SAFETY ASSESSMENT AND GENERALLY RECOGNIZED AS SAFE (GRAS) NOTIFICATION OF 2'-FUCOSYLLACTOSE FOR USE AS AN INGREDIENT IN FOODS

Notifier:
Amyris, Inc.
5885 Hollis Street, Suite 100
Emeryville, CA 94608

Date:
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1.1 Name and Address of the Notifier

Fernando Garcia
Head, Global Regulatory Affairs
Amyris, Inc.
5885 Hollis Street, Suite 100
Emeryville, CA 94608
+1 (510) 450-0761

1.2 Name of Notified Substance

The name and descriptive term of this notified food ingredient is 2'-fucosyllactose (2'-FL) produced using a genetically engineered strain of *Saccharomyces cerevisiae*.

1.3 Conditions of Intended Uses in Food

Amyris' 2'-FL is intended for use as a food ingredient in term infant formulas, toddler formulas, baby foods and drinks for toddlers and young children, various uses in conventional foods and beverages intended for children and adults. The specific uses of Amyris 2'-FL product, and the typical and maximum concentrations are detailed in Table 1.

Table 1. Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the U.S.

Food Category	Proposed Food Use	RACC[a]	Proposed Use Level (g/RACC or g/serving)	Proposed Maximum Use level (g/kg or g/L)[b]
Dairy	Unflavored Pasteurized and Sterilized milk	240 mL	0.28	1.2
	Buttermilk	240 mL	0.28	1.2
	Yogurt	225 g	1.2	5.3
	All acidophilus or fortified milks, non-fat and low-fat milk fluids, including fluid milk and reconstituted milk powder	240 mL	0.28	1.2
	Flavored milks, including chocolate milk, coffee drinks, cocoa, smoothies (dairy and fruit based), other fruit and dairy combinations, yogurt drinks and fermented milk drinks including kefir	240 mL	0.28	1.2
	Frozen dairy desserts including ice cream and frozen yogurts, frozen novelties	~70 g	1.2	17
	Milk product for pregnant women ("mum formulas") -9 to 0 months	200 mL	1.2	6.0
Dairy analogs	Milk substitutes such as soy milk and imitation milks	240 mL	0.28	1.4
	Non-dairy yogurt	225 g	1.2	5.3
Other	Syrups used to flavor milk beverages	40 g	0.28	7.0
	Dairy based pudding custards and mousses	~70 g	1.2	17
	Fruit pie filling	85 g	1.2	14.1

Table 1. Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the U.S.				
Food Category	**Proposed Food Use**	**RACC[a]**	**Proposed Use Level (g/RACC or g/serving)**	**Proposed Maximum Use level (g/kg or g/L)[b]**
	Fruit preparation such as fruit filing in bars, cookies, yogurt and cakes	~40 g	1.2	30
	Jellies and jams, fruit preserved and fruit butters	~20 g	1.2	60
Infant formulas, Follow-on formula, and baby foods	Infant formula (non-exempt formula)	100 mL	0.24	2.4
	Toddler formulas, growing-up milks (12-36 months)	100 mL	0.24	2.4
	Processed cereal-based food and baby food for infants and young children	7 to 170 g	0.084 to 2.04	12
	Other baby Foods for Infants and Young Children	7 to 170 g	0.084 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2
	Infant meal replacement products	100 mL	0.24	2.4 (0.4 g/100kcal)
	Ready-to-eat, ready-to-serve, hot cereals	15 g (dry) 110 g (ready-to-serve)	1.2	10.9 (as consumed)
	Yogurt and juice beverages ("baby drinks")	120 mL	1.2	10
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type dessert")	110 g or mL	1.2	10.9
	Baby crackers, pretzels, cookies and snacks items	7 g	0.4	57
	Milk-based drinks and similar products intended for young children	120 mL	0.14	1.2
	Milk modifiers (i.e. powder for addition in milk such as cacao-based powders, etc.)	120 mL (ready to serve)	0.14	1.2
Meal substitutes	Oral nutritional supplements and enteral tube feeding (11 years and older)	200 g or mL	4.0	20
	Milk-based meal replacement beverages or diet beverages / meal replacement drinks for weight reduction (milk-based and non milk-based)	240 mL	1.2	5.0
	Meal replacement bars for weight reduction	30g	1.2	40
Grain products	Grain bars, including snack bars, meal replacement bars, and breakfast bars	40g	0.48	12
Breakfast cereals	Ready-to-eat breakfast cereals for adults and children - puffed	15g	1.2	80
	Ready-to-eat breakfast cereals for adults and children – high-fiber	40g	1.2	30
	Ready-to-eat breakfast cereals for adults and children – biscuit types	60g	1.2	20

