, or MGH was detected in unhydrolyzed sera of rats given MGH or hesperidin. However, one animal (1 of 5) given MGH had what the authors called "a high level" of hesperidin in its serum when the serum was hydrolyzed with β-glucuronidase. The time or number of samples in which hesperidin was found in the serum was not mentioned.

Analysis of the urine showed that hesperetin was detected in the urine of animals given either hesperidin or MGH; however, the concentration was greater in the urine from rats given MGH. A second peak identified as homoeriodictyol, a metabolite of hesperidin, was also apparent. When the urine samples were examined by time of excretion, significant differences were observed. In the rats receiving the hesperidin, hesperetin-glucuronide was identified starting at 2 – 4 hours after administration, peaking at 8 – 11 hours and still being at a substantial concentration during the 11 – 27-hour collection. Urine samples from animals given MGH had a similar profile but had a higher concentration at all collection times. Hesperetin-glucuronide was detected in the 0 – 2-hour sample and reached a maximum concentration (829 µmol/L) at 8 – 11 hours after administration; however, the concentrations were only statistically greater than the hesperidin group.

Free hesperetin was also detected in the urine of both groups of animals. The $T_{max}$ for hesperetin was at 8 - 11 hours for both treatment groups, but the $C_{max}$ was 5 - 6 fold greater following the MGH administration as compared to hesperidin. The mean values of free hesperetin of the MGH group were greater at all sampling times; however, the differences were only significantly greater than the hesperidin group at 2 – 4 and 8 – 11 hours after consumption, respectively. Further, rats given MGH had low concentrations of hesperidin-glucuronide and hesperidin in their urine at the 0 – 2-hour and 2 – 4-hour sampling times, but not after; whereas the group administered hesperidin did not have any detectable hesperidin-glucuronide or hesperidin in their urine at any time after administration.

These data suggest that an increase in solubility of hesperidin (MGH) results in a dramatic increase in hesperidin (hesperetin) absorption into the body. The AUC for the animals given MGH is about 3.7 times greater than those given hesperidin. This is supported by the work of Nielsen, et al. using H7G (Fig. 6-1), which is more soluble than hesperidin. In Nielsen's study approximately 3 times the amount of hesperetin was absorbed (Nielsen, et al., 2006). Two additional studies of other flavonoids also suggest that increased solubility results in increased absorption (Piskula, Terao, 1998; Shimoi, et al., 2003).

The absorption time profile, as seen in the serum concentrations of hesperetin (hesperetin-glucuronide), indicates that the lower water solubility of hesperidin causes its delayed absorption. This is indicated by the $T_{max}$ of 6 hours after MGH administration, compared to the 9 to 12-hour $T_{max}$ after hesperidin administration. It appears that both treatments require the activity of enzymes in the lower gastrointestinal tract to hydrolyze the rutinose (rhamnose and glucose) and produce the aglycone (hesperetin). It should be noted that dietary hesperidin is usually provided as part of a citrus fruit or juice and therefore the kinetics of consumption may be different.

One interesting observation is the almost immediate presence of hesperetin-glucuronide in the serum of the MGH group, which continued until there was a major positive increase in the absorption at 3 hours after ingestion. Explanations for this observation might include that the physical structure of the MGH allows it to be partially processed by an, as yet, unknown enzyme, or non-enzymatic system. Alternatively, examination of the small intestine showed a significant amount of α-glucosidase activity and a relatively small amount of β-glucosidase. While the β-glucosidase did not display a high amount of activity *in vitro*, these enzymes might work more efficiently on MGH *in vivo* than on hesperidin, resulting in the earlier presence in the blood of hesperetin-glucuronide.

After administration of MGH, urinary concentrations of hesperetin-glucuronide and hesperetin were greater at all sampling times than those after hesperidin administration. The relative difference between the two groups for hesperetin-glucuronide became smaller and non-significant by the 8 – 11- and 11 – 27-hour urine samples. This may reflect the delayed absorption of hesperidin. The presence of relatively low concentrations of hesperidin-glucuronide and hesperidin in the urine right after administration of MGH and through the collection suggests that some hesperidin can directly enter the system in low concentrations without deglycosylation, presumably through the small intestine. However, both substances are excreted relatively early in the process suggesting that they are not reabsorbed in the kidney. Whether this correlates to the early presence in the serum of hesperetin-glucuronide or is a separate phenomenon is not known. It is interesting to note that one of the rats in the MGH group did have high concentrations of hesperidin-glucuronide in its serum, which could indicate a malabsorptive condition.

### 6.5.2 Rat Study 2 on Monoglucosyl Hesperidin

In a second unpublished study rats were used to compare the metabolism of MGH and hesperidin (Yamada, et al., 2006b, unpublished). A single 2-mL dose of 1 mmol/kg bw

of either hesperidin or GH (95.7% MGH) in water were administered by gavage. A control group of rats were given water. Subsets of animals were sacrificed at 0, 6 and 24 hours after administration, and homogenates were prepared of digestive organs and liver and kidneys. Samples were split into 2 aliquots, one not treated and the other treated with β-glucuronidase. MGH was not detected in any organ at any time during the study, indicating that either MGH does not enter the system or was degraded by 6 hours. The absence of MGH in the 24-hour collection of the feces suggests that it was completely hydrolyzed in the digestive tract. More hesperetin was detected in the feces of the MGH than the hesperidin group even though the previous study demonstrated about 3.7 times more hesperidin is assimilated from MGH as hesperidin. None of the 0 time tissue samples or samples from the untreated control group contained any detectable amounts of any of the test substances.

Taken together the studies showed that orally administered GH or hesperidin was hydroloyzed to hesperetin and absorbed in the intestinal tract as either hesperetin-glucuronide or hesperetin. It confirmed that GH was transported into the body more than hesperidin because of the high absorption efficiency. The results showed that these metabolites were not accumulated in the organs, and the amount of excretion of metabolites was larger in rats administrated GH than when administrated hesperidin due to the greater amount of GH large absorbed into the body.

### 6.5.3 Rat Study 3 on Monoglucosyl Hesperidin

As was seen in Part 6.5.1 and supported by the report of Neilson and co-workers, the increased water solubility of GH as compared to hesperidin results in a significant increase of uptake of hesperetin into the body (Nielsen, et al., 2006; Yamada, et al., 2006a). Howver, study by Matsumoto and co-workers has shown that sodium carboxymethyl cellulose (CMC-Na) can help to solubilize flavonoids and increase their absorption (Matsumoto, et al., 2004). A third study in this sequence was performed to examine the metabolism of hesperidin and GH (MGH 95.7%) in a 0.2% solution of CMC-Na. It is believed that the use of CMC-Na more closely mirrors the natural metabolism of hesperidin in a food system. In this study, 2-mL doses of a 0.2% aqueous solution of CMC-Na containing either 0.5 mmol/kg bw hesperidin or MGH were administered by gavage to rats (Mitsuzumi, et al., 2006, unpublished). The dose was based on a dose of 16 g/day of hesperidin in a human weighing 50 kg and it is 1/2 of the dose given in the previous two studies. Blood samples were collected for serum before and 1, 3, 6, 9, 12 and 27 hours after administration, and urine and feces were collected during the experimental period (27 hours) as one sample. Additionally at 9 and 27 hours after administration liver and kidneys were collected from some of the animals. All samples were divided and assayed for appropriate hesperidin-associated metabolites either directly or after hydrolysis using β-glucuronidase.

The data taken together suggests that when hesperidin and MGH are placed in a solution of 0.2% CMC-Na the metabolic profiles are essentially the same. CMC-Na was used as an emulsifier, which equalized the absorption pattern, absorption rate, pattern of distribution, and pattern of excretion. The study indicates that when the solubility of hesperidin is increased the bioavailability increases. In the previous study when hesperidin was suspended in water the $T_{max}$ of hesperetin was reached between 9 and 12 hours, as compared to 6 hours for the GH administration (Yamada et al., 2006a). In this study the $T_{max}$ for both groups was identical (9 hours). This suggests that increasing the solubility of hesperidin with CMC-Na increases the amount of hesperidin absorbed but does not reduce the time to $T_{max}$, in fact it delayed the $T_{max}$ for the GH administration. One difference, which might have some effect on the kinetics between the two studies was that in the former study 1 mmol/kg bw was given and in the latter 0.5 mmol/kg bw. In the study where a 0.5% CMC-Na solution (versus 0.2% in this study) was used with hesperidin (Matsumoto, et al., 2004) the $T_{max}$ of the two major metabolic groups were 4 and 6 hours. The reason that the $T_{max}$ was not similar between the two studies using CMC-Na to "solubilize" the hesperidin is not known; however, the concentration of hesperidin and CMC-Na, and the quantity of the doses were different. In the two studies where hesperidin was administered in orange juice to humans, the $T_{max}$ were 5.6 and 5.4 hours indicating that the natural absorption of hesperidin may be a little faster or there may be a difference in the kinetics between species.

Regardless of the mechanism by which the hesperetin was absorbed, once in the body it showed that the metabolism and tissue distribution of hesperetin after administration of hesperidin and MGH were virtually identical.

### 6.5.4 Rat Study 4 on Monoglucosyl Hesperidin

Study 3 demonstrated that CMC-Na provides a matrix where hesperidin and GH are metabolized in the same manner and at the same rate, except for the initial hydrolysis of the glucosyl unit on GH. To better understand the metabolic pathway of these two substances in the digestive tract, GH (MGH 95.7%) and hesperidin, in CMC-Na, were administered to rats, and concentrations of MGH, hesperidin and hesperetin in the digestive tract were measured (Mitsuzumi, et al., 2008, unpublished). Rats were given a single dose of 0.5 mmol/2-mL/kg bw of one of the treatments dissolved/suspended in a 0.2% solution of CMC-Na via gavage. The dose was based on a dose of 16 g/day of hesperidin in a human weighing 50 kg. After administration six rats from each administration group were sacrificed at 0.5, 1, 3, 6, 9, 12 and 24 hours. The stomach, jejunum, ileum and cecum were excised, with the digestive contents, and processed (homogenized) for analysis of MGH, hesperidin, hesperetin and their respective

glucuronide-conjugated forms. Homogenate samples were divided into two aliquots and were either left untreated or were treated with β-glucuronidase. The study was similar to the study (Part 6.5.2) in which hesperidin and GH in water were administered to rats.

These data suggest that when GH or hesperidin are dissolved/suspended in CMC-Na (as a food matrix model) and orally administered to rats, neither of the substances were metabolized in the stomach, but rather were passed through to the jejunum intact. Furthermore, it is thought that most of the administered GH was hydrolyzed to hesperidin in the jejunum followed the same metabolic pathway as orally administered hesperidin. That is to say, hesperidin enzymatically derived from GH in the jejunum is thought to reach the cecum via the ileum principally in the form of hesperidin. Additionally, it was shown that a portion of the hesperidin was hydrolyzed to hesperetin during passage through the ileum and that enzymatically released hesperetin was absorbed in the ileum. Hesperidin that reached the cecum was shown to be hydrolyzed to hesperetin. The hesperetin reaching the cecum did not appear to be absorbed and conjugated, but rather was excreted into feces. These findings strongly indicate that GH has the same metabolic fate as hesperidin in the intestinal tract of rats after hydrolysis in the jejunum. Therefore, the metabolism of GH in the intestinal tract appears essentially identical to that of hesperidin.

### 6.5.5 Metabolism of Glucosyl Hesperidin in Humans

An unpublished metabolic study, which included two separate experiments, was performed on the same group of 10 subjects as described below (Yamashita, et al., 2008, unpublished). In the first experiment 10 healthy volunteers (5 females, 5 males; 37 ± 8.77 years old, body weight 57.7 ± 10.5 kg, BMI 20.9 ± 2.19 kg/m$^2$) were given 500 mg of a commercial GH preparation (Lot No. 8C211; 78.7% MGH = 393.5 mg, 17.4% hesperidin) in 50 mL of tap water (Yamashita, et al., 2008). The subjects were instructed not to eat any food for 2 hours before ingestion of test material, and not to eat any citrus fruits or citrus- containing products for 3 days before and during the study. Additionally, 12 hours before the start of the study and throughout the study no alcohol, coffee, or tea was permitted. One subject (male) was found to have consumed a hesperidin-containing food and was deleted from the analysis. Blood samples were taken at 0.5, 1, 3, 6, 8, 16 and 25 hours after administration. Because of sample collection logistics the 16-hour sample was performed as a separate study. After a washout period of at least 1 week the subjects ingested the same amount of GH, and blood collected at the same times, including the 16-hour sample. Plasma samples were divided into 2 aliquots, which were treated or not treated with β-glucuronidase, and then the hesperidin-related substances were assayed by a standard HPLC method.

No free hesperetin or hesperidin in any form was observed in the plasma at any time. The temporal profile of the conjugated hesperetin shows no hesperetin in the blood through 3 hours after treatment. At 6 hours the concentration of conjugated hesperetin reached a maximum of about 0.6 µmol/L, which was maintained at the 8-hour sampling. There was high variability in hesperetin concentrations at these times (± 0.75 and 0.4 µmol/L, respectively). The concentration decreased to about 0.2 µmol/L at 16 hours and was almost at 0 by the 25-hour sample. The $T_{max}$ was calculated as 9.22 ± 6.00 hours. This occurred because one of the subjects reached maximum concentration at 25 hours after ingestion. The value for this subject at 16 hours was only slightly less (0.005 µmol/L) than the 25-hour sample. Of the remaining 8 subjects (one deleted for hesperidin consumption) 3 had their $T_{max}$ at 6 hours and 5 at 8 hours after consumption. The $C_{max}$ and $AUC_{0\text{-}25\ hour}$ were 0.750 ± 0.699 µmol/L, and 6.02 ± 3.76 µmol/L·hour, respectively, which demonstrates a large variability (Yamashita, et al., 2008). The conjugated hesperetin profile of the 9 subjects appears similar to that observed in the rat study where hesperidin and MGH were given in an aqueous solution of CMC-Na, except hesperetin was present at earlier times in the rat study. This might be related to concentration, because the relative amount of MGH given to the rats was several times higher than the 500 mg dose of GH in this study (Yamashita, et al., 2008).

The second experiment was designed to show the blood concentration of hesperetin during and after 14 daily doses of 393.5 mg of MGH in tap water (total 500 mg as GH; Lot No. 8C211) to the same 10 subjects used in the first study (5 female, 5 male). After a washout period of at least one week the subjects had blood samples collected at days 0, 1, 7, 14 and 21 (7 days after cessation of the administration). The GH was administered in the morning and the blood was collected 8 hours later, which is the time in the first study when most subjects had the maximum concentration in their plasma. Plasma was tested for conjugated and free hesperetin using β-glucuronidase hydrolysis. No free hesperetin or hesperidin in any form was detected in the plasma at any sampling times. No hesperetin was observed at day 0 (before consumption). Plasma values at days 1, 7, 14 and 21 (7 days after cessation of ingestion) were approximately 0.68 ± 0.41, 0.81 ± 0.50, 0.54 ± 0.25, and 0 µmol/L, respectively. There were no significant differences between the values at days 1, 7, and 14. The data demonstrates that GH does not concentrate in the blood and appears to remain at about the same concentration as that observed after a single dose. Taken together these data show that under these conditions GH and hesperidin do not enter the blood stream after a single or multiple oral doses. Additionally the only GH metabolite that was found in the blood after ingestion of MGH by humans is the conjugated form of hesperetin. Further, the conjugated hesperetin does not concentrate in the blood, even after 14 days of administration (Yamashita, et al., 2008).

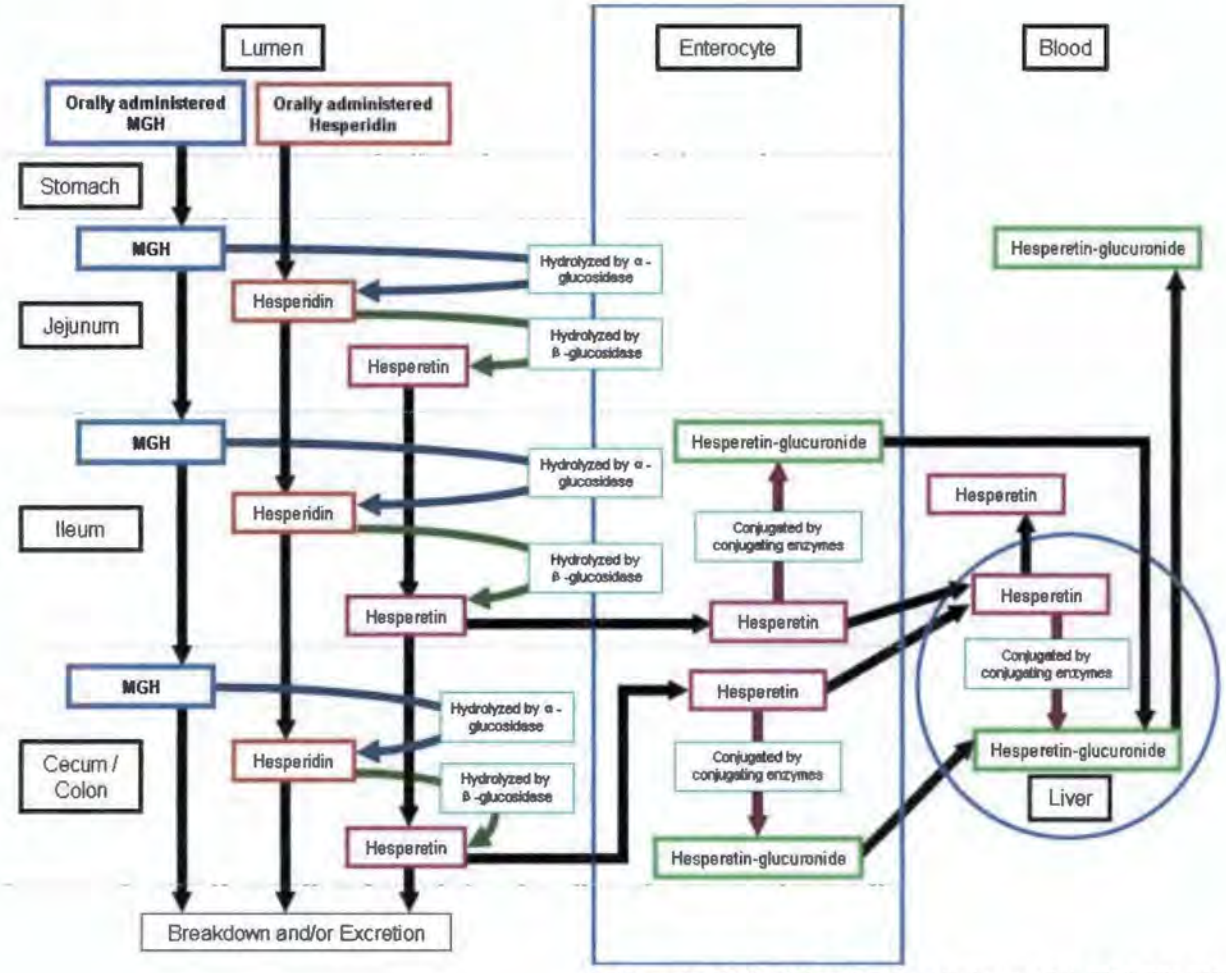
### 6.5.6 Conclusion of Rat and Human Metabolic Studies

In total, these four animal studies and one two-part human study demonstrated that GH is metabolized and absorbed into the body in the same manner as natural hesperidin. The only difference is that GH requires an initial hydrolysis step in which the added glucose moiety on the hesperidin molecule (MGH) is cleaved in the upper small intestine by α-glucosidase. In rat studies it appears that at high doses some non-conjugated hesperetin can be identified in the plasma and possibly hesperidin. However, in humans no hesperidin or non-conjugated hesperidin was observed. Consumption of 500 mg/day of GH for 14 days does not result in an acumluation of hesperetin (conjugated) in the plasma.

Figure 6-3 (below) provides a detailed schematic of the hypothesized mechanism of hesperidin and MGH metabolism from the rat and human studies (Matsumoto, et al., 2019). Data from the rat studies, where relatively high doses of hesperidin and MGH were administered suggests that little, if any, metabolic activity occurs in the stomach. In the upper small intestine MGH is hydrolyzed to hesperidin by α-glucosidase, and a small portion of the hesperidin (both natural and from MGH) is hydrolyzed by an enzymatic system to hesperetin. It would appear that in high concentrations in the rat, hesperidin and MGH directly enter the circulation, but from the human study it shows that at lower doses (500 mg GH, 394 mg MGH) this does not occur at this location, or at any other location along the digestive tract. In the ileum a similar pattern occurs in the lumen. The kinetic studies would suggest that some hesperetin may enter the enterocyte and either become conjugated intracellularly or pass into the hepatic circulation to be conjugated in the liver. This may occur primarily in conditions of high concentration of hesperidin. In the cecum (colon) all of the above activities occur; however, this is believed to be the principal site of the enzymatic conversion of hesperidin to hesperetin, and subsequent absorption of hesperetin into the enterocyte. When this occurs, as mentioned above, the hesperetin will be conjugated and enter the circulation, or pass into the portal circulation and be conjugated in the liver. The data from the human bioavailability study suggests that at the concentrations given, the uptake occurs principally in the area of the large intestine by β-glucosidase. Once it is conjugated it enters the blood stream, and except in situations where high doses are consumed, is found only in the conjugated form.

If the substances are administered in an aqueous solution, GH will be absorbed faster and to a greater extent than hesperidin; however, this appears to be more a function of the insolubility of hesperidin. When the solubility of hesperidin is equalized with GH using a matrix of CMC-Na, it behaves in a similar manner to GH. This may also occur when hesperidin is consumed with the complex matrices associated with foods.

**Figure 6-3 Diagram of Hypothesized Metabolism of Hesperidin and Monoglucosyl Hesperidin**




Matsumoto et al. 2019, Fundam.Toxicolog Sci. 6(8) 299-317.

## 6.6 Safety Studies with Hesperidin

There have been several relatively recent studies to evaluate the potential nutritional benefits of hesperidin. Search of PubMed.gov using the terms "hesperidin safety" and "hesperidin toxicity" provided a total of 58 and 176 references, respectively. This servey was last done on December 5, 2019. However, only one publication by Li et al. provided information directly on the toxicity/safety of hesperidin (see Part 6.6.5 below; Li et al., 2019). None of the other publications appeared to discuss the toxicity of hesperidin, but rather the anti-toxic effects or other beneficial nutritional effects of heperidin with and without the co-administration of other substances. A search of TOXLINE through December 5, 2019 provided much more limited but similar results. As mentioned previously, and based of the provided metabolic data, it is the contention of the Notifier that GH is substantially equivalent to hesperidin in its metabolism when orally consumed. Because the first step of GH metabolism is to

enzymatically remove the added glucose in the lumen of the small intestine, GH becomes hesperidin. From this point on the digestive, absorptive and further metabolic processes are identical to that occurring to natural hesperidin.

Because GH (MGH) is metabolized in the same manner as hesperidin any information or data about the safety of the consumption of hesperidin is relevant to the safety of GH. Therefore, safety information and data about hesperidin is included in this GRAS Notice. However, the Notifier, Hayashibara Co., Ltd., has also performed a number of animal and human studies specifically examining the safety of GH. Additionally, other entities have performed studies using GH and collected data on the safety of GH. This information is presented below. Some of these studies have been published, while others have not been published. All the studies using human subjects, to the Notifier's knowledge, have been published.

### 6.6.1 LSRO Review (SCOGS report)

Many animal studies have been conducted using hesperidin and/or hesperidin-containing complexes (Fisher, 1982; Pizzorno, Murray, 1999; Garg, et al., 2001). In 1982, the Bureau of Food, US FDA contracted with Life Sciences Research Offices, Federation of American Societies (LSRO) (contract number FDA 223-78-2100) for the "Evaluation of the Health Aspects of Hesperidin, Naringin and Citus Bioflavonoid Extracts as Food Ingredients" (Fisher, 1982; known as Select Committee of GRAS Substances (SCOGS)). Naringin and some other flavonoids are discussed in this report; however, only information related specifically to hesperidin or substances high in hesperidin will be included in this review. The conclusion of the report does say that the Select Committtee found no evidence that naringin is a hazard to public health. The document stated, "rough calculations suggest that the per capita intake of these bioflavonoids in natural sources are many times the amounts added to food or employed as nutritional supplements." Further, "the daily per capita amount of hesperidin available for consumption would be about 44 mg" from orange juice alone. This is likely an underestimation of consumption because eating portions of the fruit provides higher concentrations than juice (Fisher, 1982).

Since that the time of the LSRO report the per capita "availability" of oranges and orange juice for consumption has been reduced by approximately 25 and 50%, respectively (USDA ERS, 2018). Greater declines are seen in grapefruit consumption, while tangerines/tangelos, and lemons have generally had small increases. However, the amount of orange and orange juice consumption is several-fold greater than the other citrus fruits. The USDA reports that in 2017 the per capita "availability" of fruit and juice from oranges/temples, grapefruit, tangerines/tangelos, lemons, and limes were 21.2, 1.6, 3.4, 4.5 and 2.1 kg, respectively. "Availability" is the amount of fruit or

juice that was produced for consumption, although an unknown amount is not consumed because of processing waste, spoilage, and other factors (USDA ERS, 2018). Therefore, these numbers are likely over estimations of the actual consumption of these hesperidin- containing foods.

Several of animal safety studies cited in the 1982 LSRO review were conducted by Sunkist Growers, Inc. After checking with the FDA, the US Department of Commerce National Information Service, and Sunkist Growers, Inc. it was found that only a few of the original studies are available. Therefore, the LSRO evaluation summary is the only source of the bulk of the safety information; however, the original references are provided although many were not published. It is realized that the LSRO review is a secondary source for this safety information.

**Mutagenicity**

Hesperidin and hesperetin have been reported as non-mutagenic (Bjeldanes, Chang, 1977; Brown, et al., 1977; Brown, Dietrich, 1978; MacGregor, Jurd, 1978; Fisher 1982).

**Acute toxicity**

In acute toxicity studies of a hesperidin complex (average hesperidin content 72%), doses up to 16 g/kg bw administrated by stomach tube did not result in any deaths or noticeable change in the health or behavior of 10 young, male Long-Evans rats (age and weight not given) during the 72-hour observation period (Primorganics, 1955). Another study used two citrus extracts (lemon bioflavonoid complex; about 4 - 7 % bioflavonoids, hesperidin is in the highest concentration) and lemon-orange flavonate glycoside (not defined), which would presumably contain hesperidin. The substances were administered by gavage to young rats at a maximum dose of 24 g/kg bw with no deaths or apparent untoward effects (sex, age, strain and number of animals were not reported in the LSRO report; Primorganics, 1956; Fisher, 1982). In the LSRO report, DeEds stated, "None of the flavonoids administered to experiemental animals in single doses orally, intraperitoneally or intravenously when possible, produced signs of acute toxicity" (DeEds, 1968; Fisher, 1982). Further Singleton and Kratzer stated that the toxicity of common plant flavonoids was "negligible" (Singleton, Kratzer, 1973).

**Chronic toxicity**

Albino rats (number, sex and weight were not given in the reference) were fed up to 1% hesperidin (approx. 1g/kg bw) in a standard diet for 200 days (Wilson, DeEds, 1940). No significant differences were observed in mortality, food intake, weight gain, gross morphology, or histology. Citrus bioflavonoids containing hesperidin were fed at 0.5 to 5.0% of the diet to 6 - 8-week old chickens (strain and sex not reported) for up to 8 weeks. No changes in mortality, growth or feed efficiency were observed in animals

receiving up to 2.5%; however, a "marked reduction" (no statistical significance given) in growth and feed efficiency was noted in the 5% group (Deyoe, et al., 1962; Fisher, 1982).

Weanling female Sprague-Dawley rats (n=16) were fed diets containing 2.5% (approximately 2 - 5 g/kg bw/day) of 6 different bioflavonoids preparations, including hesperidin, for 400 days. At 70 - 75 days of feeding, half the animals were sacrificed and examined. Mean body weights of the group fed lemon bioflavonoids complex (LBC), but not the hesperidin group were significantly lower ($p < 0.05$) than the control group. Mean kidney:body weight ratios for all treated groups were lower than controls; however, only the hesperidin, naringin and orange complex concentrate groups were significantly less ($p < 0.05$) than controls. Liver:body weight ratios of rats fed LBC 2x and 6x were greater ($p < 0.05$) than controls. Only rats fed LBC 2x and 6x were examined for histopathology. Mild renal hydronephrosis was reported, but no significant hepatic changes were noted. No other differences were observed in the hesperidin group as compared to the control group after 75 days of feeding. There were no statistically significant differences in body weights, clinical chemistry, histopathology, or organ:body weight ratios, among the animals that survived to 400 days, except a significant increase ($p < 0.05$) in the liver:body weight ratio in the group receiving the orange complex (not hesperidin) (Patterson, 1960; Fisher, 1982). Several other studies cited in the LSRO evaluation examined other bioflavonoids; however, this summary addresses only products that are directly related to hesperidin.

Patterson also reported the results of the examination of the rats that continued to be fed hesperidin until sacrificed at 400 days. No significant differences were found in any variables except liver/body weight ratio. There was a 13.7% increase in liver:body weight ratio for the group fed orange bioflavonoid complex. However, histopathological examination of the heart, spleen, kidney, lower left jaw and liver revealed no abnormal changes (Patterson, 1961).

**Reproductive toxicity**

A hesperidin complex and LBC were fed to nine female mice (Palmer, Patterson, 1954; Fisher, 1982). The estimated daily consumption ranged from 1.3 to 3.6 g/kg bw. A preliminary control feeding and mating (without biloflavonoids) resulted in 8 litters born to each treatment group. After receiving the bioflavonoid in the diet, the number of litters were 8 and 6, respectively. No adverse effects were reported in animals that were continued on the hesperidin complex diet after 158 days of treatment or the LBC diet (178 days). Some of the mice were subsequently given the control diet and mated a second time; however, the LSRO reviewers commented that the data were inconsistent in the latter portion of the study so it was discounted (Fisher, 1982).

In another reproduction study, 1 male and 2 female weanling rats were fed 2 - 4% (up

to 10 g/kg bw) of three different hesperidin containing preparations, namely LBC (4% of 2x concentrate, 2% of 6x concentrate), or 2% orange bioflavonoid complex concentrate (Call, Patterson, 1960; Fisher, 1982). After reaching puberty the males were rotated between the cages and the ratio of females that bore litters were recorded. The ratios were: controls 6/6; lemon 2x 4/4; lemon 6x 4/6; and orange concentrate 5/5. The mean number of days from start to birth of litters was 67.3, 56.8, 77.8, and 62.0, respectively (Call, Patterson, 1960; Fisher, 1982).

**Clinical toxicity**

The LSRO report mentions that, "(A) number of clinical studies had been reported in which bioflavonoid preparations have been given daily for periods up to 5 years with no reported side-effects or toxic reactions." The original reference (Fostvedt, 1956) could not be found to provide more specific details. The LSRO report also states that usual doses were 150 to 600 mg of hesperidin per day (2.5 to 10 mg/kg-bw). Van Buskirk (1946; letter could not be found) is cited as reporting that one individual received 1 to 4 grams of hesperidin daily for 2 years after which the same person consumed 10 to 16 grams daily for 2 more years with "no adverse effect" (Fisher, 1982).

**Conclusion**

The LSRO Select Committee concluded that the acute toxicity of purified hesperidin (≥ 80%) or hesperidin (72%) are extremely low (Fisher,1982). Short- (200 days) and long-term (400 days) rat feeding studies with purified hesperidin at doses of about 2 - 5 g/kg bw/day failed to elicite adverse effects. Hesperidin was non-mutagenic using microbial analytic methods, and no flavonoids associated with the hesperidin complex have demonstrated mutagenic potential. Consumption of approximately 2.5 g/kg bw of hesperidin complex did not result in fertility problems in mice. Hesperidin taken in gram amounts daily by humans for months or years have demonstrated no toxicity; however, this information is anecdotal or uncontrolled. The Select Committee concluded that, "There is no evidence in the available information on hesperidin (purified or hesperidin complex) or naringin 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 reasonably be expected in the future" (Fisher, 1982).

Information that has appeared since that LSRO report reaffirms the conclusion of the Select Committee with respect to the safety of the consumption of hesperidin by humans.

### 6.6.2 *Daflon 500mg* on Mice, Rats, Primates, and Humans

A second secondary source of the safety information of hesperidin comes from a

review publication by Meyer (1994). Meyer summarizes published and unpublished articles on the use of *Daflon 500 mg*. This is a flavonoid vasoprotector venotonic agent used in the treatment of venous insufficiency. While the product contains only a relatively small amount of hesperidin (10%) as compared to diosmin (90%), it has been tested for safety in a large number of animal and human studies, and in high concentrations in a few studies. Several references were provided; however, *Dalfon 500 mg* is approved as a prescription pharmaceutical in France. Many of the references listed as "Expert Reports" are in French, are not available for review, and a majority of the published articles are in French, with only some abstracts in English. The original references are provided in the text below. No references were provided in the review article that were related to the studies of *in vitro*, or animal safety.

**Acute and Chronic toxicity**

Meyer (1994) reported summary data on standard toxicology studies in mice, and rats with a drug product (*Daflon 500 mg*). He mentions treatment of primates, but no data could be specifically identified related to primates in the review. *Daflon 500 mg* contains 450 mg of diosmin (another bioflavonoid) and 50 mg of hesperidin. *Daflon 500 mg* is approved for treatment of venous insufficiency and hemorrhoidal disease in France. The standard dose is two tablets per day (100mg hesperidin). It was stated in the article that the studies were conducted "in accordance with rules of Good Laboratory Practice" (Meyer, 1994). The $LD_{50}$ for mice and rats was greater than 3,000 mg/kg (300 mg/kg hesperidin) for the active substances. An actual $LD_{50}$ was not possible to determine because of the low toxicity of the product. The human daily therapeutic dose reported in the same article was given as two 500 mg tablets per day, which would be equivalent to 100 mg of hesperidin. The author reported that no acute toxicity was detected at 180 times the equivalent human daily therapeutic dose in mice, rats and primates (18,000 mg hesperidin per day; Meyer, 1994). Additionally, daily doses equal to 35 times the human daily dose (3,500 mg/day of hesperidin), for a period of 13 and 26 weeks, did not result in any mortality or toxicity in mice, rats and primates (Meyer, 1994). The substance did not elicit evidence of gastrointestinal problems in rats given oral doses (method of administration not reported) representing 12, 24 and 48 times the equivalent daily human therapeutic dose (1,200, 2,400 and 4,800 mg hesperidin per dose; Meyer, 1994).

The active substance was eliminated 96 hours after administration with no "untoward" accumulation in any particular organ, shown by macroscopic autoradiography in the rat."

**Geno- and Clastogenic toxicity**

Genotoxicity and clastogenic studies with *Daflon 500 mg* (50 mg hesperidin) revealed no indication of positive results in the bacterial gene mutation test (organism and

strains not reported); *in vitro* human lymphocyte chromosomal aberration test; *in vitro* eukaryote gene mutation test (HPRT locus Chinese hamster V79 cells, Ade2, and TR2 using *S. Cerevisiae* D4); *in vivo* clastogenic lesion test (micronuclei in bone marrow OF1 mouse); and DNA repair test (Hela cells) (Meyer, 1994).

**Reproduction toxicity**

Meyer (1994) also reported that *Daflon 500 mg* (50 mg hesperidin) showed no interference with reproduction in rats given an oral dose 37 times the daily therapeutic dose (3,700 mg hesperidin per day). No particular concentrations of the actives were observed in maternal genital organs.

**Clinical toxicity**

As mentioned previously *Daflon 500 mg* contains 50 mg of hesperidin and 450 mg of diosmin, another bioflavonoid (Meyer, 1994. In total, 2,850 patients were treated mid-term and long-term with two tablets of *Daflon 500 mg* (100 mg hesperidin)(Amiel, et al, 1985; Galley, 1987; Lagrue and Behar, 1987; Frileux and Gilly, 1987; Vicari, 1985; Cope, 1985; Frileux, et al., 1987; Delmont, 1985 Pointel, et al, 1988; Cospite and Florena, 1988; Peker, el al., 1988; Elbaz, 1989). In addition, some of these studies included 225 patients treated with placebo (Amiel, et al, 1985; Galley, 1987; Lagrue and Behar, 1987; Frileux and Gilly, 1987; Vicari, 1985; Cospite and Florena, 1988), and 85 with "Daflon", which is qualitatively similar to *Daflon* 500 *mg* having diosmin and hesperidin in the tablet, but not as a micronized formulation ((Vicari, 1985; Cope, 1985). Meyer reports that the percentage of side effects for *Daflon 500 mg,* placebo, and "Daflon" was 10%, 13.9%, and 13.0%, respectively. The types and incidence of the side effects in patients receiving *Daflon 500 mg* included gastrointestinal (6.9%; abdominal pain, gastric discomfort, epigastric pain, nausea, dyspepsia, vomiting and diarrhea), and autonomic (1.7%; insomnia, drowsiness, vertigo, headache, tiredness, anxiety, cramps, palpitations, and hypotension) signs and symptoms. The side effects reported for the groups treated with placebo and Daflon were similar in incidence and nature (Meyer, 1994). The percentage of subjects that dropped out of the studies because of side effects were 1.1% for *Daflon 500 mg*, 3.2% for placebo, and 4.8% for "Daflon".

One hundred and thirty-one (131) patients were treated with a single dose (two tablets) (Duchene, et al., 1988; Amiel, Barbe, 1985a; Amiel, Barbe, 1985b) or short-term treatment (one week to two months) with two tablets (Duchene, et al., 1988; Guillot, et al., 1988; Behar, et al., 1988) of *Daflon 500 mg* per day. No side effects were reported by any of these patients. In mid-term controlled trials (six weeks to two months) that included 2,494 patients receiving *Daflon 500 mg*, 11.1% had side effects of principally gastrointestinal and autonomic nature (Amiel, et al, 1985; Galley, 1987; Lagrue and Behar, 1987; Frileux and Gilly, 1987; Vicari, 1985; Cope, 1985; Peker, el

al., 1988; Elbaz, 1989). Of these patients, 1.2% stopped treatment as a result of side effects. However, the nature and incidence of side effects experienced by patients treated with placebo (n = 216) and Daflon (n = 84) were "identical" to the *Daflon 500 mg* treated group.

Long-term treated (six months to one year) patients include a group of 90 patients given *Daflon 500 mg* for 6 months and 215 patients administered *Daflon 500 mg* for 12 months (Frileux, et al., 1987; Delmont, 1985; Pointel, et al, 1988). Of these patients, 6.9%, and 9% developed side effects, respectively. Studies were performed where doses greater than two tablets per day of *Daflon 500 mg* were given. Eighteen (18) patients, 10 patients, and 18 patients were treated with a single dose of 4 tablets (Duchene, et al., 1988), a daily dose of 4 tablets for one month (Lacombe, Lelievre, 1988), and a daily dose of 6 tablets for 28 days (Lacombe, et al., 1988), respectively. No side effects were reported for any of these treatments. "No evidence was found for any drug incompatibility or interaction" (Meyer, 1994).

A total of 369 patients treated with *Daflon 500 mg*, 24 patients with "Daflon" and 44 treated with placebo, were followed for "laboratory parameters" for 2, 6 and 12 months in 5 studies (Frileux and Gilly, 1987; Cope, 1985; Frileux, et al., 1987; Delmont, 1985 Pointel, et al, 1988; Meyer, 1994). No differences were observed between the groups in patients treated for 2 (Frileux and Gilly, 1987; Cope, 1985) and 6 months (Frileux, et al., 1987; Delmont, 1985) between the start and at the end of treatment. In the one-year multicenter there were no changes to any of the values measured, except there was a significant decrease in plasma creatinine in 65.5% comparing the starting and ending values.

A final study reviewed the possibility of photosensitization of *Daflon 500 mg* (Ortonne, et al, 1986). No patients displayed evidence of a photosensitizing effect (Ortonne, et al, 1986).

In a study of the long-term effects of *Daflon 500 mg* 215 patients with various venous insufficiency were enrollled in a study (Guillot, et al., 1989). All patients were given 2 tablets of *Daflon 500 mg* per day for 1 year. Because the purpose of this submission is not to report on the clinical benefits of hesperidin or *Daflon 500 mg*, the only comment about the results are that all variables improved. Side effects were reported as rare and occurred in 20 patients. These were all judged as "probably not" related to treatment. As stated previously, *Daflon 500 mg* contains only 10% hesperidin of the active ingredients, so the exact contribution of hesperidin to any of the few effects noted are unknown. The authors stated that the safety of *Daflon 500 mg* is supported by the "rareness and mildness of the clinical side effects." It may be concluded that hesperidin is a safe and well tolerated substance when consumed by humans (Guillot,

et al., 1989).

### 6.6.3 Other Safety Studies or Reports

Kawaguchi and coworkers fed hesperidin at 0, 5 or 10% of the diet for 30 days to rats. The 10% hesperidin group had no significant differences in all of the test variables. The 10% hesperidin group consumed an average of 583 grams of food during the 30 days, which is 58.3 grams of hesperidin or about 1.9 grams per day (Kawaguchi, et al., 1997).

In their Textbook of Natural Medicines, Pizzorno and Murray reviewed various natural products. They stated that the human dosage of hesperidin for the treatment of venous insufficiency and hemorrhoids "translates to a dosage of citrus bioflavonoids, rutin and hesperidin of 3,000 to 6,000 mg daily." They also conclude that hesperidin, "appear(s) to be extremely safe and without side effects even for humans during pregnancy" (Pizzorno, Murray, 1999).

### 6.6.4 GRN Notices 000719 & 000796

An FDA "no objection" letter was filed in December of 2017, for a GRAS Notice GRN 000719 (FDA GRAS, 2017). The Notifier presents two animal studies using methyl hesperidin, a hesperidin derivative that is closely related to hesperidin. These were used to support the safety of hesperidin and provide a NOAEL (Kurata et al., 1990; Kawabe et al. 1993). Both studies treated 10 female and 10 male mice per dose group for 13 and 96 weeks, respectively, with methyl hesperidin. The "pivotal study" was identified as the 96-week oral toxicity and carcinogenicity study (Kurata et al., 1990). The dose identified as the NOAEL was 8,600 mg/kg-bw/day for female mice and 7,500 mg/kg-bw/day for males. The conclusion of the studies was that no treatment related effects were noted. Other safety studies with additional hesperidin-related substances were also presented. The Response letter also stated that published mutagenicity and genotoxicity studies on hesperidin and hesperidin showed no genotoxicity.

A second "no questions" FDA Response letter was provided for GRN 000796 (FDA GRAS, 2019). This GRAS Notice was for an Orange extract, which contains $\geq$ 85% Hesperidin, plus minor amounts of other naturally occurring flavonoids found in orange material. The intended uses were for many of the same food categories as listed in this current GRAS Notice, plus some additional foods. The maximum use amounts for all foods are the same as what is being requested for GH, namely 500 mg/serving.

The FDA GRAS Response letter provides the maximum dietary exposure to hesperidin based on exposure in the diet and on the consumption of hesperidin added

to the diet (The Notifier assumed that orange extract contained 100% hersperidin). The combined dietary exposure of the 90th percentile for the total population was 2,608 mg/p/d (39.5 mg/kg-bw/d). The dietary exposure of the 90th percentile of consumers only, and specific groups within the total population would have a greater consumption, but was not mentioned in the Response letter. As can be noted from Part 3, the hesperidin equivalent for the 90th percentile of the total, consumers, and highest consumption sub-group, is less than the amount given for the orange extract in GRN 000796.

The FDA Response letter mentioned the various studies upon which the safety of 000796 (orange extract ≥ 85%) was based. The Response letter referred to the 1982 LSRO report and some of the Daflon-500 mg data that are provided above. GRN 000796 summarizes several published human clinical studies using hesperidin and naringin, with no adverse effects. The NOAEL value of 7,500 mg/kg-bw/day (male mice) was used as a comparison to the hesperidin EDI. This NOAEL was from the Kurata and coworkers study using the methyl hesperidin referenced in GRN 000719 (Kurata, et al., 1990). Again, this value is based on the consumption by male mice for 96 weeks. This was the lower of the two genders with females consuming 8,600 mg/kg-bw/day.

The following sections include more specific information on the 13- and 96- week studies using methyl hesperidin, which the Notifier concludes, based on the metabolism data, is similar to both hesperidin and GH.

**1) 13-week subchronic toxicity study of methyl hesperidin in mice**

A 13-week subchronic toxicity study using methyl hesperidin (MH) was performed on mice (Kawabe, et al., 1993). MH is substantially equivalent to hesperidin and GH in that it has a methyl group on the C 4' of the hesperidin molecule. A total of 120 mice (60 female, 60 male) were equally divided into 6 groups that were fed a standard chow containing 0, 0.3, 0.6, 1.25, 2.5 or 5.0% of MH for 13 weeks. The MH was of a purity of > 95.5%. MH consumption is assumed to be essentially equal of consumption of hesperidin.

Two (2) deaths occurred during the study period, one each in the 1.25% and 3.0% MH groups. The authors reported that neither was related to MH consumption, but no cause of death was reported. The authors concluded that "[N]o significant treatment-related differences were found in the data for body weights, food and water consumption, hematology, clinical chemistry and organ weights." Additionally, "no effects of treatment were observed on gross and histopathological examination of

major organs" (Kawabe, et al., 1993). They concluded that there were no toxic effects when mice of either sex were fed a diet containing 5.0% MH.

Data from many sources informs that hesperidin including GH, must be hydrolyzed to hesperetin to enter the body through the gut wall. This appears to be true for many other glyconated flavonoids. No information concerning MH has been found to support or contradict this understanding. It is felt that these data, in which mice consumed up to 5% of MH in their diet, demonstrate no toxic effect at the highest dose, strongly suggesting the safety of hesperidin.

**2) 96-week subchronic carcinogenicity study of methyl hesperidin in mice**

A 96-week carcinogenicity study was performed in which MH (> 95.5%) was fed to 300 mice at concentrations of 0, 1.25% and 5.0% in a standard chow diet for 104 weeks (96 weeks treatment MH, and 8 weeks basal diet) (Kurata et al., 1990). The investigators calculated the total and average daily consumption of MH at 5.0% of the diet (Kurata, et al., 1990). Female mice consumed 8.6 g/kg bw/day, while males averaged 7.5 g/kg bw/day. Because the authors concluded that ".... no biologically significant effects were evident with respect to mortality or clinical signs", nor were there any untoward affects in haematology, clinical chemistry, and urinalysis, and no differences were seen in the numbers and types of non-neoplastic and neoplastic lesions, 7.5 g/kg bw/day was concluded to be the NOAEL, although the authors did not use this term.

### 6.6.5 Acute and subchronic oral toxicity studies of hesperidin

A safety study was recently published in which a preparation of hesperidin isolated from orange peel extract was administered to rats to assess acute and sub-chronic toxicity (Li, et al., 2019). The authors state that hesperidin is one of these uninvestigated substances, although many older studies have been reported, as provided above, using various preparations and derivatives of hesperidin (MH).

**1) Acute study**

The hesperidin sample for both acute and sub-chronic experiments were produced from dried green peels from the orange, *Citrus sinensis* (Li, et al., 2019). The dried powder was mixed with petroleum ether in a reflux condenser, filtered, dried, re-extracted with methanol, filtered, washed with hot methanol, and concentrated and dried. The dried residue was mixed with acetic acid and the precipitated crude hesperidin was filtered, washed with the same acetic acid solution, and dried (Li, et al., 2019). It should be noted that this method using organic solvents is not used for

preparation of hesperidin for GH manufacture, for other food uses, as described in the LSRO report, and or for production of the hesperidin product in GRN 000796. Rather a series of aqueous alkaline extraction and then aqueous acidification to precipitate the hesperidin are used.

Ten (10) rats were included in the acute study. The doses consisted of control (vehicle; distilled water), and 55, 175, 550, 1750, and 5000 mg/kg of the hesperidin preparation. The single dose at each concentration was given by gavage at a volume of 10 mL/kg. All animals were observed for 14 days following dosing for signs of morbidity or mortality. The results of the actue study showed that one male rat from the 5000 mg/kg group died on the 11$^{th}$ day after dosing. Using Karber's method the authors calculated a median lethal dose 50 value ($LD_{50}$) of 4837.5 mg/kg. No differences in body weight, feed or water intake were noted between control and the treated groups for either sex. There was a significant increase compared to the control group in the absolute liver and spleen weights of animals receiving the 5000 mg/kg of hesperidin. Relative liver weights were greater ($p < 0.01$) in both male and female rats treated administered 5000 mg/kg hesperidin as compared to control animals. Relative spleen weights were significantly greater in female rats in the 5000 mg/kg group was compared to the control group.

The authors concluded from the one dead male rat that male rats are more sensitive to hesperidin than female rats, and further stated that males have a longer gastric residence time because hesperidin is reported to have a delayed absorption. They do not mention that many flavonoids, including hesperidin, have a slower absorption time because the enzymes needed to hydrolyze the hesperidin to hesperitin are primarily found in the lower gut. It is also problematic that no comment was made about specifically examining the animal that died to be sure the death was not caused by a gavage failure at administration or other internal issue. Further, the authors did not state whether the significant differences in organ (absolute and relative) weights were dose-dependent or not, or within the normal range for this type and/or age of rats.

**2) Subchronic 13-week toxicity study**

The hesperidin and animals were produced and acclimated, respectively for the study as in the acute study. The animals, 15 per sex per four treatment groups, were administered, via gavage, freshly made daily doses up to 1000 mg/kg of the hesperidin preparation for 13 weeks. There was also a 28-day "reversal period" at the end of the study during which no treatment was given (Li, et al., 2019).

No animals died either during the treatment period or in the post-dosing recovery period (28-day reversal). There were no clinical signs of toxicity in any of the

treatment groups during these same study periods. No abnormalities were noted on ophthalmologic examination, functional observations, or findings at necropsy. Animals in the male and female 1000 mg/kg groups had significantly increased weight grains as compared to the control group. At the post-dosing recovery period (day 119) the weight difference was still present in both male and females.

The authors stated that there were a number of significant increases or decreases in hematologic variables at the end of the 13-week treatment, and after the 28-day reveral period in both the male and female treatment groups as compared to the control group. However, these differences were noted by the authors to be within normal biologic and laboratory limits, or the effects were 'not dose-dependent'. The same situation was noted for significant differences in blood chemistry values, which were also within normal limits, or the effects were not dose-dependent. No significant differences were observed in the urine analysis variables. No significant differences were seen in food consumption at 13 weeks or after the reversal period. Organ weights (absolute and relative) showed differences when compared to the respective control group. These included significant increases in male liver and spleen weights in the 1000 mg/kg group at 13 weeks and the reversal period. The female 1000 mg/kg group also had a significant increase in liver and spleen at 13 weeks and after the reversal period. Conversely, both male and female groups had significant decreases in relative kidney weight in the 1000 mg/kg groups after the 13-week treatment. The female group also had a significant decrease after the 28-day reversal. Male and female 1000 mg/kg groups showed a decrease in relative heart weight. Relative thymus weights of both male and female 1000 mg/kg groups were less after 13-weeks of treatment and the reversal period. Histophathologic findings were negative except for a mild inflammatory infiltration and necrosis in the colon; minimal focal to multifocal lymphocytic infiltration of the liver; moderate inflammatory cells and mild necrosis in the lungs; and minimal inflammatory cells and necrosis in the stomach.

**3) Conclusion of acute and sub-chronic studies of Hesperidin**

The production process for hesperidin (72%) using petroleum ether, methanol, and dimethylformamide is not typical of that used to make hesperidin for food-related consumption (Fisher, 1982; GRN 000796), or for GH. The authors stated that the single death of the male rat in the 1000 mg/kg group suggested selective toxicity in males because of longer gastric residence time and delayed absorbtion. However, no information was provided regarding the cause of death of the animal. While there were no affects on behavior, food and water consumption, or necropsy findings, the high dose animals of both sexes had a significant increase in weight. The authors said that this could be due to stimulation in appetite, which suggests no deleterious effects on weight and growth. The authors state that this is "in agreement with the results of

previous studies"; however, the study sited appears to be a carcinogenicity study of Ginko biloba. The increase in body weights was not related to other clinical signs, or pathologic abnormalities.

The treatment of rats with hesperidin for 13 weeks resulted in statistically significant, although minor changes in hematologic variables. The authors concluded that while statistically significant, they were judged to be not toxicologically significant because the changes were minor, the mean values were within the standard ranges for the strain, sex, and age of rats, the differences were not dose-related, and were not associated with changes in other related variables. While serum biochemistries related to kidney function (blood urea nitrogen, calcium and sodium) were significantly different after treatment with hesperidin, they were within the normal range. Further, urinalysis showed no abnormal values, nor was there any significant necropsy findings. Although there was mild inflammatory infiltration there was no data to support the biochemical findings. Glucose values were significantly reduced following administration of hesperidin for 13 weeks; however, these were not dose-dependent nor were the values outside of normal laboratory values.

Statistically significant increases in ALT in males and AST in female animals resolved after the 28-day reversal sample, and the authors said that the differences were not sex- or dose-dependent, and were within the normal range for these variables. Animals that consumed hesperidin also had a significant increase in liver weights, and corresponding histology showed slight lymphocytic infiltration. However, this was considered a reversible change. The authors noted that other researchers have reported similar lesions that develop spontaneously in rats of this strain and age. Therefore the liver-related changes observed in this 13-week toxicity study were judged not to be toxicologically significant in reference to treatment with hesperidin. Significantly elevated concentrations of Chl, Tri and Bil, and reduced Alb can indicate hepatic dysfunction. Also with hepatic damage it is usual for fat to accumulate in hepatocytes. In this study administration of hesperidin for 13 weeks did not result in increased Chl, nor were there related histopathologic changes, hepatic fat accumulation, or obstruction of the intrahepatic bile duct. The authors stated that the significantly increased triglyceride values were not dose-dependent and were within the normal laboratory range for this variable.

The authors concluded that the acute and sub-chronic toxicity studies reported in the publication did not demonstrate toxicity that was directly related to the consumption of hesperidin. This conclusion is interesting in light of the fact that they also provide a median $LD_{50}$ of 4837.5 mg/kg for the acute study, but no information on the cause of death. Further the sub-chronic oral toxicity study was given a Low Observed Adverse Effect Level (LOAEL) at 1000 mg/kg for both males and females. The Notifier disagrees with the assignment of the LOAEL, and would argue that 1000 mg/kg might

be termed a Low Observed Effect Level (LOEL) instead of a LOAEL, and even a NOAEL at 1000 mg/kg considering the fact that the authors themselves concluded that consumption of up to 1000 mg/kg "did not show any toxicity attributable to the administration of hesperidin" (Li, et al., 2019).

### 6.6.6 Conclusion of safety of hesperidin

It is felt that the publically available data and information presented in this GRAS Notice, in GRAS Notices GRN 000719 and 000796, the 13- and 96-week MH toxicity studies, and the acute and sub-chronic hesperidin toxicity studies described above, support a conclusion of safety of the consumption of hesperidin as added to the diet for the intended uses at concentrations similar to that which is being proposed for GH in this GRAS Notice. Because the metabolism of hesperidin and GH has been demonstrated to be essentially the same, the Notifier concludes that the safety of hesperidn is substantiall equivalent to that of GH, when consumed in similar amounts.

## 6.7 GH Genotoxic, Acute Oral, Four-Week Oral and Thirteen-Week Oral Toxicity, Teratogenic, and Physiologic Studies

A literature search through December 5, 2019 from PubMed.gov and the TOXLINE database was done of glucosyl or glycosyl hesperidin, alone or with toxicity or safety. The PubMed.gov search on GH revealed 32 references since 2002, and 9 on glycosyl hesperidin since 2007. None of the studies reported any toxicity or safety concerns. Several of the citations are included in this GRAS Notice supporting the safe use of GH in both animals and humans. The TOXLINE search provided only a fraction of the citations of PubMed.org and these were noted as being in the PubMed.org database.

The following information is specifically related to information on the safety of GH in animals and humans with its primary components being MGH and hesperidin. As noted in Table 2-7 GH is composed of approximately 78% MGH, 14% hesperidin, 3% di- and oligoglucoside-binding hesperidin, 3% other glycosylated flavonoids, and 2% various saccharides and carbohydrates. The di- and oligoglucoside-binding hesperidins, and other glycosylated flavonoids are metabolized in a similar manner, if not identical to MGH. The various saccharides and carbohydrates are composed of glucose and are also hydrolyzed in the small intestines and/or fermented in the large bowel.

A critical evaluation of acute and sub-chronic toxicological studies of hesperidin and GH in animals revealed that hesperidin and GH are safe; that is, no signs of toxicity were reported in any study at the highest doses tested. These studies support the conclusion that the intended use of GH can be considered GRAS under 21 CFR

§170.30 (FDA, 21 CFR §170.30). The supporting studies are discussed below. As with all the information in this GRAS Notice, the original study reports, if not published, can be provided for review by the FDA.

Two different grades of GH were used for the following safety studies, both of which contained over 75% MGH. The first is GH, the specific subject of this GRAS Notice, and is used as a food ingredient and/or food additive in Japan, Korea and Taiwan. The second grade is used as a cosmetic ingredient (GH cosmetic grade = GH-C) in several countries in the world, including the US, Japan, and were registered in the EU for use as a new chemical substance (cosmetic ingredient) in ELINCS/REACH in July 2006. To provide a comparison between the two grades, lot #0411192 of GH, which was used for the 28 and 90-day oral toxicity studies contained 78.08% MGH, 16.26% hesperidin, and 0% 7-glucosyl hesperetin; whereas lot #705160 of GH-C contained 75.7% MGH, 1.4% hesperidin, and 10.5% 7-glucosyl hesperetin (see Figure 6-1).

GH-C was used in only the bacterial mutagenicity study, and the acute toxicity study to confirm the safety of MGH, which is the main constituent of GH. All other *in vitro*, animal and human studies used GH as the test substance.

### 6.7.1 Genotoxicity studies

Unpublished -- GH-C (75.7% MGH) was examined for mutagenic activity in two standardized bacterial reverse mutation assays (OECD #471 & #472); a standard plate incorporation assay and a pre-incubation assay (Wollny, 1997, unpublished). Both experiments were conducted with and without metabolic activation (S9-mix). The first experiment was the plate incorporation assay and used the histidine-requiring *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100, TA102, and the tryptophan-requiring *Escherichia coli* strain WP2 uvrA. The second study was a pre-incubation assay, and *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and *Escherichia coli* strain WP2 uvrA were used. Five concentrations (33, 100, 333, 1,000, 2,500 and 5,000 µg/plate) of GH-C were used. The negative control was water, while the positive controls were sodium azide, 4-nitro-*o*-phenylene-diamine, methylmethane sulfonate, and 2-aminoanthracene. Final concentrations were formulated by a dilution method. All tests were done in triplicate.

The test article did not inhibit growth of the bacteria, even at the 5,000 µg/plate concentration. No apparent increase was recorded for the mean number of revertant colonies for any dose with or without metabolic activation. There was also no tendency for higher mutation rates with increasing concentrations in the range generally acknowledged to be biologically relevant. These results were seen at all doses, strains and samples with and without metabolic activation. The negative and

positive controls demonstrated that the experiment was run to proper standards. No gene mutations of base pair changes or frameshifts were observed under the experimental conditions. It was concluded that GH-C, is not mutagenic in this *Salmonella typhimurium* and *Escherichia coli* reverse mutation study (Wollny, 1997).

Published -- To evaluate the genotoxicity of GH in mammalian cells, a standard chromosomal aberration study was performed (Matsumoto, et al., 2019). The cell growth inhibition test was performed with doubling doses of the test substance from 4.9 to 5,000 µg/mL. GH (75.6%) did not induce structural or numeric aberrations in either the 6-hour (with or without metabolic activation) or 24-hour experiments (without metabolic activation). It was therefore concluded that this substance is not genotoxic for Chinese hamster cells at a concentration of up to 5,000 µg/mL under the conditions of the test (Matsumoto, et al., 2019).

Published -- A standard mouse micronucleus assay was performed using GH (Matsumoto, et al., 2019). In the test mice were treated with a single dose of GH (75.6% MGH) administered at doses of 500, 1,000 and 2,000 mg/kg-bw. None of the treatment groups had a statistically significant increase in the micronucleus frequency when compared to the negative control group, and no dose-dependent pattern of micronuclei formation was apparent. In addition, the ratio of total polychromatic erythrocytes to total erythrocytes showed no significant difference between the treatment groups and the negative control group. Based on the criteria established for each study it was concluded that at the highest dose of 2,000 mg/kg-bw, GH did not show any positive response, and therefore under the study conditions, GH is not clastogenic (Matsumoto, et al., 2019).

### 6.7.2 Acute toxicity in rats

Unpublished -- An acute oral toxicity study using GH-C was performed rats (Arcelin, 1997, unpublished). Each animal received a single 2,000 mg/kg-bw dose of GH-C by oral gavage. GH-C was dissolved in bi-distilled water at a concentration of 0.2 g/mL and administered at a volume of 10 mL/kg to each rat after fasting for approximately 16.5 hours. Animals were examined for clinical signs four times on day 1 of treatment and once daily for 14 days after administration. Body weights were recorded on day 1 before treatment and at study days 8 and 15.

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 of GH-C 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 GH-C was concluded to be greater than 2,000 mg/kg for both male and female rats, using the OECD limit dose (Arcelin, 1997).

### 6.7.3 4-week subacute toxicity in rats

Published – A subacute 4-week oral feeding study using GH (78.08% MGH) was conducted to investigate the toxicity of daily administration of GH in HanBrl:WIST (SPF) rats (Matsumoto, et al., 2019). Rats consumed the feed containing GH at concentratons of 0, 100, 2,000, and 15,000 ppm *ad libitum* for 28 days. There were no deaths during the study, or consistent, treatment-related, dose-dependent adverse effects reported on any variables evaluated including clinical signs (behavior), general physical condition, food consumption and body weight, organ weights and ratios, and gross and histopathological findings. The investigators concluded that the no-observed effect level (NOEL) was 15,000 ppm for both male and female rats (the highest concentration fed), which is equivalent to 1,205.77 and 1,279.94 mg/kg-bw/day, respectively (Matsumoto, et al., 2019). This would equal a daily consumption of approximately 72.3 and 76.8 grams of GH in adult US males (60 kg) and females (60 kg) for 28 days, respectively.

### 6.7.4 13-week sub-chronic toxicity in rats

Published -- A sub-chronic 13-week oral toxicity study of GH (78.08% MGH) was conducted rats (Matsumoto, et al., 2019). GH was mixed with microgranulated feed and pellets were prepared. The treatment diets contained GH at concentrations of 0, 4,500, 15,000, and 50,000 ppm. Animals were fed the diet for 90 days. There were no consistent, statistically significant, treatment-related and dose-dependent adverse effects on mortality (no deaths), food consumption, body weight gains, feed efficiency, behavior, clinical chemistry and urinalysis findings, and gross and histopathological findings (Matsumoto, et al., 2019). The NOEL of GH was 50,000 ppm in the feed (the highest concentration fed), which is equal to 3,084 mg/kg-bw/day for males and 3,428 mg/kg-bw/day for females. This amount of consumption would be equivalent to adult US males and females (60 kg) consuming approximately 185.0 and 205.7 grams of GH per day, respectively, for 90 days. Supporting the conclusion that GH is safe for consumption.

### 6.7.5 Developmental teratogenicity in rats

Published -- Eighty (80) impregnated females were used in the teratology study. Aqueous solutions of 0, 100, 300 or 1,000 mg/kg-bw/day GH (70.2% MGH), which is within the official specifications in Japan, were administered by gavage from days 6 to

17 of gestation (Matsumoto, et al., 2019). On day 20 of gestation the fetuses were taken by Cesarean section and the mothers euthanized. There were no consistent, statistically significant, treatment-related and dose-dependent adverse effects on mortality (there were no deaths or abortions), food consumption, body weight gains, food efficiency, behavior, clinical signs, necropsy findings, or organ weights and ratios in the mothers. Examination of the fetuses revealed no treatment-related effects on the number of corpora lutea graviditatis, number of implantations, number of live fetuses, sex ratio, number of resorptions or dead fetuses, live fetal weight, placenta weight, placental findings, external findings, visceral findings or skeletal findings. The no-observed adverse effect level (NOAEL) for GH for both mothers and fetal development was 1,000 mg/kg-bw/day, which was the highest dose tested. No teratogenicity was observed at 1,000 mg/kg-bw/day of GH (Matsumoto, et al., 2019).

### 6.7.6 24-week feeding study in rabbits

Published -- Twenty-one (21) female rabbits received 0, 150 or 4,500 mg/day of α-glucosylhesperidin in 350 mL of water daily for 24 weeks (Naiki, et al., 2012). The α-glucosylhesperidin is produced by another company, and similar to GH, principally containing MGH and hesperidin (Kometani, et al., 2008; Takumi, et al., 2010). The purpose of the study was designed to determine the effects of ingestion of α-glucosylhesperidin on the diameter and mechanical properties of the rabbit femoral artery. The authors concluded that long-term feeding of α-glucosylhesperidin results in a reduction in arterial contraction by the neurotransmitters of sympathetic nerves. This results in the normal maintenance of blood flow in the femoral artery even when there is a situation of autonomic imbalance and cold intolerance. There were no differences in body weight. The authors concluded that there were no behavioral or appearance abnormalities in the high dose animals. Further the stiffness of the femoral arteries in the control group was similar to that in both treatment groups, except for the contractile response to norepinephrine as mentioned above. They concluded that, "[T]hese results indicate the safety of the α-glucosylhesperidin use." The consumption of approximately 1,500 mg/kg-bw/day of α-glucosylhesperidin in the highest dose group would be equal to 90 g/day in a 60 kg human suggesting a high margin of safety of a substance that is highly similar to GH.

### 6.7.7 Four physiologic studies in rats

Published -- Four published studies in which GH, hesperidin or no treatment was given to genetically hypertensive (SHR) or normal rats (WKY) are reported in the literature (Ohtsuki, et al., 2002; Ohtsuki, et al., 2003; Yamamoto, et al., 2008a; Yamamoto, et al., 2008b). These studies were not specifically designed to examine the safety of short- and long-term administration of GH. However review of the studies did not

show any evidence to suggest adverse events or toxicity of GH in either strain of rat. Doses of GH were administered in the feed in two studies at 30 mg/kg-bw/day for 25 weeks, and one study at 50 mg/kg-bw/day for 8 weeks. This latter study would be the equivalent to a 60 kg human receiving 1.8 and 3 grams per day, and a cumulative dose 315 and 168 grams of GH, respectively. The fourth study was an oral single administration of GH at 10, 30 and 50 mg/kg. These data contain no information that would suggest that long-term, daily consumption of GH is a safety concern, and supports the Notifier's contention that GH is safe.

### 6.8 Nine Studies in Humans with GH

Nine (9) studies have been published in the literature in which Hayashibara GH was consumed by human subjects. All studies were performed on Japanese subjects. A review of these studies revealed that no causal adverse effects were attributed to individuals who consumed relatively large amounts of GH (1020 mg/day MGH) for 4-weeks, or consumed lower amounts of approximately 500 mg/day GH (340 mg/day MGH) for 6-24 weeks. In these studies a major portion of the subjects had high normal to slightly above the normal range for various conditions such as total cholesterol, triglycerides, hypertension, and/or body weight/BMI. The subjects were not on any medications to treat these conditions. These summaries report on any possible safety concerns, and are not intended to provide or make any health benefit claims from consumption of GH. However, in the latter 6 studies, there was a specific review of safety of GH. Each reported that GH appeared to be safe, and any significant change in the blood chemistry variables were not considered to be of any safety concern. The following are brief summaries of the studies and safety conclusions.

**1)** Miwa and co-workers administered 100 or 500 mg/day of GH in tablet form at bedtime for 6 weeks to 20 hyperlipidemic healthy male subjects per group (Miwa, et al., 2004). No subjects were on lipid lowering drugs. After treatment the subjects were followed for another 4 weeks of non-treatment. No differences were noted in dietary habits, body weight, body mass index (BMI), serum GOT, GPT, γ-GT and creatinine levels, and clinical signs related to the GH during the 6-week consumption period. There were significant changes in blood lipid variables; however, the authors concluded that the changes observed were of no safety concern. From the data it is suggested that the consumption of up to 500 mg/day of GH for 6 weeks does not result in any safety-related concerns.

**2)** Miwa and co-workers extended the original study by administering the higher daily dose (500 mg/day) of GH to twenty-five healthy adult males for 24 weeks (Miwa, et al., 2005). The GH used was manufactured by Toyo Sugar Refining Co., Ltd. under

license from Hayashibara Co., Ltd., and is essentially identical to the GH produced by Hayashibara. Six (6) of the subjects had normal serum triglyceride concentrations, while the other subjects had fasting TG concentrations of at least 110 mg/dL and were not taking lipid-lowering drugs. The subjects were further classified according to Vigna and co-workers (Vigna, et al., 1999).

No changes were seen in dietary habits, body weight, BMI, serum creatinine and uric acid concentrations during the administration period. No clinical signs related to treatment or TG classification were observed during or after the study. All the results support the conclusion that there is no indication of any safety-related concerns to either the normal subjects or those with elevated TG.

**3)** The third study consisted of three experiments reported in a single published article. These included i) 51 "mild" hypertriglyceridemic, ii) 10 normotriglyceridemic, and iii) 9 normotriglyceridemic and 13 mild hypertriglyceridemic healthy adult subjects. They were given various doses of a tea beverage containing GH (Yuasa, et al., 2005).

In the first experiment the 51 subjects were given 340 g of the tea beverage each day for 12 weeks containing either 340 mg MGH (about 500 mg GH) or with maltodextrin added as a control. Fasting blood samples were obtained at weeks 0, 4, 8, 12, and 16. None of the significant changes were of medical concern, and in fact are considered positive for the subjects. No adverse events were observed or reported in the subjects' physiology, hematology, biochemistry or general health at any time during the study

The second experiment included 6 female and 4 male healthy adult subjects whose initial triglyceride values were less than 120 mg/dL, which is considered normal. They consumed the same amount of tea and GH (340 g/day and 340 mg of MGH, respectively) for twelve weeks as was consumed in the first study. Of the dozens of hematological and biochemical assays performed only the percent of small LDL decreased, and the particle size of the LDL statistically increased as compared to baseline values. These statistical differences continued after the subjects stopped drinking the tea with GH. These data were consistent with the first experiment. No adverse effects were observed throughout the study period.

In the final experiment,13 mild-hypertriglyceridemic healthy adult subjects were given 1,020 g/day of tea containing 1,020 mg/day of MGH (approximately 1,500 mg GH per day). Another group of 9 normal-TG subjects were also given the same concentration of MGH and amount of tea. Both groups consumed it daily for 4 weeks. Following the consumption period there was a washout period of 2 weeks. The authors stated that, "(N)o adverse effects derived from the intake[s] of GH beverage were noticeable

throughout each the study." The daily and total doses ingested in the first two experiments were 340 mg and 28.56 g, respectively; whereas, the amounts in the third study was 1.02 g and 28.56 g. The authors also stated, "[T]hese results of all three studies clearly show the safety of tea beverage containing GH ....", which suggest that the GH contained in the tea is safe.

**4)** A randomized, double-blind placebo-controlled clinical study was performed by Kozuma and co-workers (Kozuma, et al., 2007). One purpose was to assess the safety daily doses of GH (35 mg of MGH) consumed in 15 mL of a low sodium soy sauce per day for 12 weeks. One hundred and seventy-nine (179) subjects with either high-normal, or mild-hypertension were randomized to receive either the soy sauce containing GH or low sodium soy sauce alone (control). Thirteen (13) were excluded or withdrew consent leaving 85 and 81, respectively, that completed the study and were evaluated for efficacy. One subject from the control group did not attend any post baseline sampling times and therefore was evaluated for safety, but not efficacy. Additionally, adverse signs and symptoms were recorded.

No physical or biochemical variables were statistically significant within or between the two groups before or after the study, except hypertension. Evaluation of adverse events showed the following results with the control group (n = 86) as compared to the GH group (n = 81), respectively: headache (23 [26.7%] vs 11 [13.6%]); upper respiratory tract infection (21 [24.4%] vs 11 [13.6%]); tinnitus (3 [3.5%] vs 0 [0%]; abdominal pain (3 [3.5%] vs 0 [0%]); diarrhea (2 [2.3%] vs 0 [0%]); nausea (2 [2.3%] vs 0 [0%]); and vertigo (1 [1.2%] vs 0 [0%]). There were fewer adverse events reported in the GH group (11) as compared to the control (32). The adverse events reported were not serious and resolved within a few days. Results of this study demonstrate the safety of a dose of 35 mg of GH daily for 12 weeks.

**5)** A safety evaluation of the administration of 2 tablets containing 500 mg GH (340 mg MGH) or placebo tablets daily for 12 weeks was performed on human subjects using a randomized, placebo-controlled, double-blind, parallel protocol (Hanawa, et al., 2008a). In this study a total of 119 subjects categorized as "non-lean" were randomized into two groups. All subjects were given three standardized meals the day before each clinical sample was taken (weeks -2, 0, 4, 8, 12 and 16). The week-16 sample was 4 weeks after stopping consumption of the GH or placebo tablets. Five (5) subjects were removed from the GH group, and 4 from the control group after randomization resulting in a total of 54 and 56 in each group, respectively. However, 5 of the 9 subjects removed from the study were included in the safety assessment.

Review of the values obtained from lipid and glucose-associated blood chemistry (23 items) revealed no consistent changes to the group that received the GH, that was not

also detected in the placebo group at 12 weeks, except for one variable. However, the authors concluded that this increase is common during the winter months of which the study was in at the 12-week sampling time. No other consistent patterns of change were noted and it did not appear that there were any overall GH-related changes. The pH, protein and glucose were significantly different between the GH and control groups, but were all within normal limits.

Three (3) specific adverse events were noted in subjects that had taken GH. These included: an increase in ALT and γ-GT at week 12; a non-specific rash at week 11; and mild amblygeustia shortly after the start of the study. The first subject had no other changes related to this condition, but they did have fatty liver syndrome and also mentioned cold-like symptoms (week 11), which may have been responsible for the rise in ALT and γ-GT. The subject had normal values through 8 weeks of GH consumption, and no other person receiving GH had increased liver enzymes the authors concluded that it was unlikely that this adverse event was related to the consumption of GH. The subject with the rash responded to treatment and continued on the study with no resumption of symptoms. The authors concluded the rash was not likely the result of GH consumption. The reduction of taste in the third subject continued throughout the study, but almost completely returned after stopping GH consumption. An association with GH consumption could not be excluded. The authors stated that these adverse events should be monitored in future studies, because they occurred during the consumption of GH. Review of all other human studies with GH have not reported any of these three conditions, and the Notifier has not been made aware of any of these through consumer, final product manufacturer, or government reporting channels. The study reported on a number of additional adverse events during the study period; however, the authors concluded that these were not related to the GH treatment. Other than the three adverse events already discussed, "[N]o other significant adverse events were found on physical, blood, or urine examination or interview" (Hanawa, et al., 2008).

This study supports the conclusion that consumption of GH (340 mg MGH/day) over a 12-week period is safe.

**6)** A second randomized, double-blind, placebo-controlled study by Hanawa et al. was published the same year (Hanawa, et al., 2008b). Ninety-three (93) non-lean and mildly hypertriglyceridemic adult volunteers were randomly divided into 4 groups, and GH was administered in tablet form (205 mg/tablet) for 4 weeks everyday. Doses groups were low-dose (250 mg/day), medium-dose (500 mg/day), high-dose (1,000 mg/day) and placebo. The result showed no untoward changes in the variables tested. Three (3) subjects from the 250-mg/day group, 1 subject from 500-mg/day group and 2 placebo subjects reported 1 adverse event each, which included cold-like

symptoms, diarrhea, astriction, pain in the right abdomen, pyelonephritis and tachycardia. The clinician examining the subject's complaints concluded that none of the reported adverse events were related to the intake of GH or placebo. No other significant adverse events were found on physical, blood, or urine examinations or interview. These data suggest that the daily consumption of 250 to 1,000 mg of GH daily is safe.

**7)** A study, including a safety evaluation by Nakagawa, et al. consisted of two parts. The first part had subjects ingesting GH daily for 12 weeks. The second part was a safety study in which 3 times the amount of GH given in the first experiment was ingested daily for 4 weeks (Nakagawa, et al., 2008).

In the first experiment a total of 85 subjects with mildly elevated serum TG concentrations were given 130 mL of an aqueous solution containing 4 g of green tea powder with either GH (340 mg MGH, approximately 500 mg GH) or 340 mg of maltodextrin (control). There were no significant changes in hematologic values in the subjects given the GH for 12 weeks. There were no significant changes in serum biochemical values between the GH and control groups. Analysis of the urine showed no significant differences that were outside of the normal limits. Nine (9) subjects who received GH and 10 subjects in the control group reported a total of 34 adverse events. However, the authors stated that, "[T]he doctor responsible has confirmed that all the cases have no relationship with the trial products" (Nakagawa, et al., 2008).

In the second safety experiment, 28 subjects were given 130 mL of the aqueous beverage contain 4 g of green tea powder 3 times a day for 4 weeks. Each 130 mL of tea drink contained GH (340 mg MGH), equaling about 1 g/day of MGH. There was no control group. Urine and blood samples for hematology and serum biochemistry were collected at study weeks 0, 4, and 6. The variables examined were essentially the same as in the previous 12-week study.

Review of hematologic, clinical biochemical, and urine findings showed no significant changes over the period of GH consumption, or 2 weeks after cessation. All values were within normal ranges. Six (6) subjects reported 1 adverse event each. The physician responsible for examining the subject's complaints judged each of these adverse events as not being related to GH consumption. The authors stated that, "[T]hroughout these trials, no adverse effects was [sic] found in physiological, hematological, biochemical parameters and in medical interviews" (Nakagawa, et al., 2008).

**8)** In a study reported by Tanaka et al., a beverage (500 mL) was provided in two experiments to different subject populations (Tanaka, et al., 2010). The first

experiment, called the "long-term" study (12 weeks of treatment and a 4-week follow up sampling), included a total of 112 subjects having elevated TG values, which were randomly divided into two groups of 56 participants each. None were receiving serum lipid lowering agents. One group received 500 mL of a beverage containing GH approximately 500 mg (equal to 340 mg of MGH). The control subjects received an identical beverage minus the GH (control). The subjects could not distinguish the two beverages from each other. Both groups consumed one bottle of the beverage (GH or control) daily for 12 weeks. Thirteen (13) subjects (7 GH, 6 control) dropped out of the study at various times. The authors stated that none of the dropout were because of the study beverage, and therefore excluded both GH and control from the analysis. Blood and urine samples were collected before the start of treatment (week 0) and at 4-week intervals until week 12. The subjects returned at week 16 (4 weeks after stopping consumption for a final panel of tests.

The values, both significant and not significant at all times were within the reference ranges provided in the publication, except for a few variables. There was a discrepancy in the number of subjects that completed the study in the GH and control groups by 1. Regarding hematology and other biochemistry, while there were several statistically significant differences between and within the two treatment groups, the investigators concluded that "all these changes were slight and within the reference range, and considered clinically acceptable ...." (Tanaka et al., 2010). Further they stated that, "[I]n the individual subjects, there were also no abnormal changes that were considered clinically significant ....". Analyses of urine showed no significant changes in either qualitative or quantitative variables. The investigators did comment that in a few subjects there were some qualitative changes from normal to abnormal, but were judged as clinically insignificant. Adverse events were collected at each visit. A total of 38 events were reported (19 GH, 19 control). The authors stated, "... symptoms were mild and resolved spontaneously or with short-term medication". None were judged to have a causal relationship to the GH beverage.

The second experiment performed, was a high-dose study where no control group was used. The 34 subjects consisted of a mix of individuals with elevated or normal TG values. The beverage (500 mL) included GH that provided 1,030 mg of MGH (approximately 1,500 mg GH), or essentially three times the dose used in the long-term study (Tanaka, et al., 2010). The high-dose experiment included a 2-week observation period before the start of intake of GH, a 4-week intake, and a 2-week post-intake observation period. Subjects were tested at 0, 2, 4 and 6 weeks. The subjects had a specific meal for dinner the night before each visit.

While significant differences were observed in serum biochemistry, hematology or urinalysis they were considered slight, within references ranges, and/or not clinically

relevant. The authors stated, "No adverse effects were found in physiological, biochemical parameters and medical interviews throughout this trial. Eleven (11) adverse events were noted during the high-dose experiment. As with the adverse events reported for the long-term study, ".... these symptoms were mild and resolved spontaneously or with short-term medication." There was no causal relationship ascribed to the beverage. The general conclusion was that, ".... the glucosyl hesperidin-containing beverage is safe in long term and excessive ingestion.

**9)** A clinical randomized placebo-controlled study was performed with 75 health subjects with moderately high BMIs and serum TG values by Ohara and co-workers. The subject consumed a tablet in which 470 mg of GH (74% MGH, 347.8 mg) was administered daily to 4 groups of 15 subjects, and placebo was given to an additional 15 subjects for 12 weeks, followed by a 4-week washout period (Ohara, et al., 2016, Ohara, et al., 2017). Note that the second Ohara reference is an erratum correcting the original paper, which gave the GH dose as 500 mg and the concentration of MGH as 75%. The tablets contained inactive excipients and the 470 mg of GH with 0, 25, 50 or 75 mg of caffeine added. One subject in the placebo group was not included in the efficacy analysis (n = 74), because of a major protocol violation, but was included in the safety assessment (n = 75). Subjects were assessed for various factors at weeks 0, 4, 8, 12, and at 4 weeks post-ingestion (week 16).

Reviewing the hematology, blood chemistry and urine analysis there were only a few significant changes between groups that the authors considered associated with safety. However, these changes were within the ranges of corresponding reference values. The total cholesterol values in the GH + 75 mg caffeine group were significantly reduced at weeks 8 and 12; however, the values were above the standard range at week 0, therefore the reduction would not be considered a negative outcome. There were a total of 29 adverse events reported during the study. The placebo group reported 7, the GH + 0 mg caffeine 6, GH + 25 mg 5, GH + 50 mg 8, and GH + 75 mg 3. All adverse events were judged to be unrelated to the consumption of the various test treatments. A review of the study demonstrated no safety concerns when 470 mg GH was consumed daily for 12 weeks.

## 6.9 Two Human Studies with a Non-Hayashibara Preparation of a GH-like Substance

In addition to human clinical studies in which subjects consumed Hayashibara GH, there have also been two clinical studies reported where another preparation of "GH" was given to humans. The Notifier of this GRAS Notice, Hayashibara Co., Ltd., cannot be sure as to the exact production process and structure of this GH substance other than what was described by the authors (Kometani, et al., 2008; Takumi, et al., 2010).

The product was termed α-glucosyl-hesperidin (Hsp-G) in one publication, and G-Hsp in the other (Kometani, et al., 2008; Takumi, et al., 2010). These papers were not published by the authors to specifically demonstrate safety. In both studies only short general statements by the authors were made regarding the issue of safety.

**1)** The study by Kometani et al. included three parts. Briefly the first experiment was a mouse model in which male mice were injected with type II chicken collagen to induce arthritis. The animals were orally administered 3 mg/0.3 mL Hsp-G 3 times a week either before collagen injection till the end of the experiment, or after development of arthritis till the end of the study (Kometani, et al., 2008). Oral administration of Hsp-G to mice did not appear to result in any safety concerns when administered before, or after the onset of arthritis.

In the second experiment 7 healthy volunteers were fasted, except for water, for 10 hours before the test. On the day of the test the volunteers consumed Hsp-G or hesperidin (equalized to 15 mg/mL hesperetin) in 100 mL water. Blood samples were collected at times 0, 0.5, 1, 2, 4, 8 and 24 hours after consumption. Kinetics of plasma concentrations of hesperetin showed that Hsp-G was more rapidly and completely absorbed than hesperidin. The AUC value was about 3 times greater for Hsp-G, which is in conformity to that observed with GH. No comments were made about any untoward effects of consumption of the hesperidin or Hsp-G.

Twenty (20) subjects with rheumatoid arthritis (RA) were enrolled in a 12-week double-blind, placebo-controlled trial. Subjects were administered beverages containing 3 g Hsp-G (n = 10) or placebo (n = 10) every morning for the duration of the 3-month trial. No changes to any medical treatments were made. One subject in the Hsp-G group was excluded from data analysis because of a change in diagnosis during the study. The authors stated that "[N]o events attributable to Hsp-G were observed during the study. Both the test (Hsp-G) and placebo beverages used in this study were believed to be acceptable to the patients, because all patients consumed the beverage every morning for 3 months without fail."

**2)** The second study was divided into three experiments (Takumi, et al., 2010). In the first experiment 1 capsule of 500 mg G-Hsp (structure is the same as Hsp-G reported in the previous study) or placebo was given with 50 mL of water to 92 female university students who were identified as being "cold sensitive" using Terasawa's cold sensitivity diagnostic criteria (Takumi, et al., 2010). The took one of the capsules on day one and the other on the second day in a randomized double-blind placebo cross-over fashion. No hematology or chemistry was performed so, as mentioned above, there was not a specific safety component. Body surface and tympanic temperatures were measured along with blood flow

In the second experiment, the 12 Japanese female subjects with cold sensitivity sat in a room at $22^0C \pm 0.5$ (50% RH) for 40 minutes. They consumed a warm beverage (200 mL) containing either 500 mg G-Hsp, or without G-Hsp. The samples were given randomly on day one and two in a double-blind, placebo-controlled cross-over manner. Body temperatures were taken at the same locations as in the first experiment, and blood flow was measured on the middle finger of the left.

The third experiment included 11 health Japanese female volunteers with cold sensitivity similar to that of the other subjects. The test samples and conditions were the same as in experiment two except the 500 mg G-Hsp was dissolved in 100 mL water at $37^0C$.

The only statement made concerning the safety of G-Hsp were two lines, "[T]he present study has uncovered a safe and available mechanism by observing the change in HR variability after administration of G-Hsp. We therefore expect that the use of G-Hsp would be safe and would help to promote health".

From what is reported in the two publications and the fact that the studies were performed on humans and approved by the related ethical review committees the authors concluded that the preparation of α-glucosylhesperidin used is safe.

### 6.10 Summary of Safety Studies

Hayashibara Co., Ltd., has presented information and data on the chemical composition, manufacturing and purification process, and food grade specifications for Glucosyl Hesperidin (GH). Additionally much safety information was presented demonstrating that hesperidin and GH are substantially equivalent in structure, and metabolism. A review of safety studies on hesperidin strongly suggests that, even at high consumption levels, it is safe (Fisher, 1982; Garg, et al., 2001). The safety of hesperidin is further supported by a recent "no questions" letter issued by the FDA for GRN 000796, an orange extract preparation (≥ 85% hesperidin) (GRN 000796), and GRN 000719, which was used to support the former GRAS Notice (FDA, GRAS, 2019; FDA GRAS, 2017). The support for the safety of these two GRAS Notices was partially based on 13-week and 96-week oral feeding studies in mice in which methyl hesperidin was consumed at up to 5% of the diet (Kurata et al., 1990; Kawabe et al., 1993). The authors concluded that the methyl hesperidin consumed was not toxic to the animals. In the 96-week study the NOAEL of the male animals was calculated as 7,500 mg/kg-bw/day in GRN 000796. Li and coworkers administed a single dose of hesperidin via gavage at up to 5000 mg/kg to rats (Li et al., 2019). While one animal in the high dose group died on day 11 of the 14-day observation period, no mention of

the results of a necropsy was made. Further, the authors reported a LOAEL at the 1000 mg/kg (highest dose) in the 13-week sub-chronic study; however, they concluded that the results of both studies support the conclusion that administration of hesperidin, "did not show any signs of toxicity attributable to the administration of hesperidin." Taken together the data from hesperidin and hesperidin derivative studies demonstrate the safety of this substance.

This assessment of the safety of hesperidin is pertinent to the safety review of GH because the two substances, MGH (the main constituent of GH) and hesperidin, are absorbed into and metabolized in the body by the same pathway (Matsumoto, et al., 2004; Yamada, et al., 2006a; Yamada, et al., 2006b, unpublished). Therefore they are substantially equivalent. As reported earlier in Part 6, the GH used for the 28 and 90-day oral toxicity studies (lot # 0411192) was composed of 78.1% MGH and 16.3% hesperidin. GH and and MGH in GH-C (cosmetic grade), are not mutagenic or clastogenic (Wollny, 2006; Matsumoto et al., 2019). Further, GH-C containing MGH, which is the main constituent of GH does not cause any untoward affects when it was tested in a number of international standard analytical studies for approval as a cosmetic ingredient (data unpublished, not shown, but available for review).

GH has been allowed and commercialized for use in food in Japan since 1998. To the Notifier's knowledge there have been no manufacturers or consumers of final products containing GH that have reported any untoward effects in the approximate 20 years since GH was first introduced into commerce.

The acute toxicity study of GH-C containing MGH resulted in an $LD_{50}$ greater than 2,000 mg/kg for both male and female rats, which was the highest dose tested (Arcelin, 1997, unpublished).

Specifically related to the safety of GH is the NOEL in the published 13-week oral feeding study with GH was 3,084 mg/kg-bw for males and 3,428 mg/kg-bw for females, the highest dose tested (Matsumoto, et al., 2019). The average amount of consumption in a 60 kg human would equal approximately 195.36 g GH/person/day, or 142.57 g hesperidin/person/day. This amount provides at least a 100 fold safety margin for the consumption of GH (as hesperidin) in the total population (1-99 years), consumers only of the 90th percentile.

A rat teratogenicity study on GH was conducted in which the dams were administered up to 1,000 mg/kg-bw day for 12 days during gestation. The NOAEL for maternal and fetal adverse effects was 1,000 mg/kg-bw/day, the highest dose tested (Matsumoto, et al., 2019).

A published study in which Japanese white female rabbits were fed up to 1,500 mg/kg-bw/day of α-glucosylhesperidin for 24 weeks was summarized. There was no indication of untoward effects, and the authors concluded that long-term consumption was safe (Naiki, et al, 2012).

There were 4 published studies presented in which normal and genetically hypertensive rats were given GH, in 2 studies, at a dose of 30 mg/kg-bw/day for 25 weeks (Ohtsuki, et al., 2002; Ohtsuki, et al., 2003). In the second 2 studies the animals received 50 mg/kg-bw/day for 8 weeks (Yamamoto, et al., 2008a; Yamamoto, et al., 2008b). No safety related effects were identified in either normal or hypertensive rats.

Nine (9) human clinical studies were conducted and have been published for a number of years in which subjects consumed up to 500 mg of GH daily for up to 24 weeks, and other subjects consumed 1,020 mg per day for 4 weeks (Miwa, et al., 2004; Miwa, et al., 2005; Yuasa, et al., 2005; Kozuma, et al., 2007; Hanawa, et al., 2008a; Hanawa, et al., 2008b; Nakagawa, et al., 2008; Tanaka, et al., 2010; Ohara, et al., 2016;) No untoward hematological or biochemical changes were observed in any of the studies. No adverse events were reported that were judged as related to the consumption of GH, except for 3 subjects with different mild symptoms in one study. The authors suggested that the observed events should be monitored in other studies; however, similar events were not reported in any of the other published studies, and have not been reported by individuals who manufacture GH, use GH to make finished products, or from consumers of the finished products in 20 years of consumption. Further there were no indications of any safety related concerns were reported in the two human studies using a substance similar to GH (Kometani, et al., 2008; Takumi, et al., 2010).

In summary, the data from these pre-clinical and clinical studies strongly supports the Notifier's conclusion that the consumption of GH is safe for its intended use.

### 6.11 Inconsistent Data/Information, and Exempt Information

6.11.1 The Notifier is not aware of any information that would be inconsistent with a conclusion that the proposed uses of GH, meeting appropriate specifications and used according to current Good Manufacturing Practice, are GRAS.

6.11.2 The Notifier does not claim any of the data or information included in this GRAS Notice is exempt from disclosure under FOIA.

### 6.12 General Conclusion of GRAS

The large number of published and generally available data on hesperidin and GH,

including *in vitro*, animal, and human studies, provides a basis to conclude that the use of GH for the intended uses described in this GRAS Notice fulfills the necessary standards for GRAS. To further support the conclusion of GRAS, in December 2009 the Notifier convened a GRAS Panel of individuals internationally recognized as qualified by scientific training and experience to independently and critically review generally available and other information provided. The Panel concluded that there was sufficient data and information to support a conclusion of GRAS, and the Notifier Self-Affirmed GH as GRAS. Since that time new safety-related studies have been published, and some relatively minor changes have been made to the raw materials, manufacturing process, and final specifications of GH. Further, the Nortifier decided to make a GRAS Notice submission to the FDA. Therefore the Notifier requested that the same individuals that were members of the original GRAS Panel review the changes and new safety-related studies, and determine if any of the changes mentioned above have an impact on their original conclusion that GH can be considered GRAS. The GRAS Panel once again concluded that GH produced consistent with cGMP, and to the specifications provided, is safe under its intended conditions of use (Appendix 2). In conclusion, the publically available information and data on the structure, composition, bioavailability, nutritional value, and pivotal human safety studies of GH, and its "substantial equivalence" to natural hesperidin, forms the basis for the conclusion of GRAS under conditions of intended use in foods, based on scientific procedures.

# Part 7 List of supporting data and information

This Part of the GRAS Notice contains the supporting data including the references and Appendices noted in the text. The Appendices include the report of the Estimated Daily Intake calculations and products in which GH is intended to be used (Appendix 1), and a report (2009) and subsequent updated letter (2019) by an independent GRAS Panel of that states that in their opinion the publically available data on hesperidin and GH supports a conclusion of GRAS by the Notifier.

## Part 7.1 References

The following contains the references used in this GRAS Notice. To make the information convenient to identify, the articles that are published, "generally available", and have direct relevance to the safety of the Notified substance are printed in bold typeface. Further, a reference with double bold "##" are not "generally available", but were referenced in Part 6 to support safety of GH in the GRAS Notice. There are several references that relate to the claim of substantial equivalence of GH to hesperidin. These will not be specifically identified in the references, but are associated with the adsorption and metabolism of GH and hesperidin.

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## 7.2 Appendix 1

Appendix 1 contains the Summary EDI calculations for the intended use of GH and GH as Hesperidin, and the dietary exposure to naturally occurring Hesperidin.

It provides the specific food codes for the intended use of GH.

May 6, 2019

To: Hayashibara Co., Ltd.

From: NutraSource

Subject: Estimated Daily Intakes (EDIs) of Glucosyl Hesperidin as Hesperidin

**Objective**

Hayashibara intends to market glucosyl hesperidin as a direct ingredient in select conventional foods. The estimated daily intakes (EDIs) of hesperidin were calculated as: 1) EDIs under the intended use, 2) EDIs of naturally occurring hesperidin from the diet, and 3) combined EDIs under the intended use and from the diet.

As shown in Table 1, Hayashibara proposes to use glucosyl hesperidin in food applications at use levels of 500 mg per serving of food. The intended food applications include yogurt, chocolate, and gumdrops (gummis) and hardy candy, coffee and tea, soft drinks, fruit and vegetable juice drinks and lemonade, fruit flavored drinks or fruit juice, and concentrates, water (bottled- sweetened or flavored; fortified) nutritional drinks and powders, energy drinks and powders, sports drinks and powders, fluid replacements, and other functional beverages.

Table 1. Intended Use and Maximum Use Levels of Glucosyl Hesperidin

| Food category | Glucosyl hesperidin, mg/ serving | Hesperidin, mg/serving |
|---|---|---|
| Yogurt | 500 | 364.9 |
| Chocolate (sweet or dark; milk chocolate candy plain) | 500 | 364.9 |
| Gumdrops (gummi) and hard candy | 500 | 364.9 |
| Beverages; coffee, tea | 500 | 364.9 |
| Soft drinks (including carbonated water sweetened) | 500 | 364.9 |
| Fruit and vegetable juice drinks and lemonade | 500 | 364.9 |
| Fruit flavored drinks or fruit juice and powders | 500 | 364.9 |
| Water (bottled - sweetened or flavored; fortified ) | 500 | 364.9 |
| Nutritional drink and powders | 500 | 364.9 |
| Energy drinks and powders | 500 | 364.9 |
| Sports drinks and powders | 500 | 364.9 |
| Fluid replacements | 500 | 364.9 |
| Functional beverages | 500 | 364.9 |

**Methods**

Using food intake data reported in the 2013-2016 NHANES, exposure levels to glucosyl hesperidin that will result from the intended uses were estimated (Tables 2 and 3). The most recent NHANES (2013-2016) compiled by the National Center for

Health Statistics and the Nutrition Coordinating Center was used to calculated the exposure estimates. The NHANES was conducted between 2013-2016 with non-institutionalized individuals in the U.S. The NHANES provides the most current food consumption data available for the American population. The food and dietary supplement record for each individual includes the gram weight and nutrient data for all foods consumed during the day of the recall. All estimates were generated with USDA sampling weights to adjust for differences in representation of subpopulations. For this study, 1 g is considered equivalent to 1 ml for soft drinks and formula diets for meal replacement. NHANES 2013-2016 dietary data of breast-fed children aged 1+ after exclusions, pregnant or lactating females, and unreliable data were used to estimate the intake of hesperidin. SAS 9.4 along with strata, plus, and day 2 dietary weights were used for analyses of mean, median, 90$^{th}$ percentile, and standard errors (SE) for hesperidin exposure. Intake was calculated as average of day 1 and day 2 intake. The sample population was limited to subjects with both day 1 and day 2 dietary data.

Glucosyl hesperidin is intended to be used at 500 mg per serving. This level corresponds to the use level of 364.9 mg hesperidin per serving of select conventional foods. The EDIs of hesperidin under the intended use and those of naturally occurring hesperidin from the diet were also calculated. Finally, the combined EDIs of hesperidin under the intended use and from the diet were estimated.

Food codes table
Food codes table displays food groupings, food codes, consumers, and consumptions for population aged 1 year or above who had both days of dietary records (Appendix). RACC denotes the reference amount customarily consumed (FDA, 2016). Food codes with a group number are those with a use level of 500 mg glucosyl hesperidin/serving of hesperidin (364.9 mg hesperidin/serving).

The NHANES flavonoid database gives hesperetin values in mg/serving by food code for NHANES 2007-2010. Naturally occurring hesperidin from the diet were obtained by using hesperetin values available from the NHANES flavonoid database. The hesperetin values from NHANES were divided by 0.495 to get dietary hesperidin values. This analysis uses 2013-2016 NHANES and some food codes in 2013-2016 are not in the NHANES 2007-2010 flavonoid database. For these food codes, the food code in 2013-2016 is mapped to a food code in 2007-2010 using food code descriptions and this hesperetin value is used.

Statistics in mg and mg/kg body weight (bw) tables
Tables displays the estimated daily intakes (mean and 90$^{th}$ percentile) in all-users (all-consumers) and all population (all-person or per capita intake). Intake is given in mg/day, and in mg per kg body weight per day. The estimated mean and 90$^{th}$ percentile are given for the total population and within consumers.

**Results**

Tables 2 and 3 summarize the EDI under the intended use of glucosyl hesperidin by the population groups; the first table presents the results of the mean of the population, as well as the 90$^{th}$ percentile, in mg/person/day and the second in mg/kg-bw/day.

**Table 2 Summary of the EDI Under the Intended Use of Glucosyl Hesperidin by Population Group, mg/person/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/d) | | All-users Intake (or consumers only, mg/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90$^{th}$ Pctl | % | n | Mean | 90$^{th}$ Pctl |
| Young children | 1-6 | 344.87±12.39 | 839.29±41.41 | 75.37 | 1,286 | 457.58±12.38 | 984.87±44.07 |
| Children | 7-12 | 517.85±19.26 | 1,146.09±45.21 | 82.42 | 1,439 | 628.32±18.41 | 1,257.40±58.96 |
| Total teenagers | 13-18 | 689.48±39.13 | 1,431.48±77.79 | 85.16 | 1,363 | 809.62±43.85 | 1,544.42±75.55 |
| Male teenagers | 13-18 | 830.57±68.50 | 1,732.51±155.41 | 87.07 | 676 | 953.94±75.30 | 1,891.15±166.13 |
| Female teenagers | 13-18 | 548.87±42.58 | 1,166.24±84.81 | 83.26 | 687 | 659.23±45.83 | 1,284.88±89.11 |
| Total adults | 19-99 | 691.49±17.58 | 1,666.67±54.68 | 78.08 | 6,893 | 885.60±19.27 | 1,840.73±52.00 |
| Male adults | 19-99 | 788.59±27.81 | 1,961.68±68.29 | 78.42 | 3,304 | 1,005.61±32.39 | 2,111.64±68.07 |
| Female adults | 19-99 | 596.94±17.82 | 1,441.03±44.31 | 77.75 | 3,589 | 767.73±19.67 | 1,564.44±35.82 |
| Total population | 1-99 | 651.00±15.82 | 1,544.72±41.69 | 78.83 | 10,981 | 825.86±17.56 | 1,725.33±47.47 |

Based on the 2013-2016 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; y = years.

**Table 3 Summary of the EDI Under the Intended Use of Glucosyl Hesperidin from Diet by Population Group, mg/kg-bw/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/kg-bw/d) | | All-users Intake (or consumers only, mg/kg-bw/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90th Pctl | % | n | Mean | 90th Pctl |
| Young children | 1-6 | 20.63±0.72 | 50.68±1.85 | 74.66 | 1,274 | 27.36±0.81 | 58.65±2.55 |
| Children | 7-12 | 14.57±0.57 | 33.09±1.37 | 81.68 | 1,432 | 17.69±0.56 | 36.01±1.92 |
| Total teenagers | 13-18 | 10.38±0.55 | 22.35±1.05 | 84.76 | 1,354 | 12.19±0.61 | 23.49±1.04 |
| Male teenagers | 13-18 | 11.76±0.89 | 23.49±1.46 | 87.05 | 675 | 13.51±0.98 | 25.10±1.74 |
| Female teenagers | 13-18 | 8.99±0.67 | 20.86±1.45 | 82.47 | 679 | 10.79±0.71 | 22.16±1.71 |
| Total adults | 19-99 | 8.55±0.21 | 19.96±0.42 | 77.51 | 6,842 | 10.95±0.24 | 22.26±0.78 |
| Male adults | 19-99 | 9.08±0.31 | 21.19±0.80 | 77.54 | 3,275 | 11.59±0.37 | 24.16±0.91 |
| Female adults | 19-99 | 8.04±0.26 | 19.02±0.42 | 77.48 | 3,567 | 10.33±0.29 | 20.72±0.63 |
| Total population | 1-99 | 10.11±0.21 | 23.73±0.48 | 78.25 | 10,902 | 12.82±0.21 | 26.49±0.51 |

Based on the 2013-2016 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percent

Tables 4 and 5 summarize the EDI under the intended use of hesperidin by the population groups; the first table presents the results of the mean of the population, as well as the 90$^{th}$ percentile, in mg/day and the second in mg/kg-bw/day.

**Table 4 Summary of the EDI Under the Intended Use of Hesperidin by Population Group, mg/person/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/d) | | All-users Intake (or consumers only, mg/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90$^{th}$ Pctl | % | n | Mean | 90$^{th}$ Pctl |
| Young children | 1-6 | 251.69 ± 9.04 | 612.51 ± 30.22 | 75.37 | 1,286 | 333.94 ± 9.03 | 718.75 ± 32.16 |
| Children | 7-12 | 377.92 ± 14.06 | 836.42 ± 33.00 | 82.42 | 1,439 | 458.55 ± 13.44 | 917.65 ± 43.03 |
| Total teenagers | 13-18 | 503.18 ± 28.56 | 1044.70 ± 56.77 | 85.16 | 1,363 | 590.86 ± 32.00 | 1127.12 ± 55.14 |
| Male teenagers | 13-18 | 606.15 ± 49.99 | 1264.39 ± 113.42 | 87.07 | 676 | 696.19 ± 54.96 | 1380.16 ± 121.24 |
| Female teenagers | 13-18 | 400.57 ± 31.07 | 851.13 ± 61.89 | 83.26 | 687 | 481.10 ± 33.45 | 937.71 ± 65.03 |
| Total adults | 19-99 | 504.65 ± 12.83 | 1,216.33 ± 39.91 | 78.08 | 6,893 | 646.31 ± 14.06 | 1,343.37 ± 38.16 |
| Male adults | 19-99 | 575.51 ± 20.29 | 1,431.63 ± 49.84 | 78.42 | 3,304 | 733.90 ± 23.64 | 1,541.07 ± 49.77 |
| Female adults | 19-99 | 435.65 ± 13.01 | 1,051.66 ± 32.25 | 77.75 | 3,589 | 560.29 ± 14.35 | 1,141.73 ± 26.14 |
| Total population | 1-99 | 475.10 ± 11.54 | 1,127.34 ± 30.43 | 78.83 | 10,981 | 602.71 ± 12.81 | 1,259.15 ± 34.64 |

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; y = years.

**Table 5 Summary of the EDI Under the Intended Use of Hesperidin by Population Group, mg/kg-bw/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/kg-bw/d) | | All-users Intake (or consumers only, mg/kg-bw/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90th Pctl | % | n | Mean | 90th Pctl |
| Young children | 1-6 | 14.91 ± 0.54 | 36.79 ± 1.33 | 74.66 | 1,274 | 19.97 ± 0.59 | 42.80 ± 1.86 |
| Children | 7-11 | 10.54 ± 0.41 | 24.05 ± 1.00 | 81.68 | 1,432 | 12.91 ± 0.41 | 26.28 ± 1.40 |
| Total teenagers | 13-18 | 7.54 ± 0.40 | 16.28 ± 0.75 | 84.76 | 1,354 | 8.89 ± 0.44 | 17.14 ± 0.76 |
| Male teenagers | 13-18 | 8.58 ± 0.65 | 17.14 ± 1.07 | 87.05 | 675 | 9.86 ± 0.71 | 18.32 ± 1.27 |
| Female teenagers | 13-18 | 6.50 ± 0.48 | 15.19 ± 1.05 | 82.47 | 679 | 7.88 ± 0.51 | 16.17 ± 1.25 |
| Total adults | 19-99 | 6.19 ± 0.16 | 14.52 ± 0.30 | 77.51 | 6,842 | 7.99 ± 0.18 | 16.25 ± 0.57 |
| Male adults | 19-99 | 6.56 ± 0.22 | 15.39 ± 0.57 | 77.54 | 3,275 | 8.46 ± 0.27 | 17.64 ± 0.67 |
| Female adults | 19-99 | 5.84 ± 0.19 | 13.86 ± 0.32 | 77.48 | 3,567 | 7.54 ± 0.21 | 15.12 ± 0.46 |
| Total population | 1-99 | 7.32 ± 0.15 | 17.21 ± 0.38 | 78.25 | 10,902 | 9.35 ± 0.15 | 19.33 ± 0.37 |

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; y = years.

Tables 6 and 7 summarize the EDIs of naturally occurring hesperetin from the diet sources: Table 6 presents the EDIs in mg/person/day and Table 7 shows the EDI values in mg/kg-bw/day. The NHANES flavonoid database reports hesperetin values; thus, the hesperetin from NHANES is divided by 0.495 to get EDIs for dietary hesperidin.

**Table 6 Summary of the EDI from Diet by Population Group, mg/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/d) | | All-users Intake (or consumers only, mg/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90th Pctl | % | n | Mean | 90th Pctl |
| Young children | 1-6 | 16.00 ± 1.60 | 52.84 ± 4.36 | 57.37 | 1,003 | 27.89 ± 2.08 | 67.70 ± 4.40 |
| Children | 7-12 | 16.46 ± 1.15 | 53.33 ± 3.60 | 58.03 | 1,047 | 28.36 ± 1.50 | 71.96 ± 5.01 |
| Total teenagers | 13-18 | 16.51 ± 1.34 | 54.15 ± 4.59 | 47.16 | 841 | 35.02 ± 2.27 | 81.58 ± 2.26 |
| Male teenagers | 13-18 | 21.06 ± 2.27 | 70.76 ± 6.26 | 46.33 | 400 | 45.46 ± 3.63 | 104.72 ± 7.73 |
| Female teenagers | 13-18 | 11.98 ± 0.88 | 38.47 ± 3.57 | 47.98 | 441 | 24.98 ± 1.40 | 60.13 ± 5.00 |
| Total adults | 19-99 | 16.10 ± 0.52 | 56.42 ± 1.48 | 56.31 | 5,015 | 28.59 ± 0.82 | 81.97 ± 3.03 |
| Male adults | 19-99 | 18.12 ± 0.81 | 64.26 ± 3.70 | 57.73 | 2,283 | 34.36 ± 1.53 | 91.17 ± 4.58 |
| Female adults | 19-99 | 14.14 ± 0.67 | 49.13 ± 2.88 | 59.79 | 2,732 | 23.65 ± 1.04 | 66.48 ± 4.08 |
| Total population | 1-99 | 16.16 ± 0.52 | 56.25 ± 1.46 | 55.75 | 7,906 | 28.98 ± 0.79 | 79.40 ± 2.49 |

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; y = years.

**Table 7 Summary of the EDI from Diet by Population Group, mg/kg-bw/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/kg-bw/d) | | All-users Intake (or consumers only, mg/kg-bw/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90th Pctl | % | n | Mean | 90th Pctl |
| Young children | 1-6 | 0.96 ± 0.09 | 3.08 ± 0.20 | 56.96 | 996 | 1.69 ± 0.12 | 4.14 ± 0.25 |
| Children | 7-11 | 0.47 ± 0.03 | 1.52 ± 0.12 | 57.85 | 1043 | 0.81 ± 0.04 | 2.01 ± 0.17 |
| Total teenagers | 13-18 | 0.26 ± 0.02 | 0.79 ± 0.07 | 46.88 | 836 | 0.56 ± 0.04 | 1.39 ± 0.11 |
| Male teenagers | 13-18 | 0.33 ± 0.04 | 1.01 ± 0.13 | 46.33 | 400 | 0.71 ± 0.06 | 1.64 ± 0.14 |
| Female teenagers | 13-18 | 0.20 ± 0.01 | 0.63 ± 0.06 | 47.44 | 436 | 0.41 ± 0.02 | 1.06 ± 0.06 |
| Total adults | 19-99 | 0.21 ± 0.01 | 0.71 ± 0.03 | 55.85 | 4979 | 0.37 ± 0.01 | 1.03 ± 0.05 |
| Male adults | 19-99 | 0.22 ± 0.01 | 0.74 ± 0.04 | 52.06 | 2265 | 0.41 ± 0.02 | 1.14 ± 0.07 |
| Female adults | 19-99 | 0.20 ± 0.01 | 0.69 ± 0.03 | 59.55 | 2714 | 0.34 ± 0.02 | 0.93 ± 0.05 |
| Total population | 1-99 | 0.29 ± 0.01 | 0.89 ± 0.04 | 55.34 | 7854 | 0.52 ± 0.02 | 1.36 ± 0.05 |

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; y = years.

Tables 8 and 9 present the combined EDIs of hesperidin under the intended use and from the diet sources: Table 8 presents the EDIs in mg/person/day and Table 9 shows the EDI values in mg/kg-bw/day.

**Table 8 Summary of the EDI of Hesperidin Under the Intended Use and Diet by Population Group, mg/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/d) | | All-users Intake (or consumers only, mg/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90th Pctl | % | n | Mean | 90th Pctl |
| Young children | 1-6 | 267.69 ± 10.01 | 642.60 ± 33.23 | 86.77 | 1,476 | 308.52 ± 9.04 | 693.36 ± 29.93 |
| Children | 7-12 | 394.38 ± 14.12 | 852.59 ± 32.13 | 90.17 | 1,565 | 437.39 ± 12.55 | 885.43 ± 41.36 |
| Total teenagers | 13-18 | 519.69 ± 28.43 | 1080.58 ± 58.91 | 91.15 | 1,488 | 570.18 ± 31.73 | 1,108.96 ± 49.29 |
| Male teenagers | 13-18 | 627.21 ± 49.60 | 1315.53 ± 104.12 | 92.60 | 739 | 677.34 ± 54.25 | 1,330.92 ± 117.63 |
| Female teenagers | 13-18 | 412.55 ± 30.96 | 869.43 ± 65.44 | 89.70 | 749 | 459.94 ± 32.81 | 906.12 ± 64.33 |
| Total adults | 19-99 | 520.75 ± 12.68 | 1,224.45 ± 36.81 | 90.36 | 7,933 | 576.28 ± 14.48 | 1,293.18 ± 38.81 |
| Male adults | 19-99 | 593.63 ± 20.33 | 1,445.61 ± 53.15 | 89.58 | 3,780 | 662.66 ± 23.67 | 1,492.83 ± 56.10 |
| Female adults | 19-99 | 449.78 ± 12.88 | 1,060.28 ± 33.43 | 91.12 | 4,153 | 493.59 ± 13.70 | 1,095.49 ± 33.33 |
| Total population | 1-99 | 491.25 ± 11.39 | 1,146.54 ± 30.28 | 90.14 | 12,462 | 544.98 ± 12.96 | 1,202.26 ± 36.56 |

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; y = years.

**Table 9 Summary of the EDI of Hesperidin Under the Intended Use and Diet by Population Group, mg/kg-bw/d**

| Population Group | Age Group (y) | All-person (or per capita) Intake (mg/kg-bw/d) | | All-users Intake (or consumers only, mg/kg-bw/d) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90th Pctl | % | n | Mean | 90th Pctl |
| Young children | 1-6 | 15.87 ± 0.59 | 37.98 ± 1.40 | 86.04 | 1,463 | 18.45 ± 0.55 | 39.84 ± 1.52 |
| Children | 7-11 | 11.01 ± 0.41 | 25.15 ± 0.99 | 89.42 | 1,557 | 12.31 ± 0.39 | 26.06 ± 1.23 |
| Total teenagers | 13-18 | 7.80 ± 0.40 | 16.49 ± 0.77 | 90.68 | 1,478 | 8.60 ± 0.44 | 17.14 ± 0.70 |
| Male teenagers | 13-18 | 8.91 ± 0.64 | 17.31 ± 1.16 | 92.58 | 738 | 9.63 ± 0.70 | 18.08 ± 1.22 |
| Female teenagers | 13-18 | 6.69 ± 0.48 | 15.28 ± 1.11 | 88.78 | 740 | 7.54 ± 0.51 | 15.85 ± 1.21 |
| Total adults | 19-99 | 6.40 ± 0.15 | 14.84 ± 0.27 | 89.65 | 7,871 | 7.14 ± 0.18 | 15.43 ± 0.36 |
| Male adults | 19-99 | 6.77 ± 0.22 | 15.75 ± 0.55 | 88.54 | 3,747 | 7.65 ± 0.26 | 16.86 ± 0.64 |
| Female adults | 19-99 | 6.04 ± 0.19 | 14.02 ± 0.34 | 90.73 | 4,124 | 6.66 ± 0.20 | 14.41 ± 0.34 |
| Total population | 1-99 | 7.61 ± 0.15 | 17.56 ± 0.32 | 89.44 | 12,369 | 8.51 ± 0.16 | 18.38 ± 0.38 |

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; y = years.

## Appendix: Food Codes Under the Intended Use

| Food Code | Food Description | RACC | Consumers | Consumptions | Intended use group |
|---|---|---|---|---|---|
| 11400000 | Yogurt, NFS | 170 | 8 | 8 | 1 |
| 11400010 | Yogurt, Greek, NS as to type of milk or flavor | 170 | 1 | 1 | 1 |
| 11410000 | Yogurt, NS as to type of milk or flavor | 170 | 15 | 15 | 1 |
| 11411010 | Yogurt, NS as to type of milk, plain | 170 | 25 | 29 | 1 |
| 11411100 | Yogurt, whole milk, plain | 170 | 46 | 66 | 1 |
| 11411200 | Yogurt, low fat milk, plain | 170 | 66 | 78 | 1 |
| 11411300 | Yogurt, nonfat milk, plain | 170 | 21 | 27 | 1 |
| 11411390 | Yogurt, Greek, NS as to type of milk, plain | 170 | 6 | 7 | 1 |
| 11411400 | Yogurt, Greek, whole milk, plain | 170 | 13 | 15 | 1 |
| 11411410 | Yogurt, Greek, low fat milk, plain | 170 | 63 | 75 | 1 |
| 11411420 | Yogurt, Greek, nonfat milk, plain | 170 | 36 | 40 | 1 |
| 11420000 | Yogurt, vanilla, NS as to type of milk | 170 | 22 | 23 | 1 |
| 11421000 | Yogurt, vanilla, whole milk | 170 | 24 | 26 | 1 |
| 11422000 | Yogurt, vanilla, low fat milk | 170 | 67 | 84 | 1 |
| 11422100 | Yogurt, vanilla, low fat milk, light | 170 | 24 | 26 | 1 |
| 11423000 | Yogurt, vanilla, nonfat milk | 170 | 18 | 21 | 1 |
| 11424000 | Yogurt, vanilla, nonfat milk, light | 170 | 5 | 5 | 1 |
| 11424500 | Yogurt, Greek, vanilla, whole milk | 170 | 8 | 9 | 1 |
| 11424510 | Yogurt, Greek, vanilla, low fat | 170 | 33 | 35 | 1 |
| 11424520 | Yogurt, Greek, vanilla, nonfat | 170 | 23 | 25 | 1 |
| 11430000 | Yogurt, NS as to type of milk, fruit | 170 | 152 | 170 | 1 |
| 11431000 | Yogurt, whole milk, fruit | 170 | 146 | 161 | 1 |
| 11432000 | Yogurt, low fat milk, fruit | 170 | 566 | 706 | 1 |
| 11432500 | Yogurt, fruit, low fat milk, light | 170 | 86 | 103 | 1 |
| 11433000 | Yogurt, nonfat milk, fruit | 170 | 103 | 114 | 1 |
| 11433500 | Yogurt, fruit, nonfat milk, light | 170 | 28 | 30 | 1 |
| 11433990 | Yogurt, Greek, NS as to type of milk, fruit | 170 | 29 | 32 | 1 |
| 11434000 | Yogurt, Greek, whole milk, fruit | 170 | 32 | 37 | 1 |
| 11434010 | Yogurt, Greek, low fat milk, fruit | 170 | 169 | 199 | 1 |
| 11434020 | Yogurt, Greek, nonfat milk, fruit | 170 | 142 | 154 | 1 |

| | | | | | |
|---|---|---|---|---|---|
| 11434090 | Yogurt, NS as to type of milk, flavors other than fruit | 170 | 20 | 26 | 1 |
| 11434100 | Yogurt, whole milk, flavors other than fruit | 170 | 15 | 15 | 1 |
| 11434200 | Yogurt, low fat milk, flavors other than fruit | 170 | 53 | 62 | 1 |
| 11434300 | Yogurt, nonfat milk, flavors other than fruit | 170 | 23 | 26 | 1 |
| 11435000 | Yogurt, Greek, NS as to type of milk, flavors other than fruit | 170 | 20 | 22 | 1 |
| 11435010 | Yogurt, Greek, whole milk, flavors other than fruit | 170 | 6 | 6 | 1 |
| 11435020 | Yogurt, Greek, low fat milk, flavors other than fruit | 170 | 29 | 33 | 1 |
| 11435030 | Yogurt, Greek, nonfat milk, flavors other than fruit | 170 | 25 | 29 | 1 |
| 91361020 | Milk chocolate candy, plain | 30 | 494 | 560 | 2 |
| 91404000 | Chocolate, sweet or dark | 30 | 142 | 167 | 2 |
| 91708000 | Gumdrops | 30 | 385 | 438 | 2 |
| 91708010 | Hard candy | 15 | 896 | 1,071 | 2 |
| 91745010 | Coffee, NS as to type | 360 | 3 | 3 | 3 |
| 91745020 | Coffee, NS as to brewed or instant | 360 | 15 | 15 | 3 |
| 92100000 | Coffee, instant, reconstituted | 360 | 817 | 1,422 | 3 |
| 92100500 | Coffee, instant, 50% less caffeine, reconstituted | 360 | 3 | 5 | 3 |
| 92103000 | Coffee, NS as to brewed or instant, decaffeinated | 360 | 1 | 1 | 3 |
| 92104000 | Coffee, brewed, decaffeinated | 360 | 452 | 750 | 3 |
| 92111000 | Coffee, instant, decaffeinated, reconstituted | 360 | 213 | 342 | 3 |
| 92111010 | Coffee, bottled/canned | 360 | 61 | 66 | 3 |
| 92114000 | Coffee, bottled/canned, light | 360 | 6 | 6 | 3 |
| 92171000 | Coffee, instant, not reconstituted | 1.5 | 31 | 41 | 3 |
| 92171010 | Coffee, instant, decaffeinated, not reconstituted | 1.5 | 7 | 8 | 3 |
| 92191100 | Tea, iced, instant, black, unsweetened | 360 | 134 | 187 | 3 |
| 92191200 | Tea, iced, instant, black, pre-sweetened with sugar | 360 | 206 | 283 | 3 |
| 92305010 | Tea, iced, instant, black, decaffeinated, pre-sweetened with sugar | 360 | 12 | 14 | 3 |
| 92305040 | Tea, iced, instant, black, pre-sweetened with low calorie sweetener | 360 | 49 | 66 | 3 |
| 92305050 | Tea, iced, instant, black, decaffeinated, pre-sweetened with low calorie sweetener | 360 | 12 | 18 | 3 |
| 92305090 | Tea, iced, instant, black, decaffeinated, unsweetened | 360 | 18 | 21 | 3 |
| 92305110 | Tea, iced, instant, green, unsweetened | 360 | 26 | 33 | 3 |
| 92305180 | Tea, iced, instant, green, pre-sweetened with sugar | 360 | 34 | 52 | 3 |
| 92305900 | Tea, iced, instant, green, pre-sweetened with low calorie sweetener | 360 | 17 | 24 | 3 |
| 92305910 | Tea, hot, herbal | 360 | 359 | 546 | 3 |
| 92305920 | Tea, iced, instant, black, unsweetened, dry | 1.4 | 3 | 4 | 3 |

| | | | | | |
|---|---|---|---|---|---|
| 92306000 | Tea, iced, instant, black, pre-sweetened, dry | 2 | 3 | 3 | 3 |
| 92307000 | Iced Tea / Lemonade juice drink | 360 | 34 | 43 | 3 |
| 92307400 | Iced Tea / Lemonade juice drink, light | 360 | 7 | 9 | 3 |
| 92307500 | Iced Tea / Lemonade juice drink, diet | 360 | 9 | 9 | 3 |
| 92307510 | Tea, iced, bottled, black | 360 | 639 | 901 | 3 |
| 92307520 | Tea, iced, bottled, black, decaffeinated | 360 | 23 | 27 | 3 |
| 92309000 | Tea, iced, bottled, black, diet | 360 | 77 | 116 | 3 |
| 92309010 | Tea, iced, bottled, black, decaffeinated, diet | 360 | 11 | 25 | 3 |
| 92309020 | Tea, iced, bottled, black, unsweetened | 360 | 17 | 27 | 3 |
| 92309030 | Tea, iced, bottled, black, decaffeinated, unsweetened | 360 | 2 | 3 | 3 |
| 92309040 | Tea, iced, bottled, green | 360 | 196 | 285 | 3 |
| 92309050 | Tea, iced, bottled, green, diet | 360 | 74 | 116 | 3 |
| 92309500 | Tea, iced, bottled, green, unsweetened | 360 | 3 | 3 | 3 |
| 92309510 | Soft drink, NFS | 360 | 22 | 22 | 4 |
| 92309520 | Soft drink, NFS, diet | 360 | 7 | 9 | 4 |
| 92400000 | Carbonated water, sweetened | 360 | 30 | 39 | 4 |
| 92400100 | Carbonated water, sweetened, with low-calorie or no-calorie sweetener | 360 | 29 | 39 | 4 |
| 92410110 | Soft drink, cola | 360 | 2,542 | 4,316 | 4 |
| 92410250 | Soft drink, pepper type | 360 | 618 | 921 | 4 |
| 92410310 | Soft drink, pepper type, diet | 360 | 105 | 196 | 4 |
| 92410360 | Soft drink, pepper type, decaffeinated | 360 | 46 | 55 | 4 |
| 92410370 | Soft drink, pepper type, decaffeinated, diet | 360 | 22 | 37 | 4 |
| 92410390 | Soft drink, fruit flavored, caffeine free | 360 | 1,934 | 2,717 | 4 |
| 92410400 | Soft drink, fruit flavored, diet, caffeine free | 360 | 217 | 305 | 4 |
| 92410510 | Soft drink, fruit flavored, caffeine containing | 360 | 634 | 979 | 4 |
| 92410520 | Soft drink, fruit flavored, caffeine containing, diet | 360 | 96 | 175 | 4 |
| 92410550 | Soft drink, ginger ale | 360 | 221 | 282 | 4 |
| 92410560 | Soft drink, ginger ale, diet | 360 | 42 | 55 | 4 |
| 92410610 | Soft drink, root beer | 360 | 366 | 475 | 4 |
| 92410620 | Fruit juice drink, citrus, carbonated | 240 | 3 | 3 | 4 |
| 92410710 | Fruit juice drink, noncitrus, carbonated | 240 | 13 | 17 | 4 |
| 92432000 | Fruit juice drink | 240 | 252 | 347 | 5 |
| 92510610 | Lemonade, fruit juice drink | 240 | 427 | 540 | 5 |
| 92510730 | Lemonade, fruit flavored drink | 240 | 439 | 507 | 5 |
| 92510955 | Fruit flavored drink | 240 | 133 | 191 | 5 |

| | | | | | |
|---|---|---|---|---|---|
| 92511015 | Fruit flavored drink, with high vitamin C | 240 | 188 | 276 | 5 |
| 92512040 | Cranberry juice drink, with high vitamin C | 240 | 123 | 150 | 5 |
| 92530410 | Fruit juice drink, with high vitamin C | 240 | 1,157 | 1,615 | 5 |
| 92530510 | Vegetable and fruit juice drink, with high vitamin C | 240 | 52 | 78 | 5 |
| 92530610 | Fruit juice drink (Sunny D) | 240 | 174 | 228 | 5 |
| 92530950 | Fruit flavored drink, powdered, reconstituted | 240 | 577 | 903 | 5 |
| 92531030 | Fruit flavored drink, with high vitamin C, powdered, reconstituted | 240 | 39 | 55 | 5 |
| 92541010 | Fruit juice drink, with high vitamin C, light | 240 | 65 | 90 | 5 |
| 92542000 | Fruit juice drink, light | 240 | 56 | 68 | 5 |
| 92550030 | Fruit juice drink, diet | 240 | 21 | 30 | 5 |
| 92550035 | Cranberry juice drink, with high vitamin C, light | 240 | 12 | 17 | 5 |
| 92550040 | Orange juice beverage, 40-50% juice, light | 240 | 21 | 24 | 5 |
| 92550110 | Lemonade, fruit juice drink, light | 240 | 19 | 22 | 5 |
| 92550350 | Pomegranate juice beverage, 40-50% juice, light | 240 | 1 | 1 | 5 |
| 92550370 | Vegetable and fruit juice drink, with high vitamin C, diet | 240 | 13 | 16 | 5 |
| 92550380 | Vegetable and fruit juice drink, with high vitamin C, light | 240 | 14 | 17 | 5 |
| 92550400 | Fruit flavored drink, with high vitamin C, diet | 240 | 14 | 16 | 5 |
| 92550405 | Fruit flavored drink, diet | 240 | 67 | 79 | 5 |
| 92550610 | Fruit flavored drink, with high vitamin C, powdered, reconstituted, diet | 240 | 14 | 20 | 5 |
| 92550620 | Fruit flavored drink, powdered, reconstituted, diet | 240 | 291 | 456 | 5 |
| 92552000 | Fruit juice drink, reduced sugar (Sunny D) | 240 | 8 | 10 | 5 |
| 92552010 | Fruit juice drink (Capri Sun) | 240 | 325 | 452 | 5 |
| 92552020 | Fruit juice drink, with high vitamin C, plus added calcium | 240 | 1 | 1 | 5 |
| 92552030 | Fruit juice drink, added calcium (Sunny D) | 240 | 80 | 96 | 5 |
| 92582110 | Fruit flavored drink, powdered, not reconstituted | 25 | 4 | 8 | 6 |
| 92804000 | Fruit flavored drink, powdered, not reconstituted, diet | 16 | 16 | 27 | 6 |
| 93404560 | Water, bottled, sweetened, with low calorie sweetener | 360 | 112 | 168 | 7 |
| 93504100 | Water, bottled, flavored (Capri Sun Roarin' Waters) | 360 | 28 | 44 | 7 |
| 94100200 | Water, bottled, flavored (Propel Water) | 360 | 14 | 16 | 8 |
| 94100300 | Water, bottled, flavored (Glaceau Vitamin Water) | 360 | 79 | 103 | 8 |
| 94210100 | Water, bottled, flavored (SoBe Life Water) | 360 | 5 | 5 | 8 |
| 94210200 | Propel Zero Water | 360 | 5 | 8 | 8 |
| 94210300 | Water, bottled, flavored, sugar free (Glaceau Vitamin Water) | 360 | 28 | 35 | 8 |
| 94220100 | Water, bottled, flavored, sugar free (SoBe) | 360 | 9 | 13 | 8 |
| 94220215 | Nutritional drink or shake, ready-to-drink (Boost) | 240 | 17 | 21 | 9 |

| | | | | | |
|---|---|---|---|---|---|
| 94220310 | Nutritional drink or shake, ready-to-drink (Boost Plus) | 240 | 7 | 16 | 9 |
| 95101000 | Nutritional drink or shake, ready-to-drink (Carnation Instant Breakfast) | 240 | 3 | 4 | 9 |
| 95101010 | Nutritional drink or shake, ready-to-drink (Ensure) | 240 | 62 | 111 | 9 |
| 95102000 | Nutritional drink or shake, ready-to-drink (Ensure Plus) | 240 | 2 | 5 | 9 |
| 95103000 | Nutritional drink or shake, ready-to-drink, sugar free (Glucerna) | 240 | 22 | 31 | 9 |
| 95103010 | Nutritional drink or shake, ready-to-drink (Kellogg's Special K Protein) | 240 | 9 | 10 | 9 |
| 95104000 | Nutritional drink or shake, ready-to-drink (Muscle Milk) | 240 | 12 | 14 | 9 |
| 95105000 | Nutritional drink or shake, ready-to-drink, light (Muscle Milk) | 240 | 3 | 4 | 9 |
| 95106000 | Nutritional drink or shake, ready-to-drink (Slim Fast) | 240 | 16 | 20 | 9 |
| 95106010 | Nutritional drink or shake, ready-to-drink, sugar free (Slim Fast) | 240 | 8 | 10 | 9 |
| 95110000 | Nutritional drink or shake, high protein, ready-to-drink (Slim Fast) | 240 | 9 | 10 | 9 |
| 95110010 | Nutritional drink or shake, ready-to-drink, NFS | 240 | 16 | 24 | 9 |
| 95110020 | Nutritional drink or shake, high protein, ready-to-drink, NFS | 240 | 37 | 55 | 9 |
| 95120000 | Nutritional drink or shake, high protein, light, ready-to-drink, NFS | 240 | 20 | 29 | 9 |
| 95120010 | Nutritional drink or shake, liquid, soy-based | 240 | 2 | 4 | 9 |
| 95120020 | Nutritional powder mix (Carnation Instant Breakfast) | 33 | 29 | 40 | 10 |
| 95120050 | Nutritional powder mix, sugar free (Carnation Instant Breakfast) | 33 | 4 | 4 | 10 |
| 95201000 | Nutritional powder mix (EAS Whey Protein Powder) | 33 | 5 | 5 | 10 |
| 95201010 | Nutritional powder mix (EAS Soy Protein Powder) | 33 | 1 | 1 | 10 |
| 95201200 | Nutritional powder mix, high protein (Herbalife) | 33 | 21 | 41 | 10 |
| 95201300 | Nutritional powder mix (Isopure) | 33 | 1 | 1 | 10 |
| 95201500 | Nutritional powder mix (Kellogg's Special K20 Protein Water) | 33 | 1 | 1 | 10 |
| 95201600 | Nutritional powder mix (Muscle Milk) | 33 | 14 | 16 | 10 |
| 95201700 | Nutritional powder mix (Slim Fast) | 33 | 7 | 12 | 10 |
| 95202000 | Nutritional powder mix, high protein (Slim Fast) | 33 | 2 | 2 | 10 |
| 95210000 | Nutritional powder mix, NFS | 33 | 4 | 4 | 10 |
| 95210020 | Nutritional powder mix, high protein, NFS | 33 | 40 | 60 | 10 |
| 95220000 | Nutritional powder mix, whey based, NFS | 33 | 66 | 84 | 10 |
| 95220010 | Nutritional powder mix, protein, soy based, NFS | 33 | 13 | 20 | 10 |
| 95230000 | Nutritional powder mix, protein, light, NFS | 33 | 20 | 33 | 10 |
| 95230010 | Nutritional powder mix, protein, NFS | 33 | 89 | 116 | 10 |
| 95230020 | Energy drink (Full Throttle) | 360 | 6 | 7 | 11 |
| 95230030 | Energy drink (Monster) | 360 | 77 | 108 | 11 |
| 95310200 | Energy drink (Mountain Dew AMP) | 360 | 1 | 4 | 11 |
| 95310400 | Energy drink (NOS) | 360 | 9 | 10 | 11 |

| | | | | | |
|---|---|---|---|---|---|
| 95310500 | Energy drink (Red Bull) | 360 | 66 | 90 | 11 |
| 95310560 | Energy drink (Rockstar) | 360 | 15 | 18 | 11 |
| 95310600 | Energy drink (SoBe Energize Energy Juice Drink) | 360 | 5 | 7 | 11 |
| 95310700 | Energy Drink | 360 | 16 | 21 | 11 |
| 95310750 | Energy drink, low calorie (Monster) | 360 | 24 | 36 | 11 |
| 95311000 | Energy drink, sugar free (Mountain Dew AMP) | 360 | 2 | 3 | 11 |
| 95312400 | Energy drink (Ocean Spray Cran-Energy Juice Drink) | 360 | 5 | 6 | 11 |
| 95312500 | Energy drink, sugar-free (Red Bull) | 360 | 16 | 23 | 11 |
| 95312560 | Energy drink, sugar free (Rockstar) | 360 | 13 | 18 | 11 |
| 95312600 | Energy drink (XS) | 360 | 3 | 4 | 11 |
| 95312700 | Energy drink, sugar free | 360 | 23 | 36 | 11 |
| 95312900 | Sports drink (Gatorade G) | 360 | 671 | 937 | 11 |
| 95313200 | Sports drink (Powerade) | 360 | 226 | 287 | 11 |
| 95320200 | Sports drink, NFS | 360 | 7 | 11 | 11 |
| 95320500 | Sports drink, low calorie (Gatorade G2) | 360 | 81 | 107 | 11 |
| 95321000 | Sports drink, low calorie (Powerade Zero) | 360 | 33 | 40 | 11 |
| 95322200 | Sports drink, low calorie | 360 | 7 | 7 | 11 |
| 95322500 | Fluid replacement, electrolyte solution | 360 | 19 | 25 | 11 |
| 95323000 | FUZE Slenderize fortified low calorie fruit juice beverage | 360 | 2 | 2 | 11 |
| 95330100 | Fruit juice, acai blend | 360 | 3 | 6 | 11 |

## 7.2 Appendix 2

**GRAS Panel Letter (2019**

01 December 2019

Alan B. Richards, PhD
Vanguard Regulatory Services, Inc.
1311 Iris Circle
Broomfield, CO 80020

Re: Safety of Glucosyl Herperidin (GH)

Dear Dr. Richards:

Greetings.

Hayashibara convened a panel of independent scientific experts ("Expert Panel," now GRAS Panel), qualified by their scientific training and experience and their national and international recognition as experts in the assessment of the safety of food ingredients and food, to conduct a GRAS determination of the proposed uses of glucosyl hesperidin (GH). The Panel independently and collectively critically evaluated publicly available information and other information deemed appropriate for the safety assessment of GH, much of which is included in the GRAS Notice (GRASN). The GRAS Panel convened by teleconference. The GRAS Panel unanimously concluded that the proposed uses of Hayashibara's GH, manufactured consistent with cGMP and meeting the food grade specifications presented by Hayashibara, are GRAS based on scientific procedures (cf. Expert Panel Report on the GRAS Status of Glucosyl Hesperidin, 15 Dec 2009).

Hayashibara has now requested the Expert (GRAS) Panel evaluate changes in the manufacture, raw materials **(cf. Table 1)** and the specifications **(cf. Table 2)** of the currently produced GH product and to determine if these changes are substantive and would require another critical evaluation of all available information of the proposed uses of the currently produced GH to determine if the proposed uses are GRAS based on scientific procedures (i.e. another GRAS determination). Or, if, in the opinion of the Expert (GRAS) Panel, the changes are not substantive and the proposed uses remain GRAS based on scientific procedures, would the Expert (GRAS) Panel prepare a letter stating their opinion.

We very carefully considered this request, critically evaluated the changes in the context of the proposed GRASN and other documents deemed appropriate (the results of an updated comprehensive search of the published literature including a recent publication on the safety

of GH) and discussed our findings in a teleconference. We unanimously conclude that the proposed changes are not substantive and the proposed uses of the currently produced GH are GRAS based on scientific procedures.

[redacted]

Professor Joseph F. Borzelleca, Ph.D., F.A.T.S., Chairman
Virginia Commonwealth University School of Medicine

01 December 2019
Date

[redacted]

Professor I. Glenn Sipes, Ph.D., F.A.T.S., F.A.A.A.S.
University of Arizona School of Medicine

04 December 2019
Date

[redacted]

Professor John A. Thomas, Ph.D., F.A.T.S., D.A.C.T.
Indiana University School of Medicine

03 December 2019
Date

**Table 1. Original Raw Materials List with Added and Modified Raw Materials**

| **Raw Materials in Original GRAS Notice** | | |
|---|---|---|
| Hesperidin | Dextrin | Cyclodextrin Glucanotransferase (CGTase; EC 2.4.1.19)[1] |
| Glucoamylase (EC 3.2.1.3)[2] | Ascorbic Acid | Sodium Hydroxide Solution |
| Magnesium Chloride | Hydrochloric Acid | Sodium Chloride |
| Ethanol | Activated Carbon | Diatomaceous Earth |
| Perlite | Ion Exchange Resins | Adsorption Separation Resin |
| **Raw Materials Added to the Updated GRAS Notice** | | |
| Sulfuric Acid | Sodium Pyrosulfite | Powdered Cellulose |
| **Modifications to the original Raw Materials** | | |
| [1] Cyclodextrin Glucanotransferase (CGTase; EC 2.4.1.19) from *Geobacillus stearothermophilus* | | |
| [2] Glucoamylase (EC 3.2.1.3) from *Rhizopus oryzae* | | |

The added raw materials, as well as the other processing aids, are removed during purification. Sodium pyrosulfite was assayed in the finished product and found to be less than 0.003g/kg, which is below the limit of detection used.

**Table 2. The Current Glucosyl Hesperidin Specifications and Original Specifications**

| Variable | Specification | Specification in Original GRAS Notice |
|---|---|---|
| Description | A pale yellow to yellow-brown powder having a slight characteristic odor. | (Appearance) Light yellow to yellowish brown powder with slight characteristic odor. |
| Identification (1) | A brown color develops. | The solution turns into brown. |
| Identification (2) | Exhibits a peak at the position corresponding to monoglucosyl hesperidin, having an absorption maximum at a wavelength of 280-286. | Identify the maximum absorption at 280 – 286 nm. |
| Monoglucosyl hesperidin (d.b.) | 75.0 – 85.0% | Not less than 70% (on the dried base) |
| Total hesperidin (d.b.) | Note less than 70% | Not less than 70% |
| Loss on drying | Not more than 6.0% | Not more than 6.0% |
| Residue on ignition | Not more than 2.0% | Not more than 2.0% |
| pH | 5.0 – 7.0 | 5.0 – 7.0 |
| Lead (as Pb) | Not more than 0.1 µg/g | Not more than 0.1 µg/g |
| Arsenic (as As) | Not more than 1.5 µg/g | (There was no Arsenic specification) |
| Total aerobic microbial count | Not more than 300 CFU/g | (Viable count) Not more than 300 CFU/g |
| Coliform organisms | Negative/0.1g | Negative/0.1g |

Specifications names written in (parentheses) means the name of the specification was changed.