GRAS Notice (GRN) No. 977 with amendments
https://www.fda.gov/food/generally-recognized-safe-gras/gras-notice-inventory

GRAS Notification for D-tagatose Produced by a Novel Enzymatic Cascade

SUBMITTED BY:
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ON BEHALF OF:

Bonumose LLC
1725 Discovery Drive, Suite 220
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SUBMITTED TO:
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
HFS-200
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I. Signed statements and certification

I.A. Submission of GRAS notice

In accordance with 21 CFR § 170 Subpart E, Bonumose claims that D-tagatose, produced from a novel enzymatic cascade, is Generally Recognized As Safe (GRAS) for use in foods and therefore exempt from pre-market approval requirements of the Food, Drug and Cosmetic Act.

I.B. Name and address of the notifier

Bonumose LLC
1725 Discovery Drive, Suite 220
Charlottesville, VA 22911

I.C. Name of the GRAS substance

Name: D-tagatose

I.D. Intended use and consumer exposure

D-tagatose will be used as a tabletop sweetener and as an ingredient in foods and beverages (including but not limited to those categories specified on p.000022 of GRN 78 and p.000004 of GRN 352), other than infant formulas and meat and poultry products. Its primary function will be as a nutritive sweetener, but it can also be used as a flavor enhancer, humectant, texturizer and stabilizer. D-tagatose will be used as a partial or full replacement for other nutritive sweeteners such as sugar, corn syrup, high-fructose corn syrup and polyols. Levels of intended use will vary between food categories depending on the amount and type of sweeteners that are to be replaced. D-tagatose use will not exceed the amount reasonably required to accomplish its intended function in foods.

The population expected to consume D-tagatose consists of members of the general population.

I.E. Basis for the GRAS determination

Bonumose determined the GRAS conclusion of D-tagatose through scientific procedures in accordance with 21 CFR §170.30(a) and (b).

I.F. Availability of information

The data and information that serve as the basis for the GRAS conclusion are appended to this Notice. Questions or requests for additional information may be directed to: Bonumose LLC, 1725 Discovery Drive, Suite 220, Charlottesville, VA 22911 [contact: Karen Weikel (Director of Nutrition Science), telephone 631-678-7720; email: kweikel@bonumose.com].

1.G. Applicability of FOIA exemption

None of the data and information in Parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. §552.

1.H. Certification

We certify that, to the best of our knowledge, our GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, available and pertinent to the evaluation of the safety and GRAS status of the use of D-tagatose.

Karen Weikel Digitally signed by Karen Weikel Date: 2020.10.26 15:24:58 -04'00'

Karen Weikel, PhD
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II. Identity, method of manufacture, specifications, and physical or technical effect of the notified substance

II.A. Name

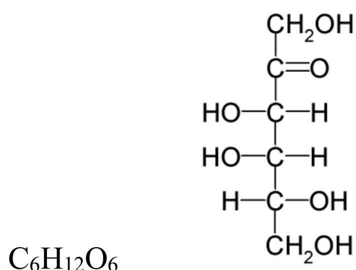
D-Tagatose. Synonyms include tagatose, D-lyxo-hexulose, α-D-tagatose, pseudo-fructose.

II.B. CAS registry number

87-81-0

II.C. Molecular and structural formula

$C_6H_{12}O_6$



II.D. Method of manufacture

Crystalline D-tagatose is manufactured from food-grade maltodextrin by an enzymatic cascade including novel enzymes immobilized in a column. The enzymatic cascade is described in more detail in Section II.E. Impurities such as solids, salts, residual enzyme, sodium sulfite and potential microorganism contaminants are removed by filtration and ion exchange (Figure 1). A frame evaporator then increases the density to 60°Bx and the syrup is purified by simulated moving-bed (SMB) affinity chromatography which removes residual sugars. A second round of evaporation increases the density further to 75°Bx. D-tagatose syrup is then pumped into a crystallizer and spray-dried using a filter dryer before collection of D-tagatose crystals. During crystallization, less pure fractions of the syrup form the mother liquor which is recycled back into SMB chromatography for further extraction of D-tagatose.

Bonumose's D-tagatose is manufactured under Good Manufacturing Practice (cGMP) using food-grade materials and processes in accordance with the applicable parts of 21 CFR §110 and 117. Process tanks and lines are cleaned with hot water and dilute sodium hydroxide if necessary, following standard procedures common to the sugar industry. Ion exchange resins used during manufacturing are food-grade and comply with 21 CFR §173.25. All processing and

filtering aids used in the manufacturing process are considered safe and suitable. Any preservatives that may be used in production are either GRAS for this intended use or comply with 21 CFR § 172, 182. In-process and final product testing are conducted to ensure the safety and quality of the final product.

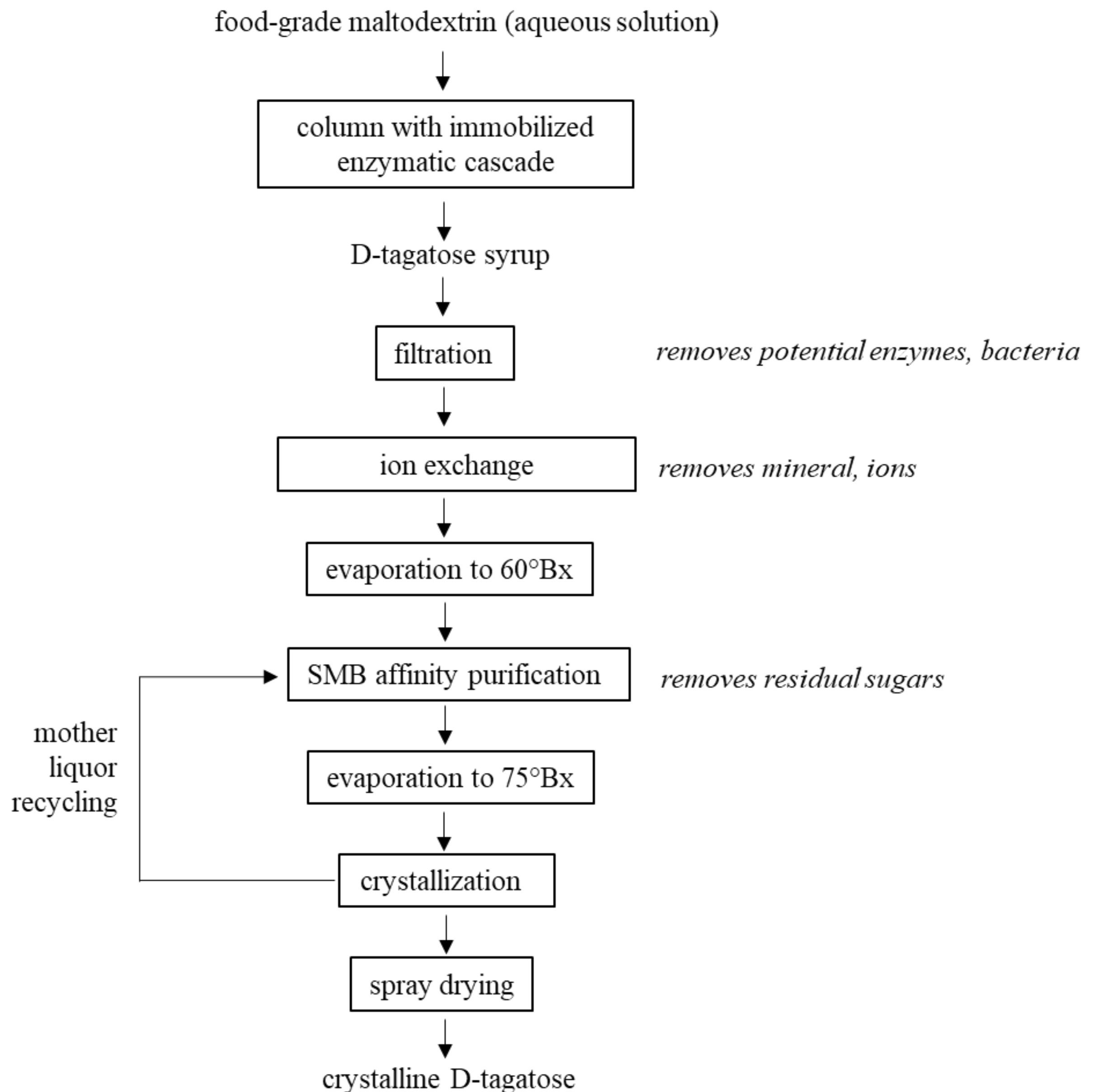


Figure 1. Manufacturing process for D-tagatose. *Italics* indicate where various potential contaminants are removed from the D-tagatose syrup throughout processing.

II.E. Enzymes used in the manufacture of D-tagatose

Enzymes have a long history of use in food production (Fernandes 2010). Similar to previous D-tagatose notices (GRN 78 and GRN 352), this notice describes the manufacture of D-tagatose using immobilized enzymes that do not appear in the final D-tagatose product. Unlike previous D-tagatose notices, some of the enzymes used in Bonumose's manufacture of D-tagatose are novel and have not been previously evaluated by FDA under the GRAS program. Although these enzymes are not incorporated into D-tagatose and therefore will not be consumed, an assessment of their safety will allay any concerns regarding this novel manufacturing process.

Evaluating the safety of an enzyme that is used in the manufacture and processing of foods requires assessment of the organism in which it originated, the organism in which it is expressed (if recombinant) and the structure of the enzyme itself. Evidence that both the source and donor organisms are non-toxigenic and non-pathogenic and that the enzyme does not actively cause harm suggests that use of the enzyme in food production is safe (Pariza and Foster 1983; Pariza and Johnson 2001). Literature searches on the safety of the source and donor organisms (and their synonyms) were conducted using Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/), Google (https://www.google.com/) and BacDive (https://bacdive.dsmz.de/) on October 8-12, 2018, January 3-4, 2019 and May 9, 2019. Each of the enzymes used in the production of D-tagatose originated in thermophilic organisms for which there is no evidence suggesting pathogenicity or toxigenicity (Sections II.E.2-II.E.8). Furthermore, the DNA from these organisms, if expressed recombinantly, used an established safe strain of *E. coli* (Section II.E.9). Bioinformatics analyses, conducted on October 18, 2018, January 3-4, 2019 and May 9, 2019 revealed that these enzymes share very little homology to known toxins and allergens (Sections II.E.2-II.E.8). Most importantly, since these enzymes are immobilized on a column, no enzymes appear in the final D-tagatose product (Sections II.E.11 and VII.B.1). Therefore, it is expected that humans who consume D-tagatose will not be exposed to the enzymes described below.

II.E.1. Bioinformatics methodology to assess enzyme allergenicity and toxicity

In addition to evaluating the safety of an enzyme's source organism and its expression host, it is also important to determine if a particular enzyme could cross-react with known allergens and possibly induce an allergenic response. Sequence homology testing was performed to determine if any of the novel enzymes used in the production of D-tagatose shared any sequence similarities with known allergens. The University of Nebraska-Lincoln's Food Allergy Research and Resource

Program (FARRP) AllergenOnline database, version 18B and 19 (released on February 10, 2019)[1] was used to conduct this search. Version 18B contains 2089 peer-reviewed sequences and 831 taxonomic protein groups while version 19 contains 2129 peer-reviewed sequences and 852 taxonomic protein groups. The database was searched using FASTA version 35.

The Food and Agriculture Organization (FAO), Codex Alimentarius, European Food Safety Authority (EFSA) and several bioinformatics experts recommend a sequence homology analysis over an 80 amino acid sliding window (all possible 80 amino acid sequences of a complete sequence are queried) to evaluate the potential allergenicity of proteins. Potential allergens are identified by an identity match of >35% (JECFA 2001; Codex Alimentarius Commission 2009; Goodman et al. 2008; Ladics et al. 2007; EFSA 2011). However, it is recognized that such a low threshold is very conservative and very rarely do proteins with less than 50% homology exhibit any cross-reactivity. Instead, some scientists recommend that any sequence identity <50% indicates low-risk allergenicity, 50-70% identity indicates moderate-risk, and >70% indicates a high risk for allergenicity (Goodman et al. 2008).

The 80 amino acid sliding window method was used to initially evaluate potential allergenicity of the enzymes used to produce D-tagatose. There has been some discussion though among experts as to whether this search or a conventional FASTA alignment (which compares overall homology) is more appropriate. Ladics and colleagues found that conventional FASTA alignment results in fewer false positives and the same amount of false negatives as the sliding window approach, although FAO and Codex have yet to change their guidelines (Ladics et al. 2007). A third approach to assessing sequence homology is to determine whether a protein shares 6-8 contiguous amino acids with a known allergen (JECFA 2001; Codex Alimentarius Commission 2009). While a 6 amino acid search can be accompanied by a high rate of false positives, an 8 amino acid search can be accompanied by a high rate of false negatives (Codex Alimentarius Commission 2009; Ladics et al. 2006; Goodman et al. 2008; Goodman 2014). For this reason, this analysis is not routinely recommended by the European Commission and JEFCA recommends that any positive matches unique to this approach be validated with further testing (JECFA 2001; EFSA 2011; European Commission 2013).

Bonumose used all three strategies to compare the novel D-tagatose enzymes to known allergens to ensure a comprehensive analysis. The conventional FASTA alignment performed on AllergenOnline[1] searched for known allergens that have more than 35% sequence homology to the queried sequence and an E value of < 1. E values are another aspect of FASTA alignment that provide some indication of the relevance of potential matches. E values depend on a variety of factors including the alignment itself as well as the size of the database searched. In general, an E

[1] www.allergenonline.org

value of greater than 1 x 10^{-7} represents a match that is likely irrelevant, whereas E values smaller than 1 x 10^{-30} represent a potentially cross-reactive allergen match (Hileman et al. 2002; referenced by http://www.allergenonline.org/databasehelp.shtml). For the sliding window search, an algorithm that performs sequential FASTA3 searches of 80 amino acid segments was used to compare the queried protein with known allergens. Similar to the conventional FASTA alignment, positive results include those matches with more than 35% sequence identity, but the E value threshold was increased to 10 (i.e. E value of < 10). Due to the increased potential for false positives resulting from a homology search of 6 contiguous amino acids, searches were performed for the matching of 8 contiguous amino acids on the AllergenOnline[1] database. Since the predictive power of matching 8 contiguous amino acids (in the absence of other identity alignments) is limited, AllergenOnline[1] does not use a specific algorithm for this approach.

In addition to using these three search methods in the AllergenOnline[1] database, complete protein sequences for the enzymes were also queried in the Allergome[2] (Mari et al. 2006) and Allerbase[3] (Kadam et al. 2017) databases. Allergome[2] utilizes the NCBI Blastp algorithm to compare the queried sequence with those in the Allergome database. These searches were configured to identify known allergens with at least 35% sequence identity to the queried sequence. There was no threshold set for E values. Allerbase[3] also uses the Blastp algorithm for its database search of 2052 experimentally-validated allergens, but these searches were not limited by either % sequence identity or E value.

Toxicity of the novel D-tagatose enzymes was evaluated by performing sequence homology analyses on the Toxic Exposome Database[4], which contains over 3,500 toxins (Wishart et al. 2015). Conventional FASTA alignment was used for these analyses, restricting results to those matches with E values of no greater than 1 x 10^{-5}.

II.E.2. 4-alpha glucanotransferase (4GT)

II.E.2.a. Name

Name: 4-alpha glucanotransferase

Synonyms: amylo-1,6-glucosidase, disproportionating enzyme, dextrin glycosyltransferase, D-enzyme, debranching enzyme maltodextrin glycosyltransferase,

[2] www.allergome.org

[3] http://196.1.114.46:1800/AllerBase/Home.html

[4] http://www.t3db.ca/biodb/search/target_bonds/sequence

amylomaltase, dextrin transglycosylase

II.E.2.b. Classification

EC number: 2.4.1.25
CAS number: 9032-09-1
Number of Amino Acids: 511
Molecular Weight: 58.4 kDa
Reaction catalyzed: Transfers a segment of a 1,4-α-D-glucan to a new position in an acceptor, which may be glucose or a 1,4-α-D-glucan

II.E.2.c. Source organism

4GT is produced by strain DSM 14523/UNI-1 (JCM 11388, NBRC 100420) of *Anaerolinea thermophila. A. thermophila* is a gram-negative, anaerobic, thermophilic bacterium that is a member of subphylum 1 of the phylum Chloroflexi (green non-sulfur bacteria). Its cells are filamentous and non-motile and do not form spores. Originally identified in a sludge blanket of an upflow anaerobic sludge blanket reactor, *A. thermophila* converts organic pollutants into methane and carbon dioxide (Sekiguchi et al. 2001, 2003; Yamada et al. 2005). There is no evidence indicating that *A. thermophila* is pathogenic or toxigenic to humans. According to the Technical Rules for Biological Agents (TRBA) in Germany, *A. thermophila* is in Risk Group 1, along with other organisms that are unlikely to cause human disease (Committee on Biological Agents 2010).

II.E.2.d. Potential allergenicity and toxicity of 4GT

Sequence analyses in which the sequence of 4GT was compared to known allergens in the FARRP AllergenOnline database[1] revealed that this protein has very low allergenicity potential and is not expected to induce any allergic reactions. Conventional FASTA alignment and alignment of the 80 amino acid sliding window did not produce any matches with more than 35% identity, nor were there any matches to 8 contiguous amino acids.

Portions of the protein sequence for 4GT showed some (>35% identity) homology to 10 sequences in the Allergome database[2]. Three of these sequences belonged to the family of Bet v 1-like proteins, 2 matched 7S vicilin, 2 matched grasses and there were single matches for lactoferrin, lectin and catalase. Although the percent identity of these matches ranged from 35%-80%, the short length of these homologous sequences resulted in large E values (ranging from 10-99). The sequence identity score was 80% for *Aspergillus fumigatus* catalase, but since the

sequence was only 10 amino acids in length, the E value was 99. Despite the potential reactivity of this protein with IgE antibodies (Singh et al. 2010), an E value of 99 suggests that it is very unlikely that 4GT would cross-react. Furthermore, this allergen did not match with 4GT in any of the other databases searched.

Alignment analysis in the Allerbase database[3] revealed that portions of the 4GT protein sequence were somewhat homologous to 3 known allergens. Two matches were for segments of enolase and 1 match was for lysosomal alpha-glucosidase. E values ranged from 0.89-7.3 and none of the matches showed more than 35% identity to 4GT, suggesting that 4GT is unlikely to cross-react with these allergens.

In the Toxic Exposome Database[4], the sequence of 4GT did not match with any known toxins.

Using three strategies for allergenicity and one for toxicity assessment, 4GT was determined to have low allergenic or toxic potential.

II.E.3. Pullulanase

II.E.3.a. Name

Name: Pullulanase
Synonyms: alpha-dextrin endo-1,6-alpha-glucosidase, amylopectin 6-glucanohydrolase, debranching enzyme, limit dextrinase, pullulan 6-glucanohydrolase

II.E.3.b. Classification

EC number: 3.2.1.41
CAS number: 9075-68-7
Number of Amino Acids: 926
Molecular Weight: 101.4 kDa
Reaction: Hydrolyzes 1,6-alpha-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the alpha- and beta-limit dextrins of amylopectin and glycogen

II.E.3.c. Source organism

A commercially available pullulanase enzyme with GRAS status and a history of use in food ingredient production is used. The safety of the source organism has been previously evaluated by FDA and it was not deemed toxic or pathogenic.

II.E.3.d. Potential allergenicity and toxicity of pullulanase

Given its GRAS status and history of use, this pullulanase has undergone an extensive safety evaluation which has indicated that it is neither toxic nor allergenic.

II.E.4. alpha-glucan phosphorylase (aGP)

II.E.4.a. Name

Name: alpha-glucan phosphorylase
Synonyms: glycogen phosphorylase, muscle phosphorylase a and b, amylophosphorylase, polyphosphorylase, amylopectin phosphorylase, glucan phosphorylase, 1,4-alpha-glucan phosphorylase, glucosan phosphorylase, granulose phosphorylase, maltodextrin phosphorylase, muscle phosphorylase, myophosphorylase, potato phosphorylase, starch phosphorylase, 1,4-alpha-D-glucan:phosphate alpha-D-glucosyltransferase

II.E.4.b. Classification

EC number: 2.4.1.1
CAS number: 9035-74-9
Number of Amino Acids: 809
Molecular Weight: 91.2 kDa
Reaction: $(1,4\text{-alpha-D-glucosyl})_{(n)}$ + phosphate ⇔ $(1,4\text{-alpha-D-glucosyl})_{(n-1)}$ + alpha-D-glucose 1-phosphate

II.E.4.c. Source Organism

aGP is produced by *Thermus sp. CCB_US3_UF1*. Similar to other species of this genus, this gram-negative, aerobic, bacterium is rod-shaped and does not release spores. This thermophilic strain was isolated from a hot spring and there is no evidence of toxicity or pathogenicity (The et al. 2015). Although this specific strain is not listed in the TRBA, all other strains of *Thermus* are a part of Risk Group 1 (Committee on Biological Agents 2010).

II.E.4.d. Potential allergenicity and toxicity of aGP

Sequence analyses in which the sequence of aGP was compared to known allergens in the FARRP AllergenOnline database[1] revealed that this protein has very low allergenicity and is not expected to induce any allergic reactions. Conventional FASTA alignment and alignment of the

80 amino acid sliding window did not produce any matches with more than 35% identity. There were also no matches to 8 contiguous amino acids.

Portions of the protein sequence for aGP showed some (>35% identity) homology to 11 sequences in the Allergome database[2]. Eight of these sequences matched portions of serum albumin, 2 matched enolase and there was a single match to lactase. Although their identity scores ranged from 35%-46%, they had large E values (ranging from 16-99) which suggests that there is very low probability that aGP cross-reacts with these allergens.

Six sequences were identified in the Allerbase database[3] that were somewhat homologous to portions of the aGP protein sequence. There were 2 matches to segments of venom allergen and 1 match to each of tetanus toxin, glucanase, beta 1,3-glucanase and preproalbumin. E values ranged from 1.1-9.1 and only 1 sequence matched aGP with >35% homology. Preproalbumin had the highest sequence identity (46%) which is still below the conservative threshold of 50%. These low similarity scores and high E values suggest that there is very low probability that aGP is a known allergen.

In the Toxic Exposome Database[4], aGP was not matched with any known toxins, but instead was only matched to 1 homologous sequence in the database (and not a toxin), glycogen phosphorylase, a synonym for aGP.

Using three strategies for allergenicity and one for toxicity assessment, aGP was determined to have low allergenic or toxic potential.

II.E.5. Phosphoglucomutase (PGM)

II.E.5.a. Name

Name: phosphoglucomutase
Synonyms: alpha-D-glucose-1,6-bisphosphate-dependent, glucose phosphomutase

II.E.5.b. Classification

EC number: 5.4.2.2
CAS number: 9001-81-4
Number of Amino Acids: 473
Molecular Weight: 52.5 kDa
Reaction: alpha-D-glucose 1-phosphate ⇔ alpha-D-glucose 6-phosphate

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II.E.5.c. Source organism

PGM is produced by DSM stain 23592 / strain GNS-1 (JCM 16980) of *Thermanaerothrix daxensis*, a filamentous, non-sporulating, gram-negative bacterium that was isolated in 2011 from an aquifer (Grégoire et al. 2011; Pace et al. 2015). Likely owing to its recent isolation, this organism has not been used extensively for the production of enzymes for food production. There is no evidence that this organism is pathogenic or toxigenic based on searches on Google, Pubmed and BacDive, and it has been given a provisional classification by TRBA as Risk Group 1 (Committee on Biological Agents 2010).

II.E.5.d. Potential allergenicity and toxicity of PGM

Sequence analyses in which the sequence of PGM was compared to known allergens in the FARRP AllergenOnline database[1] revealed that this protein has very low allergenicity and is not expected to induce any allergic reactions. Conventional FASTA alignment and alignment of the 80 amino acid sliding window did not produce any matches with more than 35% identity. There was a single match of 8 contiguous amino acids to a protein found in the Indian jujube that was cross-reactive with the latex allergen (M.-F. Lee et al. 2006), but the absence of any matches between PGM and this protein by either of the other two methods suggests that PGM will not cross-react with this allergen.

Alignment analyses in the Allergome database[2] indicated that portions of the protein sequence for PGM showed some (>35% identity) homology to 8 sequences contained in known allergens. Six of these sequences matched portions of serum albumin, and there were single matches for ovomucin and enolase. Their identity scores ranged from 39%-41%, which suggests that PGM will not cross-react since sequence identity was still <50%. Also, their large E values (ranging from 4.2-52) suggest that there is low probability that PGM is a known allergen.

Portions of the PGM protein sequence were somewhat homologous to 1 sequence identified in the Allerbase database[3], a segment of preproalbumin. However, since there was only 39% homology in the match and the E value was 7.9, it is unlikely that PGM will cross-react with this allergen.

In the Toxic Exposome Database[4], the sequence of PGM did not match with any known toxins.

Using three strategies for allergenicity and one for toxicity assessment, PGM was determined to have low allergenic or toxic potential.

II.E.6. Phosphoglucoisomerase (PGI)

II.E.6.a. Name

Name: phosphoglucoisomerase
Synonym: glucose-6-phosphate isomerase, phosphohexose isomerase, phosphohexomutase, oxoisomerase, hexosephosphate isomerase, phosphosaccharomutase, phosphohexoisomerase, phosphoglucose isomerase, glucose phosphate isomerase, hexose phosphate isomerase, D-glucose-6-phosphate ketol-isomerase

II.E.6.b. Classification

EC number: 5.3.1.9
CAS number: 9001-41-6
Number of Amino Acids: 414
Molecular Weight: 45.9 kDa
Reaction: D-glucose 6-phosphate ⇔ D-fructose 6-phosphate

II.E.6.c. Source organism

PGI produced by the ATCC BAA-163/HB27 (DSM 7039) strain of *Thermus thermophilus*, a gram-negative, non-sporulating, rod-shaped aerobic bacterium. *T. thermophilus* was the first thermophilic bacterium isolated, found in hot springs (Oshima and Imahori 1974; R. A. D. Williams et al. 1995). There is no evidence of any pathogenic or toxigenic potential of this strain and it is classified by the CDC as Biosafety Level 1 (Centers for Disease Control and Prevention 2009). Various types of enzymes from *T. thermophilus* are being used in the food and biotechnology industries such as starch-processing enzymes (4GT, xylose isomerase), lactases, esterases, proteases, phosphatases, pyrophosphatases, catalases and DNA and RNA processing enzymes (Nguyen et al. 2014; Fuciños et al. 2005; Pantazaki, A. Pritsa, D. Kyriakidis, Pritsa, and Kyriakidis 2002; Kim, Jang, and Kim 2013).

II.E.6.d. Potential allergenicity and toxicity of PGI

Sequence analyses in which the sequence of PGI was compared to known allergens in the FARRP AllergenOnline database[1] revealed that this protein has very low allergenicity and is not expected to induce any allergic reactions. Conventional FASTA alignment and alignment of the

80 amino acid sliding window did not produce any matches with more than 35% identity, nor were there any matches to 8 contiguous amino acids.

Portions of the protein sequence for PGI showed some (>35% identity) homology to 10 sequences in the Allergome database[2]. Eight of these sequences were contained in 11S globulin and single matches were made for beta galactosidase and enolase. Although their identity scores ranged from 35%-56%, the large E values (ranging from 19-60) suggests that there is low probability that PGI is a known allergen.

Alignment analyses in the Allerbase database[3] indicated that portions of the PGI protein sequence were somewhat homologous to 5 known allergen sequences. Three of these matches (E values <0.01) were for portions of glucose-6-phosphate isomerase, a synonym for PGI, but had identity scores less than 35%. The other 2 matches were for 11S globulin (E = 1.8) and glycinin G2 (E = 6.9). Although glycinin G2 had an identity score of 56%, the high E score suggests that there is unlikely to be cross reactivity with PGI.

In the Toxic Exposome Database[4], the sequence of PGI did not match with any known toxins.

Using three strategies for allergenicity and one for toxicity assessment, PGI was determined to have low allergenic or toxic potential.

II.E.7. Fructose-6-phosphate-epimerase (F6PE)

II.E.7.a. Name

Name: fructose-6-phosphate-epimerase
Synonyms: D-tagatose-6-phosphate-4-epimerase

II.E.7.b. Classification

EC number: 5.1.3.40
CAS number: none
Number of Amino Acids: 426
Molecular Weight: 47.7 kDa
Reaction: D-fructose-6-phosphate → D-tagatose-6-phosphate

II.E.7.c. Source organism

F6PE is produced by DSM stain 23592 / strain GNS-1 (JCM 16980) of *Thermanaerothrix daxensis*, a filamentous, non-sporulating, gram-negative bacterium that was isolated in 2011 from an aquifer (Grégoire et al. 2011; Pace et al. 2015). Likely owing to its recent isolation, this organism has not been used extensively for the production of enzymes for food production. There is no evidence that this organism is pathogenic or toxigenic based on searches on Google, Pubmed and BacDive, and it has been given a provisional classification by TRBA as Risk Group 1 (Committee on Biological Agents 2010).

II.E.7.d. Potential allergenicity and toxicity of F6PE

Sequence analyses in which the sequence of F6PE was compared to known allergens in the FARRP AllergenOnline database[1] revealed that this protein has very low allergenicity and is not expected to induce any allergic reactions. Conventional FASTA alignment and alignment of the 80 amino acid sliding window did not produce any matches with more than 35% identity, nor were there any matches to 8 contiguous amino acids.

Ten sequences of known allergens in the Allergome database[2] showed some (>35% identity) homology to F6PE. Eight of these sequences belonged to the SXP/RAL-2 family of proteins and there were single matches for cucumisin and an unnamed protein. Identity scores ranged from 38%-61%, and E values ranged from 28-88, suggesting that there is low probably that F6PE is a known allergen.

In Allerbase[3], segments of the F6PE protein sequence were somewhat homologous to 8 known allergen sequences. Seven of these matches were portions of serum albumin and 1 was for glucose-6-phosphate isomerase. E values ranged from 1.4-9.6 and none of the matches had more than 35% sequence identity, suggesting that F6PE is unlikely to have allergenic potential.

In the Toxic Exposome Database[4], the sequence of F6PE did not match with any known toxins.

Using three strategies for allergenicity and one for toxicity assessment, F6PE was determined to have low allergenic or toxic potential.

II.E.8. Tagatose-6-phosphate phosphatase (T6PP)

II.E.8.a. Name

Name: tagatose-6-phosphate phosphatase
Synonyms: none

II.E.8.b. Classification

EC number: none
CAS number: none
Number of Amino Acids: 219
Molecular Weight: 24.0 kDa
Reaction: D-tagatose-6-phosphate → D-tagatose

II.E.8.c. Source organism

T6PP was produced by the DSM 20745 / S 6022 strain of *Sphaerobacter thermophilus* (ATCC 49802), isolated from aerobic thermophilic sludge (Hensel, Demharter, and Hilpert 1989). *S. thermophilus* is an aerobic, gram-positive, coccus-shaped immotile bacteria (Pati et al. 2010). There is no evidence to suggest that it is toxic or pathogenic and is classified as Risk Group 1 according to the TRBA (Committee on Biological Agents 2010).

II.E.8.d. Potential allergenicity and toxicity of T6PP

Sequence analyses in which the sequence of T6PP (*Sphaerobacter thermophilus)* was compared to known allergens in the FARRP AllergenOnline database[1] revealed that this protein has very low allergenicity and is not expected to induce any allergic reactions. Conventional FASTA alignment and alignment of the 80 amino acid sliding window did not produce any matches with more than 35% identity. There were also no matches to 8 contiguous amino acids.

Portions of the protein sequence for T6PP (*Sphaerobacter thermophilus)* showed some (>35% identity) homology to 10 sequences in the Allergome database[2]. Three of these sequences were portions of chitosanase, 2 were contained in Bla g 1 protein and there were single matches for pesticidal crystal protein, cysteine protease, calcium-binding protein, grass and heat-shock protein 70. Identity scores ranged from 35%-46%, indicating the presence of low risk for cross-reactivity. This, combined with the large E values (ranging from 1.1-72) suggest that there is low probability that this T6PP is a known allergen. Pesticidal crystal protein, also known as Cry1F had 46% homology to T6PP with an E value of 1.1. While this E value is relatively low, it is still considered non-significant. Furthermore, published literature indicates that Cry1F poses little allergenic threat (Ladics et al. 2006).

Portions of the T6PP (*Sphaerobacter thermophilus)* protein sequence were somewhat homologous to 3 sequences identified in the Allerbase database[3]: putative tegumental protein, erythroagglutinin and alliin lyase. E values ranged from 8.7-9.0 and all had more than 35% identity but were still below the 50% threshold (42%, 43% and 41%, respectively). Thus it is unlikely that T6PP has high allergenic potential.

In the Toxic Exposome Database[4], the sequence of T6PP (*Sphaerobacter thermophilus)* did not match with any known toxins.

Using three strategies for allergenicity and one for toxicity assessment, T6PP was determined to have low allergenic or toxic potential.

II.E.9. Recipient organism

4GT, aGP, PGM, PGI, F6PE and T6PP are all expressed in *E. coli* BL21(DE3), a non-pathogenic and non-toxigenic strain that is very commonly used in the biotechnology industry. The strain was obtained commercially (catalog # CMC0014, Millipore Sigma) and therefore has an established and verifiable identity. The non-pathogenic and non-toxic nature of this strain has been previously reviewed in GRN 485 (pp.15-18).This strain can be handled using Biosafety Level 1 containment because it is not known to cause disease in healthy adults and present little risk to laboratory personnel and the environment (Centers for Disease Control and Prevention 2009). Genomic sequencing of BL21(DE3) revealed that this strain, similar to the K-12 strain, lacks an O antigen, a component of lipopolysaccharide that coats the surface of the bacteria (Jeong et al. 2009). (According to the NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules, K-12 *E. coli* is categorized as Risk Group 1 because it is not associated with disease in healthy adults (National Institutes of Health 2016).) Furthermore, BL21(DE3) *E. coli* has been used as the host organism for production of BbgIV Beta-galactosidase, an enzyme preparation approved for use in food in 2014 (GRN 485, pp.7).

Pullulanase is expressed in the source organism, which has little toxigenic or pathogenic potential (Section II.E.3).

II.E.10. Enzyme production process

The novel enzymes used in the production of D-tagatose derive from non-pathogenic, non-toxigenic sources and are expressed recombinantly in a non-pathogenic, non-toxigenic host. Therefore, they are produced according to the criteria for the lowest level of containment of

Good Industrial Large Scale Practice (Organisation for Economic Cooperation and Development 1992).

With the exception of pullulanase (Section II.E.3.d.), all other enzymes are produced by submerged fermentation followed by heat purification. The ingredients and protocol for the inoculum, seed fermentation, main fermentation and purification are common to those used for the production of many food enzymes. Baker's yeast extract included in the feed is GRAS (21 CFR §184.1983) and minimal food-grade antifoam (21 CFR § 173.340) is used during fermentation. Other materials include water, a carbon source, a nitrogen source, salts and minerals, pH adjustment agents and a food-grade flocculation agent. All of these materials are standard for the enzyme industry, considered safe and suitable for the specified uses and used in accordance with cGMP.

II.E.10.a. Cloning and transformation

Several cloning vectors are used for enzyme expression: Novagen pET-28 a (+) vector (catalog #69864, Millipore Sigma), Novagen pET-9 a (+) vector (catalog #69431, Millipore Sigma) and Novagen pET-29 a (+) vector (catalog #69871, Millipore Sigma). Each is used according to the manufacturer's protocol that utilizes procedures commonly used in the biotechnology industry. All three of these vectors are well-characterized and extremely stable when expressed in hosts that do not express T7 RNA polymerase (EMD Chemicals 2011; Addgene 2018a, 2018c, 2018b). The newly constructed plasmid is then transformed into the BL21(DE3) strain of *E. coli* by either electroporation or heat shock using standard protocols. These pET vectors contain the *kan* gene which confers resistance to kanamycin. Therefore, kanamycin is included on agar plates along with plasmid DNA to ensure the growth of only colonies containing the gene of interest. Transfer of the *kan* gene to the enzyme preparation is not expected because homogenization of the *E. coli* will release endogenous endonucleases and exonucleases that will degrade cellular DNA. Furthermore, these vectors have *E. coli* origins of replication and cannot replicate in other hosts. Nevertheless, we tested 3 different lots of D-tagatose for the presence of the *E. coli* kan gene (Sections II.E.11 and VII.B.1) and found that it was not present.

II.E.10.b. Fermentation

A single colony of growth from the transformed plasmid is used to prepare an overnight seed culture. Glycerol stocks are then prepared from this biomass and stored at -80°C for future fermentations. The inoculum is aseptically transferred to shake flasks to continue growth of *E. coli* with fermentation media. When sufficient biomass has accumulated, cultures from shake flasks are then transferred to a fermentor containing additional media. Temperature and pH are

held constant and protein expression is induced with isopropyl P-D-thiogalactopyranoside (IPTG). Kanamycin is included in the fermentation media to maintain stable growth of *E. coli* and fermentation continues until the desired amount of enzyme production has been reached. The use of IPTG and antibiotics during fermentation has been reviewed previously (GRN 126, pp. 000013-000014). Cell pellets are stored at -20°C until purification.

The fermentation equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken to ensure absence of foreign microorganisms and diafiltration removes residual fermentation chemicals. Since all enzymes are immobilized for D-tagatose manufacturing, remnants of the enzyme production process (*E. coli*, endotoxins, kanamycin, *kan* gene) as well as the enzymes themselves do not appear in the final product. Validation of D-tagatose quality is described in Section II.E.11.

II.E.10.c. Purification

The cells containing novel enzymes (4GT, aGP, PGM, PGI, F6PE, T6PP) are homogenized and cell debris removed through flocculation and centrifugation. The cell-free extract obtained is heated and centrifuged again to remove unstable proteins. The supernatant containing the enzyme of interest is then diafiltered, concentrated, and lyophilized. The lyophilized enzyme is stored frozen and tested for enzymatic activity before use.

II.E.10.d. Immobilization

Novel enzymes are immobilized on a resin that is compliant with 21 CFR §173.357 and regularly treated with a food-safe anti-microbial solution.

II.E.11. D-tagatose quality-control

Using an immobilized enzyme system for the production of D-tagatose ensures that materials used in this novel enzyme production do not contaminate the final product. Impurities are removed by filtration and ion exchange. Since dry sugars do not have a history of housing food-borne pathogens and sugar syrups are resistant to spoilage by surface molds and yeast due to their high concentration, microbiological contamination of D-tagatose is not expected (ICMSF 1986). Nevertheless, thorough analyses are conducted to ensure that remnants from the manufacturing process are absent from D-tagatose. Quality-control testing according to the methods and specifications outlined in Table 1 was carried out in 3 non-consecutive lots of D-tagatose. Certificates containing these test results can be found in Section VII.B.1.

There is no toxicity associated with the intake of endotoxins from either oral drugs or food, which is why there are no endotoxin regulations for these types of products. The microbiome in the human gut produces at least 1,000-fold the lethal amount of endotoxins. They exert no toxicity because the endotoxin remains in the gut lumen and does not enter the blood stream in a significant quantity (Wassenaar and Zimmermann 2018). Nevertheless, we include this analysis in our quality assessment of D-tagatose to illustrate the robustness of our enzyme purification and immobilization methodologies and also to provide an additional assurance that the use of immobilized *E. coli*-produced enzymes does not detract from the safety of D-tagatose.

To provide some perspective, the endotoxin limit for most parenteral drugs (substances that can potentially access the bloodstream) is 5 EU/kg bodyweight/hr (Dawson 2017). The D-tagatose sample from Lot 19-HN9705-TAG3 contained 4.32 EU/g D-tagatose. Although the Joint FAO/WHO Expert Committee on Food Additives (JECFA) found that no level of D-tagatose is unsafe and there is no limit on daily consumption, they do recommend a maximum single dose (per meal intake) of 30 g D-tagatose (JECFA 2006). This amount would contain 129.6 EU. For 60 kg and 70 kg humans, exposure would be 2.16 EU/kg bodyweight and 1.85 EU/kg bodyweight, respectively. As mentioned earlier, endotoxins found in food products such as D-tagatose would not enter the bloodstream in any significant quantity. However, even if they *could* access the bloodstream, these exposures are well below the limit of 5 EU/kg bodyweight/hr.

Overall, these results reflect the production of a safe, consistent product. In addition, subsequent lots of D-tagatose are analyzed to ensure absence of proteins and microbial contamination. Although contaminants related to enzyme production are not expected in the final product, any lots that contain protein are further scrutinized to ensure product safety.

Table 1. Validation of D-tagatose quality.

Analytical Parameter	Acceptable Target Range	Lot # 19-HN9705-TAG3 Results	Lot # 19-HN9705-TAG5 Results	Lot # 19-HN9705-TAG9 Results	Method of Analysis
Protein	not specified	0 μg/mL	0 μg/mL	0 μg/mL	Bradford
Aerobic Plate Count	<10,000 cfu/g	20 cfu/g*	<10 cfu/g*	< 10 cfu/g*	FDA BAM Ch. 3
Total Coliforms	< 10 cfu/g	<10 cfu/g	<10 cfu/g	< 10 cfu/g	FDA BAM Ch. 4
E. coli	< 10 cfu/g	<10 cfu/g	<10 cfu/g	< 10 cfu/g	FDA BAM Ch. 4
Salmonella species (per 25 g)	negative	negative	negative	negative	AOAC OMA 2004.03, 2011.03
Staphylococcus aureus	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g	FDA BAM Ch. 12
Endotoxin	not specified	4.32 EU/g	2.28 EU/g	3.32 EU/g	USP 85
Kanamycin Activity	none detected	none detected	none detected	none detected	Kirby-Bauer disk diffusion susceptibility test
Kan Gene	negative	negative	negative	negative	Qualitative PCR nptII
Sulfite	< 10 ppm	< 10 ppm	< 10 ppm	< 10 ppm	AOAC 990.28

*Estimated count

II.F. Specifications

D-tagatose is in compliance with the specification set forth in the Food Chemical Codex (FCC) (Eleventh Edition) monograph for D-tagatose. Table 2 indicates the methods used to determine compliance with the FCC. The atomic absorption spectrophotometric graphite furnace method for the measurement of lead impurities, as specified by FCC 11 is no longer available. Therefore, USP 233 is performed in its place, a method that utilizes ICP-MS and has been shown to be a suitable alternative in the testing of food samples, offering a lower detection limit than the graphite furnace method (Perkin Elmer 2018; Zhang et al. 1997). Certificates of these results are in Section VII.B.1. In addition to lead analysis, specifications regarding levels of arsenic, cadmium and mercury are also included in Table 2. Due to availability of materials, testing of

these heavy metals was conducted in a different set of non-consecutive production lots. Certificates of these results are in Section VII.B.2.

Table 2. D-tagatose specifications.

Analytical Parameter	Acceptable Target Range	Lot # 19-HN9705-TAG3 Results	Lot # 19-HN9705-TAG5 Results	Lot # 19-HN9705-TAG9 Results	Method of Analysis
Identification (FT-IR)	consistent with reference standard	consistent with reference standard	consistent with reference standard	consistent with reference standard	FCC 11
Purity	NLT 98% on the dried basis	99%	100%	100%	FCC 11
Lead	NMT 0.1 mg/kg	< 0.014 mg/kg	< 0.014 mg/kg	< 0.1 mg/kg	USP 233
Arsenic	< 100 ppb	< 10 ppb*	< 10 ppb*	< 10 ppb*	FDA EAM 4.7
Cadmium	< 100 ppb	< 10 ppb*	< 10 ppb*	< 10 ppb*	FDA EAM 4.7
Mercury	< 100 ppb	< 10 ppb*	< 10 ppb*	< 10 ppb*	FDA EAM 4.7
Loss on Drying	NMT 0.5%	<0.1%	0.1%	<0.5%	FCC 11
Melting Range or Temperature	133°C-137°C	134°C	134°C	133°C	FCC 11
Optical (Specific) Rotation	$[\alpha]_D^{20}$ between -4°and -5.6°	-4.9°	-5.4°	-4.6°	FCC 11
Appearance	white crystal or powder	white crystals	white crystals	fine, white crystalline powder	Visual

* These tests were conducted in Lots # 19-HN9705-TAG18, 19-HN9705-TAG22 and 19-HN9705-TAG25.

II.G. Intended technical effect

D-tagatose is proposed for use as a tabletop sweetener and food/beverage ingredient that partially or fully replaces other sweeteners such as sucrose and high-fructose corn syrup. In

addition to its function as a sweetener, D-tagatose can affect other aspects of a product through its actions as a flavor enhancer, humectant, bulking agent, texturizer and stabilizer (GRNs 78, 352). D-tagatose is a versatile ingredient that can modulate viscosity, reduce stickiness and potentially exhibit anti-microbial actions (Torrico et al. 2019; Bautista, Pegg, and Shand 2000; Kang, Park, and Lee 2013; Roh et al. 1999; Bar 2004; Skytte 2006; Taylor, Fasina, and Bell 2008). It readily crystallizes, depresses freezing point and is a very effective browning agent (Baek et al. 2008; Kwon and Baek 2014; O'Charoen et al. 2014; Ryu et al. 2003b, 2003a; Yadav et al. 2018; Skytte 2006). In a diversity of applications and parameters, D-tagatose has been shown to be an acceptable replacer of sucrose (Kang, Park, and Lee 2013; Torrico et al. 2019; Rubio-Arraez et al. 2016; Roh et al. 1998; Lagast et al. 2017; Taylor, Fasina, and Bell 2008).

Studies using powdered D-tagatose as well as D-tagatose in beverages indicate that it is stable under appropriate storage conditions. Powdered D-tagatose has been shown to be stable for at least 1 year at 20°C, 30°C and 40°C as long as relative humidity does not exceed 75% (Grant and Bell 2012). When D-tagatose was dissolved in milk (1.5%) and lemonade (1.1%), it resisted degradation for 6 months when refrigerated (4°C). Storing at 25-30°C resulted in less than 10% degradation over 6 months (Bell and Luecke 2012). In solution, D-tagatose has increased stability at low pH and low buffer concentrations (Dobbs and Bell 2010).

III. Dietary Exposure

III.A. Food sources of D-tagatose

Consumption of naturally-occurring D-tagatose is limited to a few products, particularly those with processed milk. D-tagatose is naturally found in dairy, whole wheat and fruits (apples, pineapples, oranges, raisins) in very small quantities (Levin 2002; Skytte 2006). In its most potent sources, apples and sterilized dairy products, D-tagatose is approximately 0.3%. According to Zehner (Zehner 1994), human consumption of D-tagatose (mostly from lactulose) was no more than 0.3 g/day ((Zehner 1994) as cited in GRN 352, p.00013).

III.B. Intended use levels

D-tagatose is intended to be used as a tabletop sweetener and as a food ingredient. While it may be used as a flavor enhancer, humectant, texturizer and stabilizer, its primary use will be as a sweetener. D-tagatose may be used in all foods (including but not limited to those explicitly mentioned on p.00022 of GRN 78 and p.000013 of GRN 352, and tabletop sweeteners), except infant formulas and meat and poultry products, as a full or partial replacement for added sugars (sucrose, high-fructose corn syrup). Organoleptic and sweetness needs vary among different products, as do the ingredients that contribute to these effects. Thus, the intended use levels of D-

tagatose will vary between product categories. However, since D-tagatose imparts approximately 90% of the sweetness as sucrose (Fujimaru, Park, and Lim 2012), the amount of D-tagatose will likely be close to the amount of sucrose or other sugar or sweetener it replaces (in contrast to high-intensity sweeteners, of which only a small fraction of the amount of sucrose would be used). The amount used will not exceed the amount reasonably required to accomplish its intended function (21 CFR § 182.1(b)(1)).

III.C. Estimated Daily Intakes (EDIs) of D-tagatose under the intended use

As an ingredient that may be used in many different foods, the amount of D-tagatose reasonably required to replace added sugars will vary between food categories. For some products, this may mean that 100% of added sugars are replaced with D-tagatose. For other products, a much smaller substitution may be used. Furthermore, manufacturers' variation in D-tagatose usage to produce healthy, delicious products will be coupled with consumers' self-regulation of consumption of these products, based upon individual preferences.

For the purposes of determining the EDI for D-tagatose, one must approximate an average level of use in food. The mean "maximum use level" for D-tagatose across multiple food categories is approximately 15% and 19% for GRNs 78 and 352, respectively (estimates of product density were used for those categories specified as g/mL). Because the intended use of D-tagatose in this notice is not strictly limited to those food categories or usage levels specified in previous notices (e.g. the current notice includes tabletop sweeteners which would contain 100% D-tagatose), a conservatively high estimate of D-tagatose use as a sweetener to replace added sugars in the diet would be 30% on average across food categories.

This estimate of D-tagatose use can be applied to data describing the intake of added sugars in order to determine an EDI. There are several national surveys that collect dietary data in the US including the National Health and Nutrition Examination Survey (NHANES), the Continuing Survey of Food Intakes by Individuals (CSFII), and the What We Eat in America (WWEIA) survey, which combines elements of the CSFII and NHANES. An analysis of NHANES data from 2007-2008 for all people at least two years of age found that mean added sugar intake was 77 g/day (Welsh et al. 2011). In 2011-2012, (WWEIA 2011-2012) intake among children was 81 g/day and 77 g/day among adults (Powell, Smith-Taillie, and Popkin 2016). According to WWEIA, intake in 2013-2014 was 73 g/day (Bowman et al. 2017).

Powell and colleagues divided the WWEIA 2011-2012 dataset (n=16,451) into quintiles for both children (ages 2-18 years of age) and adults (ages 19+ years of age) (Powell, Smith-Taillie, and Popkin 2016). For children, the middle quintile (41%-60%) had a mean added sugar intake of 58 g/day and the highest quintile (81%-100%) had a mean added sugar intake of 155

g/day. Among adults, the middle quintile had a mean intake of 57 g/day and the highest quintile a mean intake of 177 g/day. If 100% of food and beverage items replace an average of 30% of added sugars with D-tagatose, this means that among children, the "average" consumption of D-tagatose would be 17 g/day and the "high" consumption would be 47 g/day. Among adults, the "average" consumption would be 17 g/day and the "high" consumption would be 53 g/day (Table 3).

Table 3. Approximate EDI of D-tagatose under intended use.

Age (years)	40th-60th percentile of added sugar intake (g/day)		80th-100th percentile of added sugar intake (g/day)	
	Added sugar	D-tagatose	Added sugar	D-tagatose
2-18	58	**17**	155	**47**
19+	57	**17**	177	**53**

As mentioned in GRN 352 (p. 000015), the percentage of consumed food products that actually contain D-tagatose is likely to be much smaller than 100%. This suggests that actual intake of D-tagatose will likely be much lower than those levels shown in Table 3. Importantly, even assuming the exposures in Table 3 are overestimates, they are still safe to consume. In 2006 JECFA changed the ADI for D-tagatose to "not specified" because no level of D-tagatose was deemed unsafe (JECFA 2006). Furthermore, D-tagatose intake is unlikely to exceed these levels due to its gastrointestinal side effects (Section VI.B.4).

IV. Self-limiting levels of use

None.

V. Experience based on common use in food before 1958

None.

VI. Narrative

Escalating rates of obesity and diabetes are increasing consumer awareness about the potential risks of consuming excessive amounts of added sugars (Patterson, Sadler, and Cooper 2012). Among consumers, there is rising demand for products that retain a favorable taste but limit sugar content – products made possible by low-calorie sweeteners. From 1999-2012, intake of foods and beverages containing low-calorie sweeteners increased by 200% in children and 54% in adults. This demand is expected to grow by approximately 5% each year until 2020 (Sylvetsky et al. 2017). There are a variety of artificial low-calorie sweeteners that are approved as food additives: acesulfame-potassium, advantame, aspartame, neotame, saccharin, and sucralose, as well as natural low-calorie sweeteners such as components (e.g. Rebaudioside A,

Rebaudioside D, Rebaudioside M, etc.) from *Stevia rebaudiana* that are GRAS (American Diabetes Association 2014). D-tagatose is another natural low-calorie sweetener that is not only functional in food but provides consumers with an array of health benefits in addition to its reduced energy density.

D-tagatose is a ketohexose, an epimer of D-fructose isomerized at C-4. It is naturally found in dairy, whole wheat, white beans and various fruits (Levin 2002; Skytte 2006) and is approximately 90% as sweet as sucrose (Fujimaru, Park, and Lim 2012). D-tagatose is only partially digested in the small intestine (Laerke and Jensen 1999; Bertelsen, Jensen, and Buemann 1999; Saunders, Zehner, and Levin 1999) and undergoes fermentation in the large intestine to produce short-chain fatty acids (Bertelsen, Jensen, and Buemann 1999; Vigh and Andersen 2007). As a result of its limited digestibility, D-tagatose has no more than 1.5 kcal/g (U.S. Food and Drug Administration 1999, 2011). In addition to its reduced energy density (compared to other sugars such as sucrose), there are a number of health benefits associated with D-tagatose consumption including improved glycemic control, reduced risk for dental caries, reduced risk for cardiovascular disease, and improved gut health (21 CFR §101.80(c)(2)(ii)(B), FDA-2018-P-0874) (European Commission 2016).

VI.A. Current regulatory status

D-tagatose is GRAS for its uses in foods and beverages as described by GRNs 78 and 352 (Table 4) and FDA raised no questions on these notices. Both of these notices presented a large body of literature describing the safety of D-tagatose and FDA had no questions regarding its safety under these intended uses. In the EU, the European Food Safety Authority (EFSA) approved D-tagatose as a novel food ingredient (to be used without limit in all foods except infant formula) and in Canada D-tagatose is licensed as a Natural Health Product. Neither FDA, EFSA nor Health Canada have defined an upper limit for D-tagatose consumption and JECFA ruled in 2006 that D-tagatose had an ADI that was “not specified,” a designation given to only the safest foods that means that no level tested thus far has been unsafe (JECFA 2006). The proposed uses for D-tagatose in this notice are similar to those described previously (GRN 78, GRN 352), but this notice describes a novel enzymatic method for its manufacture.

Table 4. Regulatory summary for D-tagatose use and intake.

GRN	Notifier	Intended Use	EDI
78	Arla Foods Ingredients amba	bulk sweetener, humectant, texturizer or stabilizer in: diet and/or sugar-free carbonated beverages; pre-sweetened low-calorie ready-to-drink teas; ready-to-eat breakfast cereals; icings and frostings; low/reduced fat diet, energy or nutrient fortified bars; regular and dietetic hard candies and dietetic soft candies; regular and low-fat/non-fat frozen dairy desserts; sugar-free chewing gum; and meal replacements (GRN 78, pp.000021-000022)	90th percentile: 14.9 g/day (GRN 78, p.000027)
352	CJ Cheiljedang Inc.	food ingredient in: ready-to eat breakfast cereals; diet soft drinks; non-diet soft drinks; confectionery; formula diets for meal replacement; meal replacement drink mix (powder); cake; pie; cake mix powder; frostings; ice cream and frozen yogurt; yogurt; chewing gum (sugar-free); jelly and pudding; coffee mix powder; biscuits; cookies; and cereal bars (GRN 352 pp. 000012-000013)	90th percentile: 38.9 g/day (GRN 352, Amendment p.3)
present notice	Bonumose LLC	tabletop sweetener, ingredient in foods and beverages	80th-100th percentile: 50 g/day (avg of all age groups)

VI.B. Metabolism

The metabolism of D-tagatose has been discussed in detail previously (GRN 78 pp.000070-000072, GRN 352 pp.000017-000019) and there have not been any studies since the review of GRN 352 that provide additional metabolic insight. In brief, initial digestibility studies that were conducted in rats, mice and pigs estimated that 20-25% D-tagatose is absorbed in the small intestine, 5-10% is excreted, and approximately 70-75% is fermented in the large intestine by bacteria (Bertelsen, Jensen, and Buemann 1999; Laerke, Jensen, and Hojsgaard 2000; Laerke and Jensen 1999; Saunders, Zehner, and Levin 1999; Bar 2004). Several additional studies in humans supported these findings (Venema, Vermunt, and Brink 2005; Buemann, Toubro, and Astrup 1998; Buemann, Toubro, Holst, et al. 2000). Although a study performed in ileostomy patients concluded that 81% of D-tagatose was absorbed by the small intestine (Normén et al. 2001), the validity of these results has been called into question (including reservations

expressed by the authors themselves). Not only had these patients undergone proctocolectomies for ulcerative colitis (and therefore are not representative of healthy adults), but it is not known if assessment of ileostomy effluent is a reliable indicator of monosaccharide absorption. (In fact, another ileostomy study found that sorbitol and maltitol were absorbed to a similar extent as D-tagatose (73.2% and 75.2%, respectively) (Langkilde et al. 1994), yet it is firmly established that these polyols are very poorly digested (Bar 1990) and are significantly less calorically dense than sucrose (21 CFR §109.1 (c)(1)(i)(C).)

Much of the molecular pathway by which D-tagatose exits the intestinal lumen and is metabolized in humans remains to be elucidated. However, studies performed largely in cell culture and animal models suggest that D-tagatose is metabolized in a fashion similar to its epimer, fructose. *In vitro* results from fructokinase isolated from beef liver indicate that D-tagatose is a substrate of fructokinase (Raushel and Cleland 1973), suggesting that in those tissues containing fructokinase (liver, kidney, small intestine, pancreas (Giroix et al. 2006)), D-tagatose may be phosphorylated. D-tagatose-1-phosphate has been detected in the human liver (Buemann, Gesmar, et al. 2000) and has been shown in cell culture to be a substrate of aldolase B, producing dihydroxacetone phosphate and glyceraldehyde-3-phosphate (Rognstad 1982, 1975). Cell culture studies go on to show that D-tagatose-1-phosphate may activate glucokinase (VanSchaftingen and Vandercammen 1989; Agius 1994) and glycogen synthase (Ciudad et al. 1980, 1988) as well as inhibit glycogen phosphorylase (VandeWerve and Hers 1979). These changes may explain why D-tagatose has been shown to increase liver glycogen stores in certain animal models (Bar et al. 1999). *In vitro* data also suggests that D-tagatose's effects on glycemia may be due to inhibition of the intestinal alpha-glucosidases sucrase and maltase, but this has yet to be confirmed *in vivo* (Seri et al. 1995).

VI.C. Review of safety data

VI.C.1. Safety summary for Bonumose's manufacturing process

As described in Section II.E.11 and Table 2, D-tagatose produced by Bonumose's novel process is chemically identical to previously manufactured D-tagatose that is already GRAS (GRNs 78, 352). All D-tagatose is at least 98% pure and complies with its FCC monograph.

Section II.D. describes how the downstream processing of Bonumose's D-tagatose is similar to that used by previous manufacturers (GRNS 78, 352). The novelty of Bonumose's process lies in its use of an immobilized enzymatic cascade to convert maltodextrin to D-tagatose. Sections II.E.2-8 indicate that the enzymes used in Bonumose's novel production process derive from non-toxic and non-pathogenic organisms. As demonstrated by rigorous sequence homology testing (methodology described in Section II.E.1, results summarized in Sections II.E.2-8), none of the enzymes have significant allergenic or toxic potential. The enzymes are expressed recombinantly in a non-toxic and non-pathogenic strain of *E. coli*

(Section II.E.9) that has previously been used in food-grade enzyme preparation (p.7 GRN 485). Enzymes are then purified and immobilized for maltodextrin conversion. The efficiency of these purifications and immobilizations is demonstrated by the absence of any enzyme production materials in the final D-tagatose product (Table 1).

Altogether, these data show that Bonumose's manufacturing process produces a safe product, consistent with specifications for D-tagatose produced by other methods. To further illustrate that Bonumose's process does not present additional safety concerns, Section VI.C.1.a. briefly reviews other D-tagatose production methods and indicates how Bonumose's process does not introduce or amplify any hazards.

VI.C.1.a. D-tagatose manufacturing: Bonumose vs. others

Method 1: Galactose isomerization – enzymatic then chemical. This chemical synthesis of D-tagatose was first developed by Spherix (formerly known as Biospherics (Chea 2000)) (Levin 2002). In 1997, the rights to use D-tagatose in food was acquired by MD Foods Ingredients (which later became Arla Foods Ingredients), who filed D-tagatose's first GRAS notice with U.S. FDA, GRN 78 (Nutraingredients-USA.com 2003). In 2001, FDA issued a letter of no questions for GRN 78 and D-tagatose was approved as a novel food in the EU in 2005 (European Commission 2017). This process uses food-grade lactose as a feedstock that undergoes enzymatic hydrolysis by immobilized lactase from *A. oryzae* to D-galactose (GRN 78). Calcium hydroxide is then used to isomerize D-galactose to D-tagatose. Reaction by-products including D-galactose, talose and aldol condensation products are removed by demineralization, chromatography and crystallization such that they are not present in the final product. D-tagatose produced by this process met the criteria outlined in the FCC at that time (GRN 78).

Similarities to Bonumose's process:

- Both processes utilize an immobilized enzyme, expressed in a host commonly used for the production of food processing aids. Due to immobilization, neither the enzyme nor the host organism are present in the final product.
- Process impurities are removed by chromatography and crystallization.
- Bonumose's D-tagatose is of similar purity to the D-tagatose produced by this process. D-tagatose purity under both processes is at least 98%.

How Bonumose's process contains fewer hazards:

- This process uses lactose as a feedstock, which has the potential to contain milk allergens, whereas Bonumose's process uses maltodextrin as a feedstock which does not pose any allergenic threat.
- Since 2001, the lead specification for D-tagatose in the FCC has been reduced, such that Bonumose's D-tagatose is compliant with a lower lead limit than that produced by this method.

Method 2: Galactose isomerization – enzymatic then enzymatic. When Damhert began D-tagatose production in 2007 after Arla Foods Ingredients stopped production, it is believed that they modified the production process (Illanes et al. 2016). Damhert utilized lactose which was converted to D-galactose via beta-galactosidase derived from *Pseudoalteromonas haloplanktis*. L-arabinose isomerase from *Geobacillus stearothermophillus* then converted D-galactose to D-tagatose. Both of these enzymes were expressed in *Escherichia coli* and immobilized on alginate beads during production. Downstream processing was similar to other production methods (Francois et al. 2013).

Similarities to Bonumose's process:

- Both processes utilize an immobilized enzyme, expressed in a host commonly used for the production of food processing aids. Due to immobilization, neither the enzyme nor the host organism are present in the final product.
- Downstream processing is very similar, including ion exchange, evaporation, chromatography, crystallization and drying.

How Bonumose's process contains fewer hazards:

- This process uses lactose as a feedstock, which has the potential to contain milk allergens, whereas Bonumose's process uses maltodextrin as a feedstock which does not pose any allergenic threat.

Method 3: Galactose isomerization – chemical then enzymatic. Similar to previous methods, this process developed by CJ Cheiljedang also uses food-grade lactose as a feedstock (GRN 352). D-tagatose produced according to this method became GRAS in 2011 and is authorized as a novel food in the EU (European Commission 2017). However, rather than using lactase or beta-galactosidase to produce D-galactose, sulfuric acid facilitates this conversion. D-galactose is then converted enzymatically to D-tagatose using an immobilized enzyme preparation. This preparation consists of nonviable *Corynebacterium glutumicum* expressing L-

arabinose isomerase derived from *Thermotoga neapolitana*. Downstream processing of D-tagatose to remove impurities involves carbon filtration, ion exchange, evaporation, chromatography, crystallization and drying. D-tagatose produced by this process met the criteria outlined in the FCC at that time (GRN 352).

Similarities to Bonumose's process:

- Both processes utilize an immobilized enzyme, expressed in a host commonly used for the production of food processing aids. Due to immobilization, neither the enzyme nor the host organism are present in the final product.
- Downstream processing is very similar, including ion exchange, evaporation, chromatography, crystallization and drying.
- Similar microbiological criteria were met (Table 1).
- Similar heavy metals criteria were met. Three non-consecutive lots of Bonumose's D-tagatose contained less than 10 ppb arsenic, cadmium, mercury and lead. Certificates of Analysis can be found in Section VII.B.2.

How Bonumose's process contains fewer hazards:

- This process uses lactose as a feedstock, which has the potential to contain milk allergens, whereas Bonumose's process uses maltodextrin as a feedstock which does not pose any allergenic threat.
- The enzyme preparation in this process includes the organism *Corynebacterium glutumicum*. Bonumose's enzymes are purified before immobilization so there is even less potential for material from the expression host to be found in the final product.
- Since 2011, the lead specification for D-tagatose in the FCC has been reduced, such that Bonumose's D-tagatose is compliant with a lower lead limit than that produced by this method.

Method 4: Fructose epimerization. In 2017, CJ Cheiljedang applied for and received substantial equivalence for D-tagatose as a novel food in the EU as produced by fructose epimerization (Food Safety Authority Ireland 2017). This process uses fructose as a feedstock, which can be produced by (a) full hydrolysis and saccharification of starch into glucose, (b) isomerization of glucose, and (c) chromatographic separation of fructose from glucose. Immobilized D-fructose-4-epimerase, an enzyme derived from *Thermotoga neapolitana* and expressed in *Corynebacterium glutamicum,* converts D-fructose into D-tagatose. Because the enzyme is immobilized, it is not contained in the final product. According to the Food Safety Authority of Ireland, this D-tagatose does have a slightly different melting range and optical rotation than D-tagatose already approved as a novel food in the EU, but they nevertheless

deemed it substantially equivalent (Food Safety Authority Ireland 2017). This suggests that this process does not introduce any new or altered hazards that would affect the toxicity of D-tagatose.

Similarities to Bonumose's process:

- Both processes utilize an immobilized enzyme, expressed in a host commonly used for the production of food processing aids. Due to immobilization, neither the enzyme nor the host organism are present in the final product.

How Bonumose's process contains fewer hazards:

- Since 2011, the lead specification for D-tagatose in the FCC has been reduced, such that Bonumose's D-tagatose is compliant with a lower lead limit than that produced by this method.

In summary:

- D-tagatose produced by Bonumose as well as Methods 1-4 described above are all identical in structure and are therefore metabolized equivalently.
- D-tagatose produced by Bonumose as well as Methods 1-4 are all at least 98% pure.
- D-tagatose produced by Bonumose has a lower lead specification than that produced by Methods 1, 3 and 4 (lead specification of Method 2 is not known).
- D-tagatose produced Bonumose uses a feedstock with less allergenic potential than that produced by Methods 1-3.
- D-tagatose produced by Bonumose as well as Methods 1-4 is processed in such a way that any impurities specific to its production process are not present in the final product.

In conclusion, D-tagatose produced by Bonumose would be expected to be at least as safe as those materials used in the toxicity studies previously reviewed by FDA (GRNs 78, 352).

VI.C.2. Animal and *in vitro* studies

The intended uses and technical effects of D-tagatose in this notice are similar to those described in GRNs 78 and 352. Therefore, the safety data presented in these notices is also pertinent to the current notice. The test materials used in these toxicity studies would be expected to exert a similar lack of toxicity as Bonumose's D-tagatose because:

1. they are identical in structure (thus are metabolized equivalently),
2. they all meet the specification for D-tagatose as outlined by the FCC, and

3. Bonumose's process does not introduce or amplify any hazards.

A detailed discussion of previously reviewed safety information can be found in GRNs 78 (pp.000072-000095) and 352 (pp.000019-000027). This safety information will not be discussed in detail in the current notice but is incorporated by reference into Tables 5 and 6 in Sections VI.C.2 and 3, respectively.

Tables 5 and 6 indicate the source and production method of D-tagatose (as numbered and described in Section VI.C.1.a) used in each study. For those studies in which the source of D-tagatose is not described, publicly available information was used to estimate the source and production method. Spherix/MD Foods Ingredients/Arla Foods Ingredients (Method 1) supplied D-tagatose until 2006 (Daniells 2012). In 2007, Damhert entered the D-tagatose market (Method 2), as did CJ Cheiljedang (Method 3) in 2012 (Illanes et al. 2016). CJ Cheiljedang did not introduce Method 4 until approximately 2017 (Food Safety Authority Ireland 2017).

Tables 5 and 6 also include data from studies that were not described in previous GRAS notices (7 human studies and 9 animal studies). These studies were identified from literature searches using Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/) and Google (https://www.google.com/) on Oct. 19, 2018. A description of these more recent studies is included within Tables 5 and 6, and further details regarding the human studies follow Table 6. Overall, the findings from these studies are consistent with FDA's prior decisions regarding the safety of D-tagatose.

Table 5 includes summaries of those safety studies described in GRNs 78 and 352, as well as more recent animal studies that have evaluated the effects of D-tagatose on various health outcomes. These recent efficacy studies (Yadav et al. 2018; Police et al. 2009; J. Williams et al. 2015; Nagata et al. 2018; Collotta et al. 2018; Ensor et al. 2016; Rhimi et al. 2015; Bertelsen, Jensen, and Buemann 1999; Laerke and Jensen 1999; Laerke, Jensen, and Hojsgaard 2000; Livesey and Brown 1996) suggest that when incorporated into diets in sufficient quantity, D-tagatose may improve glycemic control, reduce dyslipidemia, and decrease risk for atherosclerosis. Importantly, Bonumose's D-tagatose is substantially equivalent to the D-tagatose used in these studies; and these studies are consistent with a larger body of evidence indicating that in experimental animals, there are no adverse events associated with D-tagatose consumption.

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Table 5. Animal and *in vitro* studies with D-tagatose.[5]

Species (n)	Dose	Duration	Primary Endpoints	Main Effects of D-tagatose	NOAEL of D-tagatose	Reference	D-tagatose supplier / feedstock / production method
rats (5M, 5F), mice	10 g/kg BW (oral gavage)	single dose	toxicity	no effects	10 g/kg BW	(Trimmer 1989). Cited previously (GRNs 78, 352)(Bar 2004)	Spherix / lactose / 1
Sprague-Dawley rats (20M, 20F)	control diet; cellulose/ fructose control diet; 5, 10, 15, 20% D-tagatose in diet	90 days	toxicity	no abnormalities at 5%; increased relative liver weight at 10, 15, 20%; some hypertrophy of hepatocytes at 15 and 20%	5% in diet	(Kruger, Whittaker, Frankos, and Trimmer 1999). Cited previously (GRNs 78, 352)(Bar 2004)	MD Foods Ingredients / lactose / 1
Sprague-Dawley rats (10M, 30M)	control diet; 20% D-tagatose in diet	28 days + 14 days for recovery	toxicity	increased liver weight and glycogen; slightly increased ALAT and ASAT	not reported	(Bar et al. 1999). Cited previously (GRNs 78, 352)(Bar 2004)	MD Foods Ingredients / lactose / 1
Sprague-Dawley rats (20M)	control diet; 5, 10, 20% D-tagatose in diet	28 days	toxicity	10, 20%: dose-dependent increase in liver weight and glycogen; no other morphological changes of liver tissue; 20%: slight increase in ALAT and ASAT	5% in diet	(Bar et al. 1999). Cited previously (GRNs 78, 352)(Bar 2004)	MD Foods Ingredients / lactose / 1
Sprague-Dawley rats (10M, 15M)	control diet; 5% D-tagatose in diet	28 days	toxicity	Fasted rats: no abnormalities; Non-fasted rats: increased liver weight; no hepatocellular growth	5% in diet	(Bar et al. 1999). Cited previously (GRNs 78, 352)(Bar 2004)	MD Foods Ingredients / lactose / 1

[5] adapted from Tables 4-6 on pp.000021-000023 of GRN 352

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Wistar rats (60F)	5-10% D-tagatose in diet	6 months	toxicity	no effects	10% in diet	(Lina and de Bie 2000). Cited previously (Bar 2004)	MD Foods Ingredients / lactose / 1
Wistar rats (60F)	5-10% D-tagatose in diet	2 weeks	toxicity	no effects	10% in diet	(Lina and de Bie 2000). Cited previously (GRNs 78, 352)(Bar 2004)	MD Foods Ingredients / lactose / 1
Wistar rats	0, 2.5, 5, 10% D-tagatose in diet; 20% fructose in diet; 10% fructose+10% D-tagatose in diet;	24 months	toxicity	0, 2.5, 5%: no morphological changes; enlarged liver in 10% tagatose (M), 20% fructose (M), 10% tagatose+10% fructose (M,F); increased nephron-calcinosis in females with tagatose and in males with 10% tagatose or 10% tagatose+10% fructose; increased incidence of adreno-medullary proliferative disease in 2.5% tagatose (M), 5, 10% tagatose (M,F), 10% tagatose+10% fructose (M,F)	10% in diet	(Lina and Bar 2003). Cited previously (GRN 352)(Bar 2004)	Arla Foods Ingredients / lactose / 1
Lewis, Fischer, Brown Norway, Lister Hooded, Sprague-Dawley and Wistar rats	20% D-tagatose in diet	4 weeks	toxicity	increased liver weight	not reported	(Appel 2002). Cited previously (GRN 352)(Bar 2004)	Arla Foods Ingredients / lactose / 1

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pigs (2)	up to 20% D-tagatose in diet	33 days	toxicity	no effects	20% in diet	(Mann 1997). Cited previously (GRN 352)(Bar 2004)	MD Foods Ingredients / lactose / 1
Sprague-Dawley rats (24F)	0, 4, 12, 20 g/kg BW/d (gastric intubation)	days 6-15 of gestation	develop-mental and reproductive toxicity	12 and 20 g/kg: maternal liver weight increased; no morphological changes in liver; no other adverse effects	20 g/kg BW/d	(Kruger, Whittaker, Frankos, and Schroeder 1999). Cited previously (GRN 352)(Bar 2004)	MD Foods Ingredients / lactose / 1
S. typhimurium (TA 1535, TA 1537, TA1538, TA98, TA100); *E.coli* (WP2uvrA)	100-5000 ug/plate	n/a	genotoxicity	no mutagenic potential	100-5000 ug/plate	(Kruger, Whittaker, and Frankos 1999). Cited previously (GRN 352)(Bar 2004)	MD Foods Ingredients / lactose / 1
Chinese Hamster Ovary cells	1250-5000 ug/ml	n/a	genotoxicity	no change in the number of chromosomal aberrations	1250-5000 ug/ml	(Kruger, Whittaker, and Frankos 1999). Cited previously (GRN 352)(Bar 2004)	MD Foods Ingredients / lactose / 1
CD-1 bone marrow	1250-5000 mg/kg BW	n/a	genotoxicity	no change in micronucleus formation	1250-5000 mg/kg BW	(Kruger, Whittaker, and Frankos 1999). Cited previously (GRN 352)(Bar 2004)	MD Foods Ingredients / lactose / 1
C57BL/6 mice (10M)	PBS + 1 g/kg BW glucose; 0.2 mL of 10 g/L + 1 g/kg BW glucose (intragastric)	single dose	glycemia	no adverse effects reported; decreased rise in blood glucose level	N/A	(Rhimi et al. 2015)	Produced in Rhimi lab

pigs (5M)	20% D-tagatose in diet	7 days	absorption of short-chain fatty acids	no adverse effects reported	20% in diet	(Bertelsen, Jensen, and Buemann 1999)	MD Foods Ingredients / lactose / 1
pigs (8M)	10% D-tagatose + 5% sucrose in diet	17 days	short-chain fatty acid production	no adverse effects reported; increased production of short-chain fatty acids	10% in diet	(Bertelsen, Jensen, and Buemann 1999)	MD Foods Ingredients / lactose / 1
Danish Landrace x Yorkshire pigs (8M)	15% sucrose in diet; 10% D-tagatose + 5% sucrose in diet	18 days	digestibility, short-chain fatty acid production, microbial activity	no adverse effects reported; digestibility was no greater than 25.8% in small intestine; increased production of short-chain fatty acid bacteria and microbial activity	10% in diet	(Laerke and Jensen 1999)	MD Foods Ingredients / lactose / 1
Danish Landrace x Yorkshire pigs (8M)	15% sucrose in diet; 10% D-tagatose + 5% sucrose in diet	18 days	*in vitro* short-chain fatty acid and bacteria production, fecal pH	no adverse effects reported; increased production of D-tagatose-degrading bacteria and short-chain fatty acids	10% in diet	(Laerke, Jensen, and Hojsgaard 2000)	MD Foods Ingredients / lactose / 1
Wild-type zebrafish (70)	control diet; high-cholesterol diet; high-cholesterol diet + 50% D-tagatose; high-cholesterol diet + 50% fructose	21 days	blood lipids, inflammation	90% survival in high-cholesterol and high-cholesterol+D-tagatose groups; no change in total cholesterol; decreased triglycerides and CETP activity; decreased infiltration of lipid and inflammatory cells in liver; reduced embryonic toxicity of oxidized LDL	50% in diet	(Yadav et al. 2018)	CJ Cheiljedang / fructose / 4

Sprague-Dawley rats (6)	control diet; 3% D-allulose, D-fructose, D-tagatose or D-sorbose in diet	28 days	blood lipids, lipogenic enzymes	no adverse effects reported; no effects	3% in diet	(Nagata et al. 2018)	CJ Cheiljedang / fructose / 4
pigs (2M)	20% sucrose in diet; 10% sucrose + 10% D-tagatose in diet; 20% D-tagatose in diet	33 days	digestibility, short-chain fatty acid product	no adverse effects reported; increased production of short-chain fatty acids	20% in diet	(Bertelsen, Jensen, and Buemann 1999)	MD Foods Ingredients / lactose / 1
C57BL/6-LDLr$^{-/-}$ mice (10M)	water treatment 2x week (oral gavage): 0-0.853 g/kg BW/dose glucose, fructose, D-tagatose; 0-0.15 g/kg BW/dose polydatin	56 days	blood lipids	no adverse effects reported; reduced total cholesterol, triglycerides	1.7 g/kg BW/week	(Ensor et al. 2016)	CJ Cheiljedang / lactose / 3
Wistar rats (30M)	10.5% sucrose in diet; 10.5% D-tagatose in diet	21 days (adapta-tion) + 40 days	body weight, net metabo-lizable energy	no adverse effects reported; decreased energy density	10.5% in diet	(Livesey and Brown 1996)	Spherix / lactose / 1

ApoE$^{-/-}$ mice (10M)	control diet; high-cholesterol diet; high-cholesterol diet with 34% D-tagatose; high-cholesterol diet with 34% sucrose; high-cholesterol diet with 34% D-tagatose and dihydromyricetin; high-cholesterol diet with 34% sucrose and dihydro-myricetin	71 days	blood lipids, atherosclerotic lesions	no adverse effects reported; decreased body weight, total cholesterol and triglycerides; fewer lesions	34% in diet	(J. Williams et al. 2015)	CJ Cheiljedang / lactose / 3

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LDLr$^{-/-}$ mice (5-6M, 5-6F)	control diet; 35% D-tagatose or sucrose in diet	112 days	blood lipids, glycemic control, atherosclerotic lesions	no adverse effects reported; when compared to the sucrose diet: decreased fasting glucose (M,F) and fasting insulin (M); decreased body weight, total cholesterol, LDL cholesterol, VLDL cholesterol, triglycerides (M,F); decreased inflammation of adipocytes (F); decreased lesion formation and lipid content (M,F)	35% in diet	(Police et al. 2009)	Damhert / lactose / 2
C57BL/6 mice (6M)	control diet; 30% D-tagatose in diet or water; 30% fructose in diet or water	168 days	blood lipids, glycemic control, oxidative stress, inflam-mation	no adverse effects reported; no effect/reduced lipids, glycemia compared to control/fructose-fed mice; reduced oxidative stress and inflammation compared to fructose-fed	30% in diet	(Collotta et al. 2018)	CJ Cheiljedang / fructose / 4

M: male; F: female; N/A: not applicable

VI.C.3. Human Studies

Table 6 includes safety studies described in GRNs 78 and 352, as well as more recent human studies that have evaluated the effects of D-tagatose on various health outcomes. These recent studies are briefly described in this section, but overall, they indicate that D-tagatose was well-tolerated. For the purposes of this notice, use of the term "well-tolerated" is consistent with its use by the authors of these studies and indicates that if there were gastrointestinal (GI) side effects, they were mild or moderate in nature even at high intake levels. These side effects were not unexpected, but rather consistent with effects commonly associated with excessive consumption of other poorly digestible carbohydrates ((Grabitske and Slavin 2009) as cited in GRN 352 p.000026).

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Table 6. Human studies with D-tagatose.[6]

Subjects (n)	Dose	Duration	Primary Endpoints	Main Effects of D-tagatose	NOAEL of D-tagatose	Reference	D-tagatose supplier / feedstock / production method
healthy men (12)	15 g D-tagatose or sucrose, 3x daily	28 days	liver health, plasma uric acid	no effects on liver volume, glycogen concentration, plasma uric acid; moderate laxation and strong bloating observed	45 g/day	(Boesch et al. 2001). Cited previously (GRNs 78, 352)	MD Foods Ingredients / lactose / 1
healthy men (6)	30 g D-tagatose	single dose	serum uric acid	increased serum uric acid (within normal range)	30 g/day	(Diamantis and Bar 2001). Cited previously (GRN 352)	Arla Foods Ingredients / lactose / 1
hyperuricemic men (12)	15 g D-tagatose	single dose	serum uric acid	increased serum uric acid by 2.5%	15 g/day	(Diamantis and Bar 2002). Cited previously (GRN 352)	Arla Foods Ingredients / lactose / 1
NIDDM adults (8)	15 g D-tagatose, 3x daily	365 days	plasma uric acid, glycemic control	no effect on plasma uric acid; decreased rise in post-prandial blood glucose; GI symptoms observed in all subjects (intensity not specified in GRN 352)	45 g/day	(T.W. Donner 2006). Cited previously (GRN 352)	Arla Foods Ingredients / lactose / 1

[6] adapted from Table 7 on p.000025 of GRN352

healthy adults (4M, 4F); NIDDM adults (4M, 4F)	5, 10, 25, 75 g D-tagatose	single dose	plasma uric acid	healthy M: small rise in uric acid levels; no reported GI intolerance	75 g/day	(Saunders et al. 1999). Cited previously (GRNs 78, 352)	Spherix / lactose / 1
healthy adults (4M, 4F); NIDDM adults (4M, 4F)	healthy: 25 g D-tagatose or glucose, 3x daily; NIDDM: 25 g D-tagatose, 3x daily (8 weeks) or no sugar	8 weeks	plasma uric acid, glycemic control, blood lipids	no effect on uric acid, glycemic control or blood lipids; 7/8 subjects experienced flatulence and 6/8 experienced diarrhea (intensity not specified)	75 g/day	(Saunders et al. 1999). Cited previously (GRNs 78, 352)	Spherix / lactose / 1
healthy men (8)	water; 30 g D-tagatose or D-fructose	single dose	serum uric acid, glycemic control, energy expenditure	increased serum uric acid (within normal range); decreased post-prandial glucose and insulin; decreased fasting insulin; decreased RQ compared to fructose (no change in energy expenditure); decreased rise in lactate compared to fructose; well-tolerated GI symptoms	30 g/day	(Buemann, Toubro, Holst, et al. 2000). Cited previously (GRNs 78, 352)	MD Foods Ingredients / lactose / 1

healthy men (73)	30 g D-tagatose	single dose	GI tolerance	well-tolerated GI symptoms	30 g/day	(Buemann, Toubro, and Astrup 1999). Cited previously (GRNs 78, 352)	MD Foods Ingredients / lactose / 1
healthy men (50)	20 g D-tagatose, sucrose or lactitol	single dose	GI tolerance	well-tolerated GI symptoms	20 g/day	(A. Lee and Storey 1999). Cited previously (GRNs 78, 352)	MD Foods Ingredients / lactose / 1
healthy men (19)	29 g D-tagatose or sucrose	single dose	food intake	decreased subsequent intake; well-tolerated GI symptoms	29 g/day	(Buemann, Toubro, Raben, et al. 2000). Cited previously (GRNs 78, 352)	MD Foods Ingredients / lactose / 1
healthy adults (4M, 4F); NIDDM adults (6M, 4F)	75 g D-tagatose	single dose	glycemic control	healthy: decreased rise in insulin levels; NIDDM: decreased rise in glucose levels; GI symptoms observed in all subjects (intensity not specified)	75 g/day	(T.W. Donner, Wilber, and Ostrowski 1999). Cited previously (GRNs 78, 352)	Spherix / lactose / 1
NIDDM adults (6M, 4F)	0-30 g D-tagatose	single dose	glycemic control	decreased rise in glucose levels; GI symptoms were mild, infrequent, transient	30 g/day	(T.W. Donner, Wilber, and Ostrowski 1999). Cited previously (GRNs 78, 352)	Spherix / lactose / 1
hyperglycemic adults (18M, 15F)	5 g D-tagatose; erythritol + 0.004 g sucralose	single dose	glycemic control	decreased post-prandial glucose at 120 minutes; no effect on insulin; well-tolerated GI symptoms	5 g/day	(Kwak et al. 2013)	CJ Cheiljedang / lactose / 3
healthy adults (27M, 25F)	5 g D-tagatose; erythritol + 0.004 g sucralose	single dose	glycemic control	no effect on glycemic control; well-tolerated GI symptoms	5 g/day	(Kwak et al. 2013)	CJ Cheiljedang / lactose / 3

healthy adults (17)	10 g D-tagatose; erythritol + 0.004 g sucralose	single dose	glycemic control	no effect on glucose; decreased post-prandial insulin; well-tolerated GI symptoms	10 g/day	(Kwak et al. 2013)	CJ Cheiljedang / lactose / 3
NIDDM adults (10)	5 g D-tagatose; erythritol + 0.004 g sucralose	single dose	glycemic control	decreased post-prandial glucose; well-tolerated GI symptoms	5 g/day	(Kwak et al. 2013)	CJ Cheiljedang / lactose / 3
ileostomy patients (4M, 2F)	15 g sucrose in yogurt (1x daily) with or without 15 g D-tagatose	2 days	small bowel absorption	81% absorption in small intestine; no GI discomfort reported, 1 subject reported an increase in wet mass excretion	15 g/day	(Normén et al. 2001)	Arla Foods Ingredients / lactose / 1
healthy men (8)	30 g D-tagatose or sucrose/ day	14 days	hydrogen production, energy expenditure, blood lipids	increased hydrogen production by 35%; no change in fasting glucose; decreased fasting insulin (pooled samples); no change in total cholesterol, body weight, blood pressure; decreased triglycerides (pooled samples); no change in energy expenditure; well-tolerated GI symptoms	30 g/day	(Buemann, Toubro, and Astrup 1998). Cited previously (GRNs 78, 352)	Spherix / lactose / 1
healthy adults (12M, 18F)	7.5 or 12 g D-tagatose/day; 7.8 g fructo-oligo- sacc-harides (FOS); 7.6 g D-tagatose+ 7.5 g FOS; 15.1 g sucrose	14 days	short-chain fatty acid production	increased number of stools; increased production of acetate and lactobacilli; well-tolerated GI symptoms	12 g/day	(Venema, Vermunt, and Brink 2005)	Arla Foods Ingredients / lactose / 1

healthy adults (16)	10 g D-tagatose 3x daily	14 days	short-chain fatty acid and bacteria production	increased number of short-chain fatty acids and D-tagatose consuming bacteria; no reported GI intolerance	30 g/day	(Bertelsen, Jensen, and Buemann 1999)	MD Foods Ingredients / lactose / 1
NIDDM adults (46-52)	2.5, 5.0 or 7.5 g D-tagatose /day	180 days	glycemic control, blood lipids	7.5 g/day: decreased HbA1c, fasting glucose, body weight; no change in blood lipids; well-tolerated GI symptoms	7.5 g/day	(Ensor et al. 2014)	Spherix / lactose / 1
NIDDM adults (172-207)	15 g D-tagatose or 1.5 g Splenda, 3x daily	300 days	glycemic control, blood lipids	decreased HbA1c and fasting glucose; decreased LDL in months 6-10; increased triglycerides in months 8, 10; no change in insulin or body weight; well-tolerated GI symptoms	45 g/day	(Ensor et al. 2015)	Spherix / lactose / 1
NIDDM adults (4M, 4F)	15 g D-tagatose, 3x daily	365 days	glycemic control, blood lipids	decreased body weight; increased HDL; no change in HbA1c, fasting glucose, fasting insulin, total cholesterol, LDL cholesterol, blood pressure; well-tolerated GI symptoms	45 g/day	(Thomas W. Donner, Magder, and Zarbalian 2010)	Spherix / lactose / 1

NIDDM: non-insulin dependent diabetes mellitus (type 2 diabetes); M: male; F: female; GI: gastrointestinal; HbA1c: hemoglobin A1c

Kwak and colleagues (Kwak et al. 2013) used a double-blind crossover study to analyze the effects of low doses of D-tagatose in healthy (n=52), hyperglycemic (n=33) and diabetic (n=10) Koreans. In diabetics, consumption of 5 g D-tagatose reduced blood glucose levels 60 minutes (peak of curve) post-meal, which resulted in a smaller area under the curve (AUC). In a subset of healthy subjects (n=17) that consumed 10 g D-tagatose, post-prandial glucose levels were slightly lower than those produced by the sucralose-erythritol control, but not significantly so (Kwak et al. 2013). In these healthy adults, D-tagatose produced lower rises (lower AUCs) in insulin and C-peptide (an indicator of endogenous insulin production) compared to those who consumed the control, and also had lower levels of insulin and C-peptide 30 minutes (peak of curve) after consumption. Overall, this study shows that even low doses D-tagatose can attenuate post-prandial blood glucose levels in patients with type 2 diabetes or insulin levels in healthy adults. Both doses of D-tagatose were well-tolerated in this population. None of the subjects consuming the lower dose reported any side effects and among the high-dose group, transient watery stools were reported in 2 subjects and indigestion reported in 2 subjects.

In a population of patients with mild cases of diabetes, Ensor and colleagues (Ensor et al. 2014) supplemented subjects with 2.5 g, 5.0 g or 7.5 g D-tagatose 3 times a day in water daily for 6 months. Those receiving the highest dose (n=47) experienced significant reductions in levels of HbA1c, a long-term indicator of blood glucose control. This dose was also associated with decreased fasting blood glucose levels at 3 and 6 months, while both the 5.0 g and 7.5 g treatments decreased body weight after 3 months of treatment. These low doses of D-tagatose were well-tolerated with minimal mild adverse events related to GI distress. In fact, the number of adverse events increased as the D-tagatose dose decreased. Severe adverse events only occurred in a small number of patients, with a single subject experiencing one of the following symptoms: nausea, retching, vomiting, fatigue, lethargy and anxiety. Although this study lacks a control group, it does suggest that minimal amounts of D-tagatose 3 times daily is enough to significantly improve long-term glycemic control.

In a 10-month, randomized, double-blinded placebo-controlled trial of overweight male and female patients with mild type 2 diabetes (6.6%<HbA1c<9.0%; managed with lifestyle modification), Ensor and colleagues (Ensor et al. 2015) found that compared to daily consumption of 1.5 g Splenda (placebo – matched to D-tagatose for sweetness), consumption of 15 g D-tagatose (n=185) 3 times daily significantly reduced HbA1c and fasting blood glucose levels. D-tagatose also significantly reduced total and low-density lipoprotein (LDL) cholesterol levels but increased triglyceride levels. Baseline levels of HDL were higher in the placebo group compared to the D-tagatose group, and both treatment groups experienced a decrease in the first 4 months, followed by an increase from months 4-10. A safety analysis showed that there were more adverse events in the placebo group, while the D-tagatose group had more GI-specific adverse events. Nevertheless, 15 g D-tagatose daily was relatively well-tolerated, with most symptoms being either mild or moderate intensity. Overall, this study shows that glycemic control and blood lipid levels improve as the duration of D-tagatose consumption increases.

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This same dose of 15 g D-tagatose three times a day was used in another long-term prospective study of 8 (4 male, 4 female) patients with type 2 diabetes (4 on sulfonylureas, 1 on metformin and troglitizone; all on medication for at least 10 months) (Thomas W. Donner, Magder, and Zarbalian 2010). Unlike other D-tagatose studies in which the majority of patients with mild diabetes (HbA1c < 9%) could manage their condition with lifestyle modification (Ensor et al. 2014, 2015), this population consisted of patients with more severe cases of diabetes (HbA1c = 11%) who required medication. Fifteen g D-tagatose three times daily for 12 months did not alter baseline levels of HbA1c, fasting glucose, insulin, total or LDL cholesterol levels. Over the course of the study, body weight decreased and HDL levels increased, but the absence of a control group in this pilot study makes the results more difficult to interpret. Among those subjects who completed the study, D-tagatose was relatively well-tolerated. Two subjects initially enrolled in the study but experienced GI discomfort (diarrhea, flatulence, bloating) and chose not to participate further. Another subject with asthma withdrew due to a persistent cough. The remaining 8 subjects who completed the study initially experienced mild diarrhea, flatulence and bloating but this resolved in all but one subject who experienced flatulence for 6 months. Generally, GI discomfort was avoided if no more than 15 g D-tagatose was taken at a time and doses were separated by at least 3 hours.

In a randomized double-blind crossover study with 30 healthy adults (12 men, 18 women), Venema and colleagues evaluated the effects of D-tagatose on gut bacterial growth and SCFA production (Venema, Vermunt, and Brink 2005). Subjects consumed 5 different treatments, each for 14 days, with 14-day washout periods in-between. In 30 g of raspberry jam eaten daily at breakfast, they consumed 1) 7.5 g D-tagatose, 2) 12.5 g D-tagatose, 3) 7.8 g fructo-oligo saccharides (FOS), 4) 7.6 g D-tagatose + 7.5 g FOS or 5) sucrose. Fecal samples were collected at the end of each treatment period for direct analysis as well as *in vitro* incubations to monitor SCFA production. All arms of the study were well-tolerated and there were no significant differences in adverse events between any of the treatment groups. The D-tagatose + FOS group tended to have more GI-related events than either of the D-tagatose or FOS groups alone, but D-tagatose alone only elicited GI symptoms in 4 subjects. No changes in bowel function were noted in the first week of the treatment period, but during the second week of treatment, D-tagatose consumption increased the number of stools compared to sucrose. The high D-tagatose treatment as well as FOS resulted in thin stools, whereas the other treatments did not affect consistency. In men, there was more *Lactobacilli* produced in the 12.5 g D-tagatose group than the 7.5 g D-tagatose group, although no difference was observed in women. *In vitro* incubations of feces showed that more SCFAs were produced in each of the treatments containing D-tagatose compared to FOS, and more was produced in the 12.5 g D-tagatose treatment than sucrose treatment. Consistent with these profiles, further evidence of fermentation was found in that that both doses of D-tagatose were more effective in lowering pH than FOS and sucrose. Using an *in vitro* model of the large intestine, Venema and colleagues also found

that D-tagatose treatment increased the numbers of *Lactobacilli* as well as the SCFAs lactate and butyrate.

Similar findings were reported by Bertelsen and colleagues in a study of 16 healthy subjects that consumed 10 g D-tagatose 3 times daily for 2 weeks (Vigh and Andersen 2007; Bertelsen, Jensen, and Buemann 1999). No gastrointestinal intolerances were reported and researchers found that compared to the unadapted state, adaptation to D-tagatose increased SCFAs. Consistent with the profile of SCFAs in the adapted state and the increased production of butyric acid, these samples also had more lactic acid bacteria such as *Lactobacilli*, bacteria associated with maintenance of a healthy gut and inhibition of tumor growth (Manning and Gibson 2004).

Finally, in Normén's study of ileostomy patients (described in Section VI.B.1.), no gastrointestinal discomfort was experienced although 1 patient observed increased excretion of wet matter (Normén et al. 2001).

VI.C.4. Summary of D-tagatose effects on gastrointestinal tolerance

Human studies complement those in experimental animals and suggest that D-tagatose consumption offers numerous health benefits including an improvement in glycemic control, a reduction of dyslipidemia and actions as a prebiotic. These studies also indicate that all doses tested thus far are safe, a conclusion supported by FDA (by the absence of an upper limit) and JECFA (by an ADI that was "not specified") (JECFA 2006).

However, similar to other poorly-digestible carbohydrates ((Grabitske and Slavin 2009) as cited in GRN 352 p.000026) high doses of D-tagatose can produce GI distress (diarrhea, nausea, thirst, appetite loss, bloating, borborygmi, flatulence) depending upon the amount consumed at one time (T.W. Donner, Wilber, and Ostrowski 1999; Ensor et al. 2014, 2015; Thomas W. Donner, Magder, and Zarbalian 2010; A. Lee and Storey 1999; Boesch et al. 2001). (These symptoms are not unexpected and may be linked to D-tagatose's actions as a prebiotic, as discussed on p.000026 of GRN 352.) In a study of type 2 diabetes patients, all patients experienced GI symptoms upon consumption of 75 g D-tagatose, but when the dosage was dropped to 30 g or less, only 30% of the patients experienced any symptoms and those that did described them as "mild" (T.W. Donner, Wilber, and Ostrowski 1999). Another study in patients with type 2 diabetes found that supplementation with 15 g D-tagatose 3 times daily produced mild GI upset during the first couple of weeks, but these symptoms disappeared for the remainder of the year-long study (Thomas W. Donner, Magder, and Zarbalian 2010), suggesting that an acclimation period increases intake tolerance.

While excessive consumption of D-tagatose does not pose any health risk, self-imposed limitations on D-tagatose consumption tend to be driven by a desire to attenuate possible GI distress. In 2006 the NOAEL level was removed in favor of a “not specified” ADI, but JECFA did recommended that single doses of D-tagatose be limited to 30 g (JECFA 2006).

VI.C.5. D-tagatose considerations for special populations

Owing to its similarity in structure to fructose, D-tagatose has undergone additional scrutiny for those conditions specifically affected by fructose intake. Excessive consumption of fructose, an isomer of D-tagatose, can elevate uric acid levels, increasing risk for gout. Previous D-tagatose notices have addressed this concern and shown that in both healthy adults and those with type 2 diabetes, changes in uric acid levels, liver size and phosphate accumulation that may accompany D-tagatose consumption are not clinically significant (GRN 78 pp.000090-000094, GRN 352 pp.000023-000024) (Saunders et al. 1999; Buemann, Toubro, Holst, et al. 2000; Buemann, Gesmar, et al. 2000; Boesch et al. 2001). In addition, a study in men predisposed to gout suggested that 15 g of D-tagatose a day was even safe for hyperuricemic patients (JECFA 2006), although additional research using higher doses of D-tagatose are needed to determine if there is a limit for how much D-tagatose hyperuricemic patients can consume.

Hereditary fructose intolerance is a genetic condition in which an inability to metabolize fructose-1-phosphate can lead to hypoglycemia and hyperuricemia upon consumption of fructose (Ali, Rellos, and Cox 1998). This condition is typically discovered shortly after birth and an avoidance of all fructose in the diet is recommended. According to the Boston University Hereditary Fructose Intolerance laboratory, pure preparations of D-tagatose are tolerated by those with this condition (Hereditary Fructose Intolerance Laboratory at Boston University n.d.). However, the current ADI of “not specified” from JECFA does not apply to those with hereditary fructose intolerance since more research is needed to fully evaluate the needs of this population.

VI.D. Summary

VI.D.1. Established safe uses

D-tagatose has been used safely in foods and beverages for nearly 2 decades and has been the subject of two “no objections” GRAS notices (GRNs 78, 352). Each of these notices contained a plethora of published and unpublished literature documenting the safety of D-tagatose. This information was used by JECFA to support an ADI of “not specified” for D-tagatose, the safest classification possible (JECFA 2006).

VI.D.2. Safety of D-tagatose produced by a novel enzymatic cascade established by scientific procedures

Data from human clinical studies as well as experimental animal models indicate that D-tagatose is a rare sugar that offers a number of health benefits including improved glycemic control, reduction of cardiovascular disease risk, reduction of risk for tooth decay and improved gut health (Sections VI.C.2-3, 21 CFR §101.80(c)(2)(ii)(B), FDA-2018-P-0874)(European Commission 2016). Numerous human and animal studies indicate that there are no adverse effects of D-tagatose consumption and it has not yet been tested at a level deemed unsafe (Sections VI.C.2-3). Similar to other poorly-digestible carbohydrates, excessive consumption of D-tagatose can induce GI symptoms such as flatulence, bloating and diarrhea, but these are generally avoidable if D-tagatose is consumed in moderation (no more than 30 g/serving). The conservative overestimate of EDI specified in the current notice ("average" exposure = 17 g/day; "high" exposure = 50 g/day) is within the well-tolerated range and is fortunately also within the range associated with health benefits.

Unlike previous GRNs that manufacture D-tagatose from lactose by catalyzing a conversion from D-galactose to D-tagatose, this notice utilizes a cascade of novel enzymes to produce D-tagatose from food-grade maltodextrin. As described in Sections II.E. and IV.C.1., each of these novel enzymes is derived from a non-toxic, non-pathogenic source, expressed recombinantly in a non-toxic, non-pathogenic host and immobilized in a column. Bioinformatics analyses reveal that none of these enzymes share any structural similarities to known allergens or toxins. Moreover, since the enzymes are immobilized, they do not contaminate the final product and will not be consumed. Production of these enzymes and D-tagatose is carried out under cGMP using common food and biotechnology industry materials and processes. The final D-tagatose product is rigorously tested to ensure purity and chemical identity as well as the absence of contaminants. This GRAS conclusion is based on the data and information generally available and consented opinion about the safety of D-tagatose.

The following safety evaluation fully considers the composition, intake, and toxicological properties of D-tagatose as well as appropriate corroborative data.

1. Analytical data from multiple lots indicate that D-tagatose complies reliably with the established food-grade product specifications and meets all applicable purity standards (Section VII.B.1).

2. Bonumose's D-tagatose will be used as a tabletop sweetener and as an ingredient in foods and beverages (similar uses as GRNs 78 and 352).

3. In previous GRAS notices (GRNs 78 and 352), the safety of D-tagatose was established in animal, *in vitro* and human studies. Additional animal and human studies published subsequent to FDAs review of GRN 352 continue to support the safety of D-tagatose as a food ingredient.

4. No adverse effects of D-tagatose have been observed in experimental animals or humans. The only side effect of excessive D-tagatose consumption is GI discomfort, a common symptom for other poorly digestible carbohydrates. Limiting single doses of D-tagatose to 30 g is recommended to avoid GI distress.

5. The conservatively high estimated EDI from the proposed use of D-tagatose is within the recommended levels for reaping health benefits and avoiding GI symptoms. It is assumed that Bonumose's D-tagatose will replace currently marketed D-tagatose, so cumulative exposures are not expected.

The following evaluation considers the safety of the manufacturing process, including the novel enzyme cascade and production organisms.

1. Production of D-tagatose requires the use of novel immobilized enzymes (Sections II.D-E) derived from the following non-toxic, non-pathogenic thermophilic organisms: *Anaerolinea thermophila, Thermus sp. CCB_US3_UF1, Thermus thermophilus, Thermanaerothrix daxensis* and *Sphaerobacter thermophilus*. Not only are these organisms considered safe to use, but several have a long history of use in the food and biotechnology industries. Enzymes are expressed in a BL21(DE3) strain of *E. coli,* a non-toxic, non-pathogenic strain commonly used in the biotechnology industry.

2. Structural analyses on three allergen databases (AllergenOnline, Allerbase, Allergome) and one toxin database (Toxic Exposome Database) indicated that none of the novel enzymes were likely to be potential allergens or cross-react with potential allergens.

3. The novel enzymes used in the manufacturing process of D-tagatose are immobilized on a column, which results in no residual microorganism and/or enzymes in the GRAS substance, and thus no consumer exposure.

4. Bonumose's D-tagatose is manufactured under cGMP using common food industry materials and processes in accordance with the applicable parts of 21 CFR §110 and 117.

Overall, there is no evidence in publicly available databases nor published literature suggesting that D-tagatose or any of the enzymes used in its production may cause significant adverse events. It is concluded that D-tagatose produced by a novel enzymatic cascade and immobilized in a column, resulting in no consumer exposure to the enzymes or their production organisms, is considered GRAS by scientific procedures.

VI.D.3. Discussion of information inconsistent with GRAS determination

We are not aware of information that would be considered inconsistent with the finding that the proposed use of D-tagatose in foods and beverages, meeting appropriate specifications and used according to cGMP, is GRAS.

VII. List of generally available data and supporting information

VII.A. References

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VII.B.1. Quality-control and specification analyses of D-tagatose

Analyses of D-tagatose were carried out as described in Sections II.E.11, II.F, Table 1 and Table 2. Documentation of the results shown in Tables 1 and 2 is included herein and support the conclusion that D-tagatose produced by a novel enzymatic cascade is safe for consumption and compliant with the FCC monograph for D-tagatose. This appendix includes:

a. Protein quantification protocol and results
b. Microbiological analysis results
c. Endotoxin analysis results[1]
d. Kanamycin activity protocol and results[1]
e. *Kan* gene expression results
f. Specification and sulfite analysis results

Microbiological analyses of three non-consecutive lots of D-tagatose indicate results for aerobic plate count, total coliforms, *E. coli*, *Salmonella* species and *Staphylococcus aureus* are all within an acceptable range. Sulfite levels are less than 10 ppm, indicating that any sulfite used during production was removed from the syrup by ion exchange. The absence of any protein in D-tagatose indicates that all of the enzymes used as processing aids remain immobilized in the column and do not appear in the final product. As expected, materials used in and generated during enzyme production are also absent from D-tagatose. This is illustrated by low endotoxin levels, absence of *E. coli*'s kanamycin-resistant gene nptII and absence of detectable kanamycin activity.

Typical analysis of kanamycin levels in a product would be carried out using LC-MS. However, given the similarity in structure between D-tagatose and kanamycin, this type of chromatography would not provide reliable detection. Therefore, the presence of kanamycin was assessed indirectly by measuring antibiotic activity of D-tagatose. In its food additive specifications, JECFA describes the assessment of antibacterial activity using the disk diffusion method (JECFA 2006). The Kirby-Bauer disk diffusion susceptibility test was used to screen D-tagatose for the presence of kanamycin by incorporating a positive control for kanamycin into the protocol (described herein) and interpretation of the results was carried out according to industry standards (Hudzicki 2009; CLSI 2018). The kanamycin positive control exerted substantial growth inhibition while no growth inhibition was observed for D-tagatose and the negative control, indicating that D-tagatose does not have detectable kanamycin activity.

[1] At FDA's request, the word "confidential" has been removed from the footer of the laboratory reports for endotoxin levels and kanamycin activity because these results are not confidential. Other than this removal, the laboratory reports have not been edited in any way.

All other methods listed in Table 1 are routinely used in the industry. Documented results for all of the quality-control analyses are included herein. Raw data for the Bradford analysis performed by Bonumose LLC and endotoxin analysis performed by Associates of Cape Cod are stored electronically and in paper-format at the offices of Bonumose LLC.

References

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Protein Quantification

Objective: The purpose of this analysis is to determine the amount of protein present in a sample of monosaccharide.

Summary: For regulatory purposes, three samples from non-consecutive lots will be analyzed as per GRAS/NDIN notices. Thereafter, subsequent lots will be analyzed for quality-control purposes. All of the enzymes used in monosaccharide production are non-toxic and non-pathogenic. To ensure that all monosaccharide production is efficient and free from contamination by enzymes, protein levels in the final products are quantified.

Justification of Method: The Bradford assay is an established method for the measurement of protein and has been used previously for the determination of protein content in a GRAS notification (GRN 485). When added to a protein solution, Coomassie brilliant blue G-250 dye associates with the basic and aromatic amino acids of the protein. The unbound, acidic G-250 dye has a maximum absorbance at 470 nm, but when the dye binds to the protein, the maximum absorbance shifts to 595 nm. Bovine serum albumin (BSA) is a reference protein that is used to generate a standard curve with which to compare test samples.[1]

Bonumose reuses an immobilized cascade of enzymes many times during monosaccharide production. The detection limit of this assay is 5 ug/mL protein, making it suitable for the detection of protein that may leach from the immobilized enzyme cascade.

Materials

Test article: monosaccharide

Reference article: Bovine Serum Albumin, Biotechnology grade

Supplier: VWR

Catalog Number: 0332-25G

Lot Number: 186305681

1 x Bradford Reagent

Supplier: Oz Biosciences

Catalog Number: BA00050

Deionized water

Nanodrop One C

Supplier: ThermoFisher

Serial Number: A241810013

Method

This protocol is that described by the manufacturer of the reagent (Oz Biosciences). Complete the *Protein Quantification Worksheet* for each assay. Rather than using a microplate reader, reaction will be completed in a microcentrifuge tube and absorbance measured with the Nanodrop.

- Allow 1x Bradford reagent to warm to room temperature and invert several times to mix.
- Prepare fresh 0.1 mL of 2 mg/mL BSA (BSA stock solution). After dissolving large stock of BSA in deionized water, verify concentration on Nanodrop, then dilute to 2 mg/mL and verify concentration on Nanodrop. Use this concentration and the table below as a guide to make the following standards. Any solution remaining after testing should be stored at 4°C.

Name	uL BSA solution	uL deionized water	BSA concentration (ug/mL)
Standard A	10 µL 2000 µg/mL BSA	390	50
Standard B	7.5 µL 2000 µg/mL BSA	742.5	20
Standard C	375 µL 20 µg/mL BSA	375	10
Standard D	375 µL 10 µg/mL BSA	375	5
Standard E	0	375	0

- Prepare 1.5 mL (final volume) of 550 mg/mL solution of monosaccharide in deionized water. Centrifuge 13000 rpm 5 seconds. Be sure to account for the volume of the solute

during preparation. This stock solution will be used to measure protein levels at 4 different concentrations (Table 1). Any solution remaining after testing should be stored at 4°C.

- Add BSA standards, deionized water and monosaccharide solution to labeled microcentrifuge tubes as indicated in Table 1. Each condition should be performed in triplicate.
- For every 2 treatments:
  - Add 100 µL of 1x Bradford reagent to each tube.
  - Briefly vortex.
  - Let reaction sit at RT for 5 min.
  - Measure and record the absorbance at 595nm.
- Repeat previous step for next 2-3 treatments when at least 2.5 minutes have transpired since addition of previous Bradford reagent.
  - Since these samples are performed in tubes rather than on a microplate, limiting the absorbance readings to only 6-9 samples will minimize the potential confounding effect of extended exposure to Bradford reagent (all samples should be read within 10 min).

Table 1. Linear Bradford assay experimental setup

BSA Standard	µL deionized water	µL monosaccharide solution	µL 1x Bradford reagent	Final concentration of BSA (µg/mL)	Final concentration of D-tagatose (mg/mL)
100 µL Standard A	0	0	100	50	0
100 µL Standard B	0	0	100	20	0
100 µL Standard C	0	0	100	10	0
100 µL Standard D	0	0	100	5	0
100 µL Standard E	0	0	100	0	0
0	89.77	10.23	100	0	56.25
0	79.55	20.45	100	0	112.5
0	59.1	40.9	100	0	225
0	0	100	100	0	550
15 µL Standard A	64.55	20.45	100	7.5	112.5
40 µL Standard A	39.55	20.45	100	20	112.5
70 µL Standard A	9.55	20.45	100	35	112.5

- Prepare a standard curve and calculate the concentration of the monosaccharide sample based on the linear equation of the calibration curve.

- The detection limit of this assay is 5 µg/mL. Therefore, in unspiked samples of monosaccharide only calculated protein concentrations ≥ 5 µg/mL are considered "detectable".

Report: The completed *Protein Quantification Worksheet* should include the following information:

- Identification of the test and reference articles
- Raw absorbance values
- Standard curve generated by BSA
- Calculated quantification of protein in samples.

The *Protein Quantification Worksheet* is stored electronically on Bonumose's Dropbox folder in the "Quality-Control" sub-folder, along with the raw data and calculations.

References

1. Oz Biosciences. *Bradford-Protein Assay Kit Instructional Manual*.; 2019.

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Complete this worksheet in accordance with *Protein Quantification Protocol.*

Test facility: Bonumose LLC
1725 Discovery Drive, Suite 220
Charlottesville, VA 22911

Test article(s):

Name	Sample #	Lot #	Date of Receipt
D-Tagatose	2	19-HN9705-TAG3	2/25/20

Appearance: white solid

Purity: ≥ 98%

Storage conditions: dry container at room temperature

Production facility: Boca Raton Innovation Center, Boca Raton, FL, USA

Reference article:

Name: Bovine Serum Albumin, biotechnology grade

CAS Number: 9048-46-8

Catalog Number: 0332-25G

Lot Number: 18G3056081

Appearance: white solid

Purity: 96%

Storage conditions: 4°C

Supplier: VWR International LLC
100 Matsonford Road
Radnor Corporate Center
Building One, Suite 200
P.O. Box 6660

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Radnor, PA 19087

Reagent:

Name: 1x Bradford Reagent

Catalog Number: BA00050

Lot Number: BA180-812

Storage conditions: 4°C

Supplier: Oz Biosciences Inc.
4901 Morena Blvd
Suite 501
San Diego, CA 92117

Results:

Verification of stock BSA concentration (2 mg/mL) using Nanodrop: 2.01 mg/mL

BSA Reference Standard

Sample	Triplicate	A^{595}	Average A^{595}	Standard curve linear trendline equation
50 µg/mL BSA (Standard A)	A	1.44	1.34	y = 0.0206 + 0.3503
	B	1.45		
	C	1.12		
20 µg/mL BSA (Standard B)	A	0.79	0.82	
	B	0.83		
	C	0.83		
10 µg/mL BSA (Standard C)	A	0.63	0.69	
	B	0.41		
	C	1.02		
5 µg/mL BSA (Standard D)	A	0.43	0.39	
	B	0.31		
	C	0.44		
0 µg/mL BSA (Standard E)	A	0.22	0.27	
	B	0.31		
	C	0.27		

Protein Quantification Worksheet
updated 3/2020

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Test Article(s):

Sample #: 2 Lot #: 19-HN9705-TAG3	Triplicate	A^{595}	Protein Conc. (µg/mL)	Average Protein Conc. (µg/mL)
56.25 mg/mL monosaccharide	A	0.27	-3.90	-5.03
	B	0.25	-4.87	
	C	0.22	-6.33	
112.5 mg/mL monosaccharide	A	0.25	-4.87	-4.87
	B	0.22	-6.33	
	C	0.28	-3.41	
225 mg/mL monosaccharide	A	0.29	-2.93	-2.60
	B	0.29	-2.93	
	C	0.31	-1.96	
550 mg/mL monosaccharide	A	0.31	-1.96	-2.12
	B	0.33	-0.99	
	C	0.28	-3.41	
112.5 mg/mL monosaccharide + 7.5 µg/mL BSA	A	0.65	14.55	10.83
	B	0.52	8.24	
	C	0.55	9.69	
112.5 mg/mL monosaccharide + 20 µg/mL BSA	A	0.85	24.26	26.20
	B	0.93	28.14	
	C	0.89	26.20	
112.5 mg/mL monosaccharide + 35 µg/mL BSA	A	1.15	38.82	38.66
	B	1.22	42.22	
	C	1.07	34.94	

If protein is detectable in any unspiked monosaccharide sample:

- For those unspiked monosaccharide samples with detectable protein
    - avg. of mean protein concentration: _____ μg/mL

If protein is not detectable in any unspiked monosaccharide sample:

- protein concentration = 0 μg /mL

Sample	Lot	Protein Concentration (μg/mL)
2	19-HN9705-TAG3	0

Date of data collection: _2/28/20____

Date of worksheet completion: __3/3/20___

Assay performed by: _Karen Weikel_____________

Signature: Date: 4/9/2020

Results verified by:

Signature: Date: 09Apr2020

Protein Quantification Worksheet
updated 3/2020

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Complete this worksheet in accordance with *Protein Quantification Protocol.*

Test facility: Bonumose LLC
1725 Discovery Drive, Suite 220
Charlottesville, VA 22911

Test article(s):

Name	Sample #	Lot #	Date of Receipt
D-Tagatose	3	19-HN9705-TAG5	2/26/20

Appearance: white solid

Purity: ≥ 98%

Storage conditions: dry container at room temperature

Production facility: Boca Raton Innovation Center, Boca Raton, FL, USA

Reference article:

Name: Bovine Serum Albumin, biotechnology grade

CAS Number: 9048-46-8

Catalog Number: 0332-25G

Lot Number: 18G3056081

Appearance: white solid

Purity: 96%

Storage conditions: 4°C

Supplier: VWR International LLC
100 Matsonford Road
Radnor Corporate Center
Building One, Suite 200
P.O. Box 6660

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Radnor, PA 19087

Reagent:

Name: 1x Bradford Reagent

Catalog Number: BA00050

Lot Number: BA180-812

Storage conditions: 4°C

Supplier: Oz Biosciences Inc.
4901 Morena Blvd
Suite 501
San Diego, CA 92117

Results:

Verification of stock BSA concentration (2 mg/mL) using Nanodrop: 2.01 mg/mL

BSA Reference Standard

Sample	Triplicate	A^{595}	Average A^{595}	Standard curve linear trendline equation
50 µg/mL BSA (Standard A)	A	1.44	1.34	y = 0.0206 + 0.3503
	B	1.45		
	C	1.12		
20 µg/mL BSA (Standard B)	A	0.79	0.82	
	B	0.83		
	C	0.83		
10 µg/mL BSA (Standard C)	A	0.63	0.69	
	B	0.41		
	C	1.02		
5 µg/mL BSA (Standard D)	A	0.43	0.39	
	B	0.31		
	C	0.44		
0 µg/mL BSA (Standard E)	A	0.22	0.27	
	B	0.31		
	C	0.27		

Protein Quantification Worksheet
updated 3/2020

Test Article(s):

Sample #: 3 Lot #: 19-HN9705-TAG5	Triplicate	A^{595}	Protein Conc. (µg/mL)	Average Protein Conc. (µg/mL)
56.25 mg/mL monosaccharide	A	0.32	-1.47	-2.60
	B	0.24	-5.35	
	C	0.33	-0.99	
112.5 mg/mL monosaccharide	A	0.30	-2.44	-2.93
	B	0.33	-0.99	
	C	0.24	-5.35	
225 mg/mL monosaccharide	A	0.29	-2.93	-3.09
	B	0.24	-5.35	
	C	0.33	-0.99	
550 mg/mL monosaccharide	A	0.26	-4.38	-3.41
	B	0.28	-3.41	
	C	0.30	-2.44	
112.5 mg/mL monosaccharide + 7.5 µg/mL BSA	A	0.51	7.75	7.11
	B	0.50	7.27	
	C	0.48	6.30	
112.5 mg/mL monosaccharide + 20 µg/mL BSA	A	0.82	22.80	24.58
	B	0.86	24.74	
	C	0.89	26.20	
112.5 mg/mL monosaccharide + 35 µg/mL BSA	A	1.00	31.54	33.00
	B	1.07	34.94	
	C	1.02	32.51	

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If protein is detectable in any unspiked monosaccharide sample:

- For those unspiked monosaccharide samples with detectable protein
  - avg. of mean protein concentration: _____ μg/mL

If protein is not detectable in any unspiked monosaccharide sample:

- protein concentration = 0 μg /mL

Sample	Lot	Protein Concentration (μg/mL)
3	19-HN9705-TAG5	0

Date of data collection: _2/28/20____

Date of worksheet completion: __3/3/20___

Assay performed by: _Karen Weikel_____________

Signature: [redacted] Date: 4/9/2020

Results verified by: [redacted]

Signature: [redacted] Date: 09Apr2020

Protein Quantification Worksheet
updated 3/2020

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Complete this worksheet in accordance with *Protein Quantification Protocol*.

Test facility: Bonumose LLC
1725 Discovery Drive, Suite 220
Charlottesville, VA 22911

Test article(s):

Name	Sample #	Lot #	Date of Receipt
D-Tagatose	1	19-HN9705-TAG9	3/16/20

Appearance: white solid

Purity: ≥ 98%

Storage conditions: dry container at room temperature

Production facility: Boca Raton Innovation Center, Boca Raton, FL, USA

Reference article:

Name: Bovine Serum Albumin, biotechnology grade

CAS Number: 9048-46-8

Catalog Number: 0332-25G

Lot Number: 18G3056081

Appearance: white solid

Purity: 96%

Storage conditions: 4°C

Supplier: VWR International LLC
100 Matsonford Road
Radnor Corporate Center
Building One, Suite 200
P.O. Box 6660

bonumose

Radnor, PA 19087

Reagent:

Name: 1x Bradford Reagent

Catalog Number: BA00050

Lot Number: BA180-812

Storage conditions: 4°C

Supplier: Oz Biosciences Inc.
4901 Morena Blvd
Suite 501
San Diego, CA 92117

Results:

Verification of stock BSA concentration (2 mg/mL) using Nanodrop: 2.126 mg/mL (Guidance table in protocol for preparation of standards was adjusted accordingly.)

BSA Reference Standard

Sample	Triplicate	A^{595}	Average A^{595}	Standard curve linear trendline equation
50 µg/mL BSA (Standard A)	A	1.33	1.40	y = 0.022 + 0.3168
	B	1.43		
	C	1.43		
20 µg/mL BSA (Standard B)	A	0.86	0.81	
	B	0.75		
	C	0.83		
10 µg/mL BSA (Standard C)	A	0.53	0.51	
	B	0.49		
	C	0.51		
5 µg/mL BSA (Standard D)	A	0.44	0.43	
	B	0.39		
	C	0.45		
0 µg/mL BSA (Standard E)	A	0.30	0.30	
	B	0.30		
	C	0.31		

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Test Article(s):

Sample #: 1 Lot #: 19-HN9705-TAG9	Triplicate	A^{595}	Protein Conc. (µg/mL)	Average Protein Conc. (µg/mL)
56.25 mg/mL monosaccharide	A	0.28	-1.67	-1.82
	B	0.28	-1.67	
	C	0.27	-2.13	
112.5 mg/mL monosaccharide	A	0.29	-1.22	-1.22
	B	0.30	-0.76	
	C	0.28	-1.67	
225 mg/mL monosaccharide	A	0.30	-0.76	-0.61
	B	0.32	0.15	
	C	0.29	-1.22	
550 mg/mL monosaccharide	A	0.32	0.15	0.15
	B	0.34	1.05	
	C	0.30	-0.76	
112.5 mg/mL monosaccharide + 7.5 µg/mL BSA	A	0.49	7.87	6.36
	B	0.46	6.51	
	C	0.42	4.69	
112.5 mg/mL monosaccharide + 20 µg/mL BSA	A	0.79	21.51	22.87
	B	0.85	24.24	
	C	0.82	22.87	
112.5 mg/mL monosaccharide + 35 µg/mL BSA	A	1.11	36.05	36.81
	B	1.09	35.15	
	C	1.18	39.24	

If protein is detectable in any unspiked monosaccharide sample:

- For those unspiked monosaccharide samples with detectable protein
  - avg. of mean protein concentration: _____ µg/mL

If protein is not detectable in any unspiked monosaccharide sample:

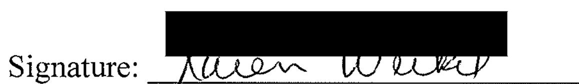
- protein concentration = 0 µg /mL

Sample	Lot	Protein Concentration (µg/mL)
1	19-HN9705-TAG9	0

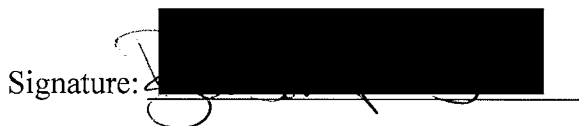
Date of data collection: _4/7/20____

Date of worksheet completion: __4/7/20___

Assay performed by: _Karen Weikel_____________

Signature:  Date: 4/7/2020

Results verified by:

Signature:  Date: 04/07/2020

Corrected Report Date: 28 April 2020
Customer Number: BON00
IC Number: 0220-098
Test Date: 3 March 2020 and 24 March 2020

References:

United States Pharmacopeia (USP) 42, NF 37 (2019), Chapter <85> *Bacterial Endotoxins Test*

Document Number P_SOP_CTS_0183, Contract Test Service (CTS)

Document Number M_CTS_CS_0929, *Turbidimetric Testing in the Pyros® Kinetix and Pyros® Kinetix Flex (PK Flex) for Contract Test Service (CTS)*

Title 21 Code of Federal Regulations, Chapter 1 - Food and Drug Administration, Department of Health and Human Services, Part 210, *Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs; General*

Title 2[redacted] ood and Drug Administration, Department of Health and Human Serv[redacted] *ractice for Finished Pharmaceuticals*

Analyst – Brenner Guerra CTS Analyst I	Date	Peer Reviewer – Maureen Woodill	Date

QA Reviewer – Rochelle Simmons — Date

124 Bernard E. Saint Jean Drive ■ East Falmouth, Massachusetts 02536 ■ Tel: (800) 848-3248 ■ (508) 540-3444 ■ Fax: (508) 540-8680

ASSOCIATES OF
CAPE COD
INCORPORATED
Specialists in Endotoxin and Glucan Detection

BACTERIAL ENDOTOXINS TEST (BET) RESULTS
Characterization – Kinetic Turbidimetric Method

Report Date: 26 March 2020
Customer Number: BON00
IC Number: 0220-105
Test Date: 3 March 2020 and 4 March 2020

Reagents:

Pyrotell®-T: Lot number 519-09-939-T. Pyrotell®-T was reconstituted with Glucashield® buffer and used to test all dilutions.

Glucashield® Buffer: Lot number 1207062.

LAL Reagent Water (LRW): Lot number AE28251284. LRW was used to reconstitute the endotoxin, prepare dilutions of the endotoxin and the sample, and serve as the negative control.

Instrumentation: Pyros Kinetix® Flex Incubating Kinetic Tube Reader; 96 tube capacity held at 37°C ± 0.5°C; CIN002334.

Control Standard Endotoxin (CSE): Lot number 155, *Escherichia coli* O113:H10. The potency of the CSE is 12 EU/ng when measured against Reference Standard Endotoxin (RSE), lot number H0K354 (United States Pharmacopeia, USP) with Pyrotell®-T lot number 519-09-939-T. The sensitivity of the assay is the lowest concentration of CSE used to construct the calibration curve. A series of two-fold dilutions between 0.001 and 0.032 EU/mL was tested in triplicate and the results were used to construct the calibration curve.

Preparation and Testing of Sample: A 0.5 mg/mL sample solution was prepared by adding 2.364 mL LRW to 1.1820 g of sample. The sample was diluted with LRW in two-fold serial dilutions ranging from 1:2 to 1:256 and tested, in duplicate, to find minimum, non-interfering dilutions. Positive product controls were tested, in parallel, on sample dilutions fortified with additional endotoxin equivalent to 0.008 EU/mL. The sample to lysate ratio was 1:1.

Results: The characterization assay is used to determine the conditions necessary to overcome interference (dilution and sample pretreatment) and the amount of endotoxin present in a sample. All parameters of the USP <85> assay were met as shown in the table below.

Assay Parameter	USP Requirement	Pass/Fail
Correlation Coefficient	$\lvert r \rvert \geq 0.980$	Pass
Negative Control (LRW)	< Lowest Standard	Pass
Positive Product Control	50% - 200%	Pass

Sample Identification	Endotoxin Concentration	Dilution(s) Reported
The Endotoxin Limit is Not Provided		
D-tagatose Sample 3 Lot Number 19HN9705TAG5	2.28 EU/g	1:128

References:

United States Pharmacopeia (USP) 42, NF 37 (2019), Chapter <85> *Bacterial Endotoxins Test*
Document Number P_SOP_CTS_0183, Contract Test Service (CTS)
Document Number M_CTS_CS_0929, *Turbidimetric Testing in the Pyros® Kinetix and Pyros® Kinetix Flex (PK Flex) for Contract Test Service (CTS)*
Title 21 Code of Federal Regulations, Chapter 1 - Food and Drug Administration, Department of Health and Human Services, Part 210, *Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs; General*
Title 21 Code of Federal Regulations, Chapter 1 - Food and Drug Administration, Department of Health and Human
g Practice for Finished Pharmaceuticals

Analyst – Brenner Guerra Date
CTS Analyst I

Peer Reviewer – Maureen Woodill Date
CTS Supervisor

QA Reviewer – Sarah L. Roth
26MAR2020
Date

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ASSOCIATES OF
CAPE COD
INCORPORATED

Specialists in Endotoxin and Glucan Detection

BACTERIAL ENDOTOXINS TEST (BET) RESULTS
Characterization – Kinetic Turbidimetric Method

Report Date: 12 May 2020
Customer Number: BON00
IC Number: 0320-069
Test Date: 25 April 2020

Reagents:

Pyrotell®-T: Lot number 520-01-005-T. Pyrotell®-T was reconstituted with Glucashield® buffer and used to test all dilutions.

Glucashield® Buffer: Lot number 1207066.

LAL Reagent Water (LRW): Lot number AE28251284. LRW was used to reconstitute the endotoxin, prepare dilutions of the endotoxin and the sample, and serve as the negative control.

Instrumentation: Pyros Kinetix® Flex Incubating Kinetic Tube Reader; 96 tube capacity held at 37°C ± 0.5°C CIN001983.

Control Standard Endotoxin (CSE): Lot number 155, *Escherichia coli* O113:H10. The potency of the CSE is 10 EU/ng when measured against Reference Standard Endotoxin (RSE), lot number H0K354 (United States Pharmacopeia, USP) with Pyrotell®-T lot number 520-01-005-T. The sensitivity of the assay is the lowest concentration of CSE used to construct the calibration curve. A series of two-fold dilutions between 0.001 and 0.032 EU/mL was tested in triplicate and the results were used to construct the calibration curve.

Preparation and Testing of Sample: A 0.5 g/mL test solution was prepared by adding 4.62 mL of LRW to 2.31 g of sample. The sample was diluted with LRW in two-fold serial dilutions ranging from 1:50 to 1:200 and tested, in duplicate, to find minimum, non-interfering dilutions. Positive product controls were tested, in parallel, on sample dilutions fortified with additional endotoxin equivalent to 0.008 EU/mL. The sample to lysate ratio was 1:1.

Results: The characterization assay is used to determine the conditions necessary to overcome interference (dilution and sample pretreatment) and the amount of endotoxin present in a sample. All parameters of the USP <85> assay were met as shown in the table below.

Assay Parameter	USP Requirement	Pass/Fail
Correlation Coefficient	$\lvert r \rvert \geq 0.980$	Pass
Negative Control (LRW)	< Lowest Standard	Pass
Positive Product Control	50% - 200%	Pass

Sample Identification	Endotoxin Concentration	Dilution(s) Reported
No Endotoxin Limit was provided. (2 g/unit)		
D-tagatose Sample 1 Lot number 19-HN9705-TAG9	3.32 EU/g	1:100

References:

United States Pharmacopeia (USP) 42, NF 37 (2019), Chapter <85> *Bacterial Endotoxins Test*
Document Number P_SOP_CTS_0183, Contract Test Service (CTS)
Document Number M_CTS_CS_0929, *Turbidimetric Testing in the Pyros® Kinetix and Pyros® Kinetix Flex (PK Flex) for Contract Test Service (CTS)*
Title 21 Code of Federal Regulations, Chapter 1 - Food and Drug Administration, Department of Health and Human Services, Part 210, *Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs; General*
Title 21 Code of Federal Regulations, Chapter 1 - Food and Drug Administration, Department of Health and Human ...*ring Practice for Finished Pharmaceuticals*

...ay 2020

Analyst – Shona... Date
CTS Analyst I

Peer Reviewer – Maureen Woodill Date
CTS Supervisor

QA Reviewer – Autumn Wood 12 MAY 2020
Date

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Detection of Kanamycin in a Tagatose Product

Eurofins Microbiology Laboratories, Inc.
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Lancaster, PA 17601

717-556-3725
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6.0 FINAL REPORT APPROVAL 6

1.0 OBJECTIVE

The overall purpose of this study is to screen for the presence of kanamycin in a tagatose sample using a modified Kirby-Bauer disk diffusion method.

2.0 PROTOCOL OVERVIEW

A tagatose sample was assayed for the potential presence of kanamycin by preparing a solution of the sample and saturating it onto blank antimicrobial disks. Prepared disks were placed on the surface of agar plates inoculated with *Escherichia coli*. Plates were incubated and any zone of inhibition developed was compared to a known kanamycin control disk.

3.0 MATERIALS AND METHODS

3.1 Challenge microorganism preparation

The following challenge microorganism was prepared for this study:

- *Escherichia coli* (ATCC #25922)

The culture was prepared from a lyophilized preparation (KWIK-STIK™, Microbiologics, St. Cloud, MN) according to manufacturer's instructions or from stock plates. The culture was transferred to Tryptic Soy Agar (TSA, Neogen, Lansing, MI) and incubated at 35 ± 2°C for 24 ± 2 hours. The culture was used to make a suspension measuring approximately 0.5 according to the McFarland turbidity standard in sterile diluent and was swabbed onto the surface of Mueller-Hinton Agar plates (MHA, Neogen). Plates were then allowed to dry approximately 2-3 minutes before applying test disks.

3.2 Preparation of samples

The following tagatose product was provided by Bonumose LLC:

- D-Tagatose Lot # 19-HN9705-TAG3 02/22/2020

A suspension of the product sample was prepared as a 50% solution by adding 5 g to 5 mL of sterile deionized water and vortexing to mix. Blank antimicrobial disks (Oxoid, CT0998B) were saturated with 25 µL of the suspension. Sample disks were allowed to dry for 4 hours at room temperature in a biosafety cabinet (Model A2, Labconco, Kansas City, MO).

3.3 Kanamycin testing

Prepared solution disks were applied in duplicate to the surface of the culture plate along with a negative control disk (blank disk prepared with 25 µL sterile water and dried 4 hours in a biosafety cabinet) and a 30 µg kanamycin positive control disk (Oxoid, CT0026B). Samples were incubated at 35 ± 2°C for 18 hours. After incubation, plates were visually examined for the presence of a clear zone of inhibition around each disk. If the tagatose preparation showed a zone of inhibition similar to the positive control, it was deemed to be positive for the presence of kanamycin.

4.0 RESULTS AND SUMMARY

Table 1: Zone of Inhibition Results

	Zone of Inhibition			
Tagatose Sample	Test Disk		Negative Control Disk	Positive Control Disk (30 µg Kanamycin)
	1	2		
1 (TAG 3)	None	None	None	26 mm

The results of the antimicrobial susceptibility testing, using a modified disk diffusion method, show that the tagatose product, D-Tagatose Lot # 19-HN9705-TAG3, had no observable antimicrobial activity against the bacteria *E.coli*, shown to be susceptible to kanamycin. The results indicate an absence of measurable kanamycin in the product using this test method.

5.0 REFERENCES

AOAC International. 2018. Official Methods of Analysis, Online Edition.

American Public Health Association. 2015. Compendium of Methods for the Microbiological Examination of Foods, 5th Edition.

Clinical and Laboratory Standards Institute. 2012. Performance Standards for Antimicrobial Susceptibility Testing, M100-S22.

Oxoid. 2018. Antimicrobial Susceptibility Test Discs, package insert X7215C, X7215E.

U.S. Department of Agriculture. 2018. Microbiology Laboratory Guidebook, Online Edition.

U.S. Food and Drug Administration. 2018. Bacteriological Analytical Manual, Online Edition.

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6.0 FINAL REPORT APPROVAL

Prepared By:

04/14/2020

Rochelle Gerges
Special Projects Lab Technician II
Eurofins Microbiology Laboratories, Inc.

Date

Approved By:

04/28/2020

Date

Manish Shekhawat
Business Unit Manager
Eurofins Microbiology Laboratories, Inc.
Lancaster, PA

Study Sponsor:

Karen Weikel Digitally signed by Karen Weikel Date: 2020.04.28 20:55:09 -04'00'

Karen Weikel
Bonumose LLC

Date

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Detection of Kanamycin in a Tagatose Product

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2430 New Holland Pike
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1.0 OBJECTIVE

The overall purpose of this study is to screen for the presence of kanamycin in a tagatose sample using a modified Kirby-Bauer disk diffusion method.

2.0 PROTOCOL OVERVIEW

A tagatose sample was assayed for the potential presence of kanamycin by preparing a solution of the sample and saturating it onto blank antimicrobial disks. Prepared disks were placed on the surface of agar plates inoculated with *Escherichia coli*. Plates were incubated and any zone of inhibition developed was compared to a known kanamycin control disk.

3.0 MATERIALS AND METHODS

3.1 Challenge microorganism preparation

The following challenge microorganism was prepared for this study:

- *Escherichia coli* (ATCC #25922)

The culture was prepared from a lyophilized preparation (KWIK-STIK™, Microbiologics, St. Cloud, MN) according to manufacturer's instructions or from stock plates. The culture was transferred to Tryptic Soy Agar (TSA, Neogen, Lansing, MI) and incubated at 35 ± 2°C for 24 ± 2 hours. The culture was used to make a suspension measuring approximately 0.5 according to the McFarland turbidity standard in sterile diluent and was swabbed onto the surface of Mueller-Hinton Agar plates (MHA, Neogen). Plates were then allowed to dry approximately 2-3 minutes before applying test disks.

3.2 Preparation of samples

The following tagatose product was provided by Bonumose LLC:

- D-Tagatose Lot # 19-HN9705-TAG5 02/25/2020

A suspension of the product sample was prepared as a 50% solution by adding 5 g to 5 mL of sterile deionized water and vortexing to mix. Blank antimicrobial disks (Oxoid, CT0998B) were saturated with 25 µL of the suspension. Sample disks were allowed to dry for 4 hours at room temperature in a biosafety cabinet (Model A2, Labconco, Kansas City, MO).

3.3 Kanamycin testing

Prepared solution disks were applied in duplicate to the surface of the culture plate along with a negative control disk (blank disk prepared with 25 µL sterile water and dried 4 hours in a biosafety cabinet) and a 30 µg kanamycin positive control disk (Oxoid, CT0026B). Samples were incubated at 35 ± 2°C for 18 hours. After incubation, plates were visually examined for the presence of a clear zone of inhibition around each disk. If the tagatose preparation showed a zone of inhibition similar to the positive control, it was deemed to be positive for the presence of kanamycin.

4.0 RESULTS AND SUMMARY

Table 1: Zone of Inhibition Results

	Zone of Inhibition			
Tagatose Sample	Test Disk		Negative Control Disk	Positive Control Disk (30 µg Kanamycin)
	1	2		
1 (TAG 5)	None	None	None	25 mm

The results of the antimicrobial susceptibility testing, using a modified disk diffusion method, show that the tagatose product, D-Tagatose Lot # 19-HN9705-TAG5, had no observable antimicrobial activity against the bacteria *E.coli*, shown to be susceptible to kanamycin. The results indicate an absence of measurable kanamycin in the product using this test method.

5.0 REFERENCES

AOAC International. 2018. Official Methods of Analysis, Online Edition.

American Public Health Association. 2015. Compendium of Methods for the Microbiological Examination of Foods, 5th Edition.

Clinical and Laboratory Standards Institute. 2012. Performance Standards for Antimicrobial Susceptibility Testing, M100-S22.

Oxoid. 2018. Antimicrobial Susceptibility Test Discs, package insert X7215C, X7215E.

U.S. Department of Agriculture. 2018. Microbiology Laboratory Guidebook, Online Edition.

U.S. Food and Drug Administration. 2018. Bacteriological Analytical Manual, Online Edition.

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6.0 FINAL REPORT APPROVAL

Prepared By:

04/14/2020

Rochelle Gerges
Special Projects Lab Technician II
Eurofins Microbiology Laboratories, Inc.

Date

Approved By:

04/28/2020

Date

Manish Shekhawat
Business Unit Manager
Eurofins Microbiology Laboratories, Inc.
Lancaster, PA

Study Sponsor:

Karen Weikel Digitally signed by Karen Weikel Date: 2020.04.28 20:52:32 -04'00'

Karen Weikel
Bonumose LLC

Date

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Detection of Kanamycin in a Tagatose Product

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1.0 OBJECTIVE

The overall purpose of this study is to screen for the presence of kanamycin in a tagatose sample using a modified Kirby-Bauer disk diffusion method.

2.0 PROTOCOL OVERVIEW

A tagatose sample was assayed for the potential presence of kanamycin by preparing a solution of the sample and saturating it onto blank antimicrobial disks. Prepared disks were placed on the surface of agar plates inoculated with *Escherichia coli*. Plates were incubated and any zone of inhibition developed was compared to a known kanamycin control disk.

3.0 MATERIALS AND METHODS

3.1 Challenge microorganism preparation

The following challenge microorganism was prepared for this study:

- *Escherichia coli* (ATCC #25922)

The culture was prepared from a lyophilized preparation (KWIK-STIK™, Microbiologics, St. Cloud, MN) according to manufacturer's instructions or from stock plates. The culture was transferred to Tryptic Soy Agar (TSA, Neogen, Lansing, MI) and incubated at 35 ± 2°C for 24 ± 2 hours. The culture was used to make a suspension measuring approximately 0.5 according to the McFarland turbidity standard in sterile diluent and was swabbed onto the surface of Mueller-Hinton Agar plates (MHA, Neogen). Plates were then allowed to dry approximately 2-3 minutes before applying test disks.

3.2 Preparation of samples

The following tagatose product was provided by Bonumose LLC:

- D-Tagatose Lot # 19-HN9705-TAG9 02/29/2020

A suspension of the product sample was prepared as a 50% solution by adding 5 g to 5 mL of sterile deionized water and vortexing to mix. Blank antimicrobial disks (Oxoid, CT0998B) were saturated with 25 µL of the suspension. Sample disks were allowed to dry for 4 hours at room temperature in a biosafety cabinet (Model A2, Labconco, Kansas City, MO).

3.3 Kanamycin testing

Prepared solution disks were applied in duplicate to the surface of the culture plate along with a negative control disk (blank disk prepared with 25 µL sterile water and dried 4 hours in a biosafety cabinet) and a 30 µg kanamycin positive control disk (Oxoid, CT0026B). Samples were incubated at 35 ± 2°C for 18 hours. After incubation, plates were visually examined for the presence of a clear zone of inhibition around the each disk. If the tagatose preparation showed a zone of inhibition similar to the positive control, it was deemed to be positive for the presence of kanamycin.

4.0 RESULTS AND SUMMARY

Table 1: Zone of Inhibition Results

	Zone of Inhibition			
Tagatose Sample	Test Disk		Negative Control Disk	Positive Control Disk (30 µg Kanamycin)
	1	2		
1 (TAG 9)	None	None	None	25 mm

The results of the antimicrobial susceptibility testing, using a modified disk diffusion method, show that the tagatose product, D-Tagatose Lot # 19-HN9705-TAG9, had no observable antimicrobial activity against the bacteria *E.coli*, shown to be susceptible to kanamycin. The results indicate an absence of measurable kanamycin in the product using this test method.

5.0 REFERENCES

AOAC International. 2018. Official Methods of Analysis, Online Edition.

American Public Health Association. 2015. Compendium of Methods for the Microbiological Examination of Foods, 5th Edition.

Clinical and Laboratory Standards Institute. 2012. Performance Standards for Antimicrobial Susceptibility Testing, M100-S22.

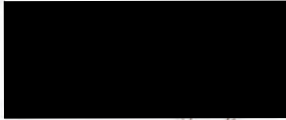
Oxoid. 2018. Antimicrobial Susceptibility Test Discs, package insert X7215C, X7215E.

U.S. Department of Agriculture. 2018. Microbiology Laboratory Guidebook, Online Edition.

U.S. Food and Drug Administration. 2018. Bacteriological Analytical Manual, Online Edition.

6.0 FINAL REPORT APPROVAL

Prepared By:



04/02/2020

Rochelle Gerges
Special Projects Lab Technician II
Eurofins Microbiology Laboratories, Inc.

Date

Approved By:

04/03/2020

Manish Shekhawat
Business Unit Manager
Eurofins Microbiology Laboratories, Inc.

Date

Study Sponsor:

4/3/2020

Karen Weikel
Bonumose LLC

Date

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REPORT OF ANALYSIS

Customer: **Bonumuse LLC**
1725 Discovery Drive Suite 220
Charlottesville, VA 22911
Attn:Karen Weikel

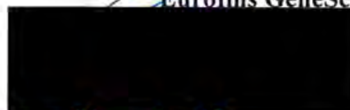
Date Received: 02/26/20
Report Date: 03/04/20

‡Description: SAMPLE #2 LOT# 19-HN9705-TAG3 PRODUCED 2-22-20
Lab Number: CI46604
Commodity: D-TAGATOSE

Analysis	Result	Units	Analyzed
PCR Qualitative - nptII	Negative	NA	03/04/20

‡The results shown in this report relate solely to the item submitted for analysis.

ISO/IEC 17025



Eurofins GeneScan

Dr. Frank Spiegelhalter
Executive Vice President

eurofins | GeneScan

REPORT OF ANALYSIS

(Revised 03/04/20)

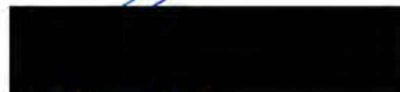
Customer:	**Bonumuse LLC** **1725 Discovery Drive Suite 220** **Charlottesville, VA 22911** **Attn:Karen Weikel**
Date Received:	02/26/20
Report Date:	03/04/20
‡Description:	SAMPLE #3 LOT# 19-HN9705-TAG5 PRODUCED 2-25-20
Lab Number:	CI46605
Commodity:	D-TAGATOSE

Analysis	Result	Units	Analyzed
PCR Qualitative - nptII	Negative	NA	03/04/20

‡The results shown in this report relate solely to the item submitted for analysis.

ISO/IEC 17025

Eurofins GeneScan



Dr. Frank Spiegelhalter
Executive Vice President

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REPORT OF ANALYSIS

Customer: **Bonumuse LLC**
1725 Discovery Drive Suite 220
Charlottesville, VA 22911
Attn:Karen Weikel

Date Received: 03/16/20
Report Date: 03/19/20

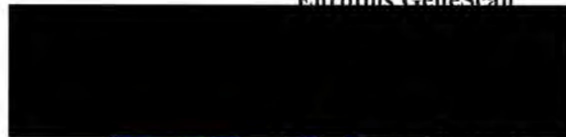
‡Description: SAMPLE #1 LOT# 19-HN9705-TAG9 PRODUCED 2129
Lab Number: CI49652
Commodity: D-TAGATOSE

Analysis	Result	Units	Analyzed
PCR Qualitative - nptII	Negative	NA	03/19/20

‡The results shown in this report relate solely to the item submitted for analysis.

ISO/IEC 17025

Eurofins GeneScan



Dr. Frank Spiegelhalter
Executive Vice President

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Bonumose LLC
1725 Discovery Drive
Charlottesville, VA 22911

Received: 2/25/2020
Reported: 4/09/2020 10:48
Project# 2002375-02Re
PO# 100219-1

Attn: Karen Weikel

Certificate of Analysis

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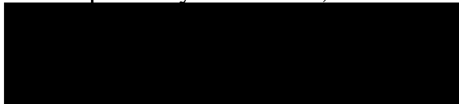
Note: This report replaces the CofA from 03/12/2020 13:18. This report was issued to separate each sample onto an individual report.

Sample Identification: D-tagatose Sample 2, Lot 19-HN9705-TAG3

Analysis:	Specification	Result	Method
Identification, FTIR	To Pass Test	Passes Test	FCC 11
Assay, Dried Basis	98% Min	99%	FCC 11
Lead	0.1 mg/kg Max	< 0.014 mg/kg	USP <233>
Loss on Drying	0.5% Max	< 0.1%	FCC 11
Melting Range or Temperature	133-137°C	134°C	FCC 11
Optical (Specific) Rotation	-4° to -5.6°	-4.9°	FCC 11
Appearance	Report	White Crystals	Visual
Sulfite	Report	< 10 ppm	AOAC 990.28

Respectfully Submitted,



Kelly Peshman, M.S.
Group Leader
Scientist

| Eurofins S-F Analytical Laboratories | 2345 South 170th Street | New Berlin, WI 53151 |
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| ISO/IEC-17025:2017 Accredited A2LA Cert # 4204.01 | State of Wisconsin Dept of Natural Resources Certified Laboratory Id: 241249360 |
| DEA Registration Number RE0533249 | FDA Registration Number 3014704316 |

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Bonumose LLC
1725 Discovery Drive
Charlottesville, VA 22911

Received: 2/25/2020
Reported: 4/09/2020 10:49
Project# 2002375-03Re
PO# 100219-1

Attn: Karen Weikel

Certificate of Analysis

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Note: This report replaces the CofA from 03/12/2020 13:18. This report was issued to separate each sample onto an individual report.

Sample Identification: D-tagatose Sample 3, Lot 19-HN9705-TAG5

Analysis:	Specification	Result	Method
Identification, FTIR	To Pass Test	Passes Test	FCC 11
Assay, Dried Basis	98% Min	100%	FCC 11
Lead	0.1 mg/kg Max	< 0.014 mg/kg	USP <233>
Loss on Drying	0.5% Max	0.1%	FCC 11
Melting Range or Temperature	133-137°C	134°C	FCC 11
Optical (Specific) Rotation	-4° to -5.6°	-5.4°	FCC 11
Appearance	Report	White Crystals	Visual
Sulfite	Report	< 10 ppm	AOAC 990.28

Respectfully Submitted,

Kelly Peshman, M.S.
Group Leader
Scientist

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Bonumose LLC
1725 Discovery Drive
Charlottesville, VA 22911

Received: 3/16/2020
Reported: 3/25/2020 07:46
Project# 2003237-01
PO# 031320-01

Attn: Karen Weikel

Certificate of Analysis

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Sample Identification: D-tagatose Sample 1, Lot 19-HN9705-TAG9 100g

Analysis:	Specification	Result	Method
Identification, FTIR	To Pass Test	Passes Test	FCC 11
Assay, Dried Basis	98% Min	100%	FCC 11
Lead	0.1 mg/kg Max	< 0.1mg/kg	USP <233>
Loss on Drying	0.5% Max	< 0.5%	FCC 11
Melting Range or Temperature	133-137°C	133°C	FCC 11
Optical (Specific) Rotation	-4° to -5.6°	-4.6°	FCC 11
Appearance	Report	Fine, White Crystalline Powder	Visual
Sulfite	Report	< 10 ppm	AOAC 990.28[1]

[1]Note: Subcontracting Partners: Eurofins Scientific Inc. (Des Moines)

Respectfully Submitted,

Kelly Peshman, M.S.
Group Leader
Scientist

VII.B.2. Heavy metal analyses of D-tagatose

As mentioned in Sections II.F. and VI.C.1.a., heavy metal analysis by ICP-MS was carried out in 3 non-consecutive lots of D-tagatose. The results are summarized in Table 7 and documentation is included herein. These data continue to support the conclusion that D-tagatose produced by a novel enzymatic cascade is safe for consumption.

Table 7. Heavy metal analyses of D-tagatose.

Analyte	Acceptable Target Range	Lot # 19-HN9705-TAG18 Results	Lot # 19-HN9705-TAG22 Results	Lot # 19-HN9705-TAG25 Results	Method of Analysis
Arsenic	< 100 ppb	< 10 ppb	< 10 ppb	< 10 ppb	FDA EAM 4.7
Cadmium	< 100 ppb	< 10 ppb	< 10 ppb	< 10 ppb	FDA EAM 4.7
Lead	< 100 ppb	< 10 ppb	< 10 ppb	< 10 ppb	FDA EAM 4.7
Mercury	< 100 ppb	< 10 ppb	< 10 ppb	< 10 ppb	FDA EAM 4.7

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REPORT #: 397390
PROJECT ID: NY08812-2003-005
REPORT DATE: 8/24/20
PRINT DATE: 8/24/20

ASR GROUP (FL)
3998 FAU BOULEVARD SUITE # 100
BOCA RATON, FL 33431
ATT: MICHELLE TITTL

PO #: 4500721394

LAB #: 1066631 **DATE RECEIVED:** 3/25/20

PRODUCT: GRANULATED TAGATOSE
PACKAGE: IN A WHIRL PAK
LOT/BATCH #: 19-HN9705-TAG18
SAMPLE ID: 1

ANALYTE	RESULT	UNITS	METHOD REFERENCE
METALS BY ICP-MS			FDA EAM 4.7
ARSENIC	<10.0	ppb	
CADMIUM	<10.0	ppb	
MERCURY	<10.0	ppb	
LEAD	<10.0	ppb	

Hesham A Elgaali
Laboratory Director

END OF REPORT

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Certificate of Analysis

REPORT #: 397391
PROJECT ID: NY08812-2003-005
REPORT DATE: 8/24/20
PRINT DATE: 8/24/20

ASR GROUP (FL)
3998 FAU BOULEVARD SUITE # 100
BOCA RATON, FL 33431
ATT: MICHELLE TITTL

PO #: 4500721394

LAB #: 1066632 **DATE RECEIVED:3/25/20**

PRODUCT: GRANULATED TAGATOSE
PACKAGE: IN A WHIRL PAK
LOT/BATCH #: 19-HN9705-TAG22
SAMPLE ID: 2

ANALYTE	RESULT	UNITS	METHOD REFERENCE
METALS BY ICP-MS			FDA EAM 4.7
ARSENIC	<10.0	ppb	
CADMIUM	<10.0	ppb	
MERCURY	<10.0	ppb	
LEAD	<10.0	ppb	

Hesham A Elgaali
Laboratory Director

END OF REPORT

This report may not be reproduced for advertising or trade purposes over our signature or in connection with our name without prior written approval. Opinions or analytical result reflect sample as received. When sampling is contracted to Certified Laboratories, statistical methods or methods as defined by regulatory agencies are applied but we make no guarantee that this sample is representative of the product/lot as whole.

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Certificate of Analysis

REPORT #: 397392
PROJECT ID: NY08812-2003-005
REPORT DATE: 8/24/20
PRINT DATE: 8/24/20

ASR GROUP (FL)
3998 FAU BOULEVARD SUITE # 100
BOCA RATON, FL 33431
ATT: MICHELLE TITTL

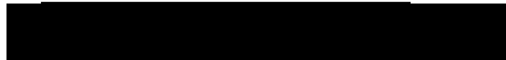
PO #: 4500721394

LAB #: 1066633 **DATE RECEIVED:3/25/20**

PRODUCT: GRANULATED TAGATOSE
PACKAGE: IN A WHIRL PAK
LOT/BATCH #: 19-HN9705-TAG25

SAMPLE ID: 3

ANALYTE	RESULT	UNITS	METHOD REFERENCE
METALS BY ICP-MS			FDA EAM 4.7
ARSENIC	<10.0	ppb	
CADMIUM	<10.0	ppb	
MERCURY	<10.0	ppb	
LEAD	<10.0	ppb	



Hesham A Elgaali
Laboratory Director

END OF REPORT

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From:	Karen Weikel
To:	Kampmeyer, Christopher
Cc:	Andrey Nikiforov; Julia Parker; Ed Rogers
Subject:	GRAS Notice for D-tagatose for Use as an Ingredient in Multiple Food Categories
Date:	Monday, February 1, 2021 12:12:07 PM

Dear Mr. Kampmeyer,

Dr. Nikiforov at Toxicology Regulatory Services passed along your question about Bonumose's D-tagatose GRAS Notice (000977). D-tagatose is not intended for use in any products under the jurisdiction of the United States Department of Agriculture.

Please let us know if you have any additional questions.

Best,
Karen Weikel

--
Karen Weikel, PhD
Director of Nutrition Science
Bonumose LLC
1725 Discovery Drive, Suite 220
Charlottesville, VA 22911
www.bonumose.com
kweikel@bonumose.com

From: Karen Weikel <kweikel@bonumose.com>
Sent: Tuesday, March 16, 2021 3:32 PM
To: Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov>
Cc: Andrey Nikiforov <ANikiforov@toxregserv.com>; Julia Parker <jparker@toxregserv.com>; Ed Rogers <erogers@bonumose.com>; Dan Wichelecki <daniel@bonumose.com>
Subject: [EXTERNAL] GRAS Notice 000977: D-tagatose Request for Clarification

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Mr. Kampmeyer,

We understand that one of your review teams requested information about the source/production organism for pullulanase in Bonumose's GRAS notice for D-tagatose.

As indicated in our GRAS notice, the pullulanase used in D-tagatose production is already GRAS. Bonumose considers the source/production organism for that pullulanase to be confidential information, and we also understand that FDA does not want any confidential information.

What would you suggest to be the best way to handle this?

Thank you for your help,

Karen Weikel

--

Karen Weikel, PhD

Director of Nutrition Science

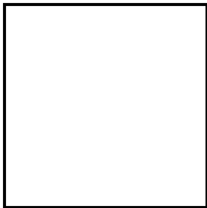
Bonumose, Inc.

1725 Discovery Drive, Suite 220

Charlottesville, VA 22911

www.bonumose.com

kweikel@bonumose.com



--
Karen Weikel, PhD
Director of Nutrition Science
Bonumose, Inc.
1725 Discovery Drive, Suite 220
Charlottesville, VA 22911
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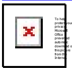
kweikel@bonumose.com



Kampmeyer, Christopher

From:	Karen Weikel <kweikel@bonumose.com>
Sent:	Wednesday, March 17, 2021 9:12 AM
To:	Kampmeyer, Christopher
Cc:	Andrey Nikiforov; Julia Parker; Ed Rogers; Dan Wichelecki
Subject:	Re: [EXTERNAL] GRAS Notice 000977: D-tagatose Request for Clarification

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Hi Chris,

Absolutely. I confirm that the source organism is nonpathogenic and nontoxigenic.

Please let me know if any more questions arise.

Best,
Karen

On Wed, Mar 17, 2021 at 9:10 AM Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov> wrote:

> Hi Karen,
>
> Because the source organism for the pullulanase is considered to be confidential, could you please verify that the source organism is nonpathogenic and nontoxigenic?
>
> Thank you,
>
> Chris
>
> **Chris Kampmeyer, M.S.**
>
> *Staff Fellow (Biologist)*
>
> *Division of Food Ingredients*
>
> **Center for Food Safety and Applied Nutrition**
>
> **Office of Food Additive Safety**
>
> **U.S. Food and Drug Administration**
>
> christopher.kampmeyer@fda.hhs.gov

From: Karen Weikel
To: Kampmeyer, Christopher
Cc: Bonnette, Richard; Julia Parker; Andrey I. Nikiforov; Ed Rogers
Subject: [EXTERNAL] GRN 000977 Clarification
Date: Monday, September 13, 2021 2:58:07 PM

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Mr. Kampmeyer,

We have identified a discrepancy in a D-tagatose manufacturing detail between its description in Bonumose's GRAS Notification for D-tagatose Produced by a Novel Enzymatic Cascade, GRN 000977, and the actual D-tagatose manufacturing process.

Page 7, Section II.D. of GRN 000977 states, "D-tagatose syrup is then pumped into a crystallizer and spray-dried using a filter dryer before collection of D-tagatose crystals." The word "spray" was incorrectly inserted into this sentence and is also incorrectly included in Figure 1 on p.8 of the notice. D-tagatose has never been and will not be spray-dried in the foreseeable future. D-tagatose has always been dried using a filter dryer. The D-tagatose samples that were analyzed according to GRN 000977 were dried using a filter dryer, and commercially-produced D-tagatose will be dried using a filter dryer.

Both spray-drying and filter drying are established safe processes that are commonly used in food preparation. When performed according to GMP (as our process is) use of one method instead of the other would not alter the safety profile of D-tagatose. The specifications in Table 2 (p.26) of GRN 000977 also represent D-tagatose that is filter dried, as demonstrated by the high purity specification (99%-100%). Such high purity would not have been possible if spray drying were performed, due to the presence of an excipient.

We sincerely apologize for any confusion this may have caused and are happy to answer any questions you have. Please include this correspondence in the file for GRN 000977 so that the records are complete and correct.

Best,

Karen Weikel

--
Karen Weikel, PhD
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