Table 1. Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the U.S.				
Food Category	**Proposed Food Use**	**RACC[a]**	**Proposed Use Level (g/RACC or g/serving)**	**Proposed Maximum Use level (g/kg or g/L)[b]**
	Hot cereals for adults and children	40g (dry) (~240g prepared)	1.2	4.8 (as consumed)
Beverage	Flavored drinks	360 mL	0.28	0.8
	Energy drinks	360 mL	0.28	0.8
	Fitness and thirst quenchers, sport and isotonic drinks / sport, isotonic drinks	360 mL	0.28	0.8
	Fruit drink, including vitamin and mineral-fortified products	240 mL	0.28	1.2
	Fruit juices / fruit juices and nectar	240 mL	0.28	1.2

[a] RACC = Reference Amounts Customarily Consumed per Eating Occasion in the U.S. Code of Federal Regulations (21 CFR 101.12)

[b] Proposed maximum use level is presented on g/kg basis for solids, and g/L basis for liquids and forms. The basis for the calculation of Estimated Daily Intake is presented in Tables 9a and 9b

1.4 Statutory Basis for GRAS Conclusion

The use of 2'-FL as an ingredient in food at the levels described herein has been determined to be safe and generally recognized as safe (GRAS), based on scientific procedures, in accordance with the Federal Food, Drug and Cosmetic Act (FFDCA), Section 201(s) and Section 170.30 of Part 21 of the Code of Federal Regulations (21 CFR 170.30).

Amyris organized a panel of experts (the "GRAS Panel") qualified by training and experience to evaluate the safety of food and food ingredients. This GRAS Panel evaluated the safety of the 2'-FL food ingredient, the intended conditions of use, and the proposed intake based on generally available and accepted information. Conclusions of the GRAS status for 2'-FL for the uses described in Table 1, are based on data provided by Amyris and publicly available literature. The safety dossier incorporates publicly available information regarding the safety of 2'-FL including published reports of toxicological and clinical studies, and estimates of the potential human exposure to 2'-FL resulting from its intended use. Amyris and the GRAS Panel independently concluded that the uses of 2'-FL described herein are safe and GRAS based on scientific procedures.

1.5 Exemption from Premarket Approval of the FFDCA

Amyris determined that the proposed food ingredient uses of 2'-FL, described in Table 1, are exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (FFDCA) because Amyris has determined the proposed uses to be safe and GRAS (21 CFR §170.30). This determination was made in compliance with the Substances Generally Recognized as Safe regulation (Federal Register, Vol. 81, No. 159, 54960, August 17, 2016; 21 CFR §170.203).

The uses of 2'-FL described herein have been determined by Amyris as safe and GRAS for the proposed levels of inclusion in term infant formulas and toddler formulas and in various commercial food and beverage uses based upon scientific procedures, and thus, these uses of 2'-FL are excluded from the definition of a food additive, and not subject to the premarket approval requirements of Section 201(s) of the FFDCA, and may be used in the U.S. without the promulgation of a food additive regulation by the United States Food and Drug Administration (FDA) under 21 CFR.

1.6 Availability of Information

The data and information that serve as the basis for this GRAS Notification regarding the conclusion of the GRAS status of the food ingredient use of Amyris 2’-FL are available to the United States Food and Drug Administration (FDA) upon request as follows:

(i) Amyris agrees to make the data and information available to FDA; and

(ii) Amyris will allow FDA to review and copy the data and information as provided at 21 CFR §170.225(c)(7).

1.7 Freedom of Information Act (FOIA)

None of the data and information in this GRN is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

1.8 Certification

This GRAS notice was compiled in accordance with the rules and regulations set out in 21 CFR Part 170, Subpart E. The Notifier certifies to the its knowledge, this GRAS notice is a complete, representative, and balanced submission, including both favorable and unfavorable information that is known to the Notifier and pertinent to the evaluation of the safety and GRAS status of the food ingredient uses of Amyris’ 2’-FL.

1.9 Name, Position, and Signature of Certifier

May 11, 2020

Fernando Garcia
Head, Global Regulatory Affairs
Amyris, Inc.
5885 Hollis Street, Suite 100
Emeryville, CA 94608
+1 (510) 450-0761
Garcia@amyris.com

PART 2: IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT. (21 CFR§ 170.230)

2.1 Chemical Identity and Structure of 2'-FL

Common Name: 2'-fucosyllactose or 2'-fucosyl-D-lactose

Abbreviation: 2'-FL

IUPAC Name: α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranoside

Chemical Abstracts Service Registry Number (CASRN): 41263-94-9

Chemical Formula: $C_{18}H_{32}O_{15}$

Molecular Weight (MW): 488.44 g / mole (mol)

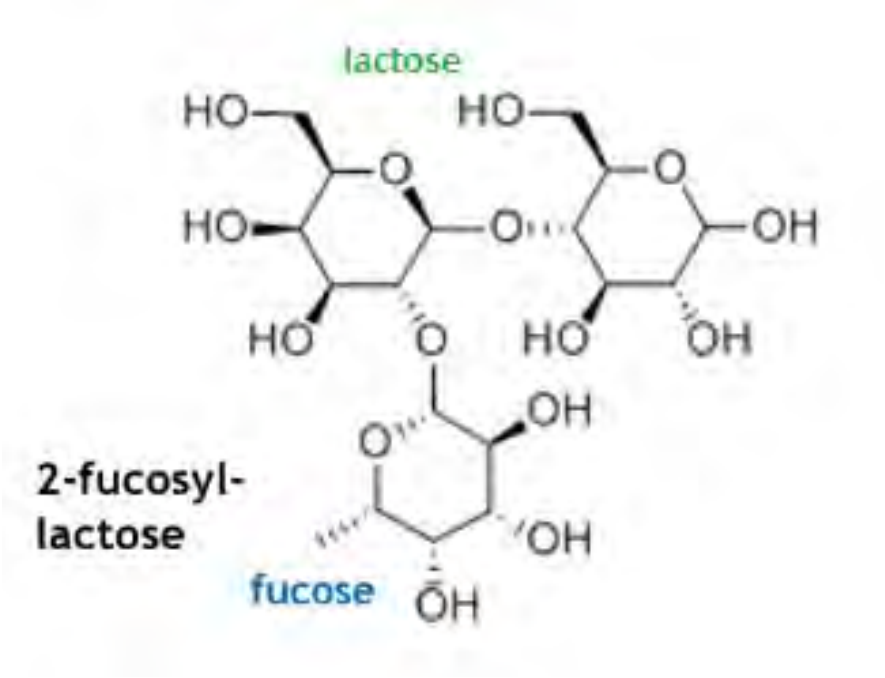

Figure 1. Chemical Structure of 2'-FL

2.2 Physical and Chemical Properties

2'-FL is a naturally occurring oligosaccharide consisting of lactose (galactose and glucose) and fucose that is found in human breast milk. The chemical structure of 2'-FL was determined (shown in Figure 1) in 1954 through nuclear magnetic resonance techniques and x-ray crystallography (Castanys-Munoz et al., 2013). 2'-FL is a naturally-occurring oligosaccharide found in human breast milk, and one of the most abundant human milk oligosaccharides (HMOs). Amyris' 2'-FL, manufactured by Amyris, is produced by fermentation with *Saccharomyces cerevisiae* ("*S. cerevisiae*") strain CEN.PK113-7D. Analytical grade human breast milk 2'-FL from Carbosynth (by high-performance anion-exchange chromatography with pulsed amperometric detection [HPAEC/PAD], Carbosynth 2017) was used as the reference material. The chemical and physical properties for the Carbosynth 2'-FL and the Amyris 2'-FL are provided in Table 2. A comparison of the proton NMR spectra for the Carbosynth 2'-FL and the Amyris 2'-FL demonstrate structural equivalence (Appendix A).

Table 2. Chemical and Physical Properties of Reference 2'-FL and Amyris 2'-FL			
Property	**Carbosynth 2'-FL**	**Amyris 2'-FL**	**Method**
Molecular weight	488.44 AMU	488.44 AMU	Appendix A
Structural analysis	Conforms to Structure	Conforms to Structure	Proton NMR spectra (Appendix A)
Appearance (Form)	Powder	Powder	Visual
Appearance (Color)	White	White to off-white/ivory	Visual
Abbreviations: AMU = atomic mass unit; N/A = Not Applicable; NMR = Nuclear Magnetic Resonance.			

2.3 Manufacturing Process

Construction of Production Strain: *S. cerevisiae* strain CEN.PK113-7D was genetically engineered to contain the biosynthetic pathway for 2'-FL through site-specific genomic integrations of deoxyribonucleic acid (DNA) constructs via homologous recombination at stable, non-essential regions of the genome. Promoters and terminators used to express the genes are native to *S. cerevisiae*, and include but are not limited to, promotors of GAL1 and GAL10 proteins, and terminators of PGK1 and CYC1. A summary of the enzymes and their respective functions in Amyris' production strain can be found in Table 3. DNA constructs consisting of genomic DNA homologous to the upstream and downstream DNA sequence of the desired integration site are inserted into the yeast genome via standard methods described in Rothstein (1991). A single DNA construct may contain one-to-four open reading frames, which consist of a native yeast promotor and terminator and a gene of interest required for 2'-FL production. DNA constructs with more than one open reading frame may contain spacer DNA obtained from amplified genomic DNA of *E. coli* K-12 to prevent interference during transcription. Spacer DNA constructs are used as structural DNA elements inside of the engineered integrations as they do not have sequence homology to yeast chromosomes. Spacer DNA does not express heterologous proteins as they do not encode functional protein sequences and/or do not include promoters expected to allow expression in yeast.

Safety and Suitability of Production Strain

The incorporated DNA to produce 2'-FL is sourced from biosafety level 1 organisms not associated with any known allergens or toxins with the exception of one unclassified genome. This genome was compiled from a groundwater metagenome sample. This phylum consists of primarily uncultured bacteria that likely rely on other microbes in the environment for vital nutrients and are therefore very unlikely to be human pathogens. The parental strain, *S. cerevisiae*, is a stable haploid yeast and therefore does not undergo mating-type switching or mating events (Jensen et al. 1983). The production strain is rendered haploid negative (HO-) by deletion of the HO gene. Replacement with a DNA construct ensures that the production strain remains haploid. The identification of the production strain is confirmed through polymerase chain reaction (PCR) analysis of the inserted DNA construct. Whole genome sequencing of the production strain was used to confirm that the DNA construct was correctly inserted. As the DNA construct was inserted by homologous recombination, the introduced genetic elements are stable, and the production strain does not contain any plasmid or other exogenous mobile genetic elements. The strain stability is demonstrated by using primary and secondary cell banks and comparing productivities. Extended seed trains are routinely tested to ensure retention of phenotype over generations of the production strain. The production strain is not toxigenic or pathogenic, and does not contain or produce any known pathogenicity-related

proteins, toxins, allergens, or pyrogens. Antibiotic resistance markers are not present in the production strain. The yeast cells are separated from the product and inactivated by heat treatment during the recovery and purification steps. Residual yeast, recombinant protein, and DNA are removed during the purification steps. Protein (<0.004%, Appendix B) and yeast DNA (<0.001ng DNA /g 2'-FL, Appendix C) are not detectable in the final product, indicating effective removal of the production strain. The production strain is consistently tested for contaminating bacteria and strain performance according to internal standard operating procedures (SOPs).

Safety and Suitability of Parental Strain: *S. cerevisiae* CEN.PK113-7D, also known as brewer's yeast or baker's yeast, has an extensive history of safe use in the food industry. In the U.S., according to 21 CFR §172.896, dried yeast, including *S. cerevisiae*, is permitted for use in food. Protein isolated from *S. cerevisiae* and the dried cell walls of *S. cerevisiae* (baker's yeast glycan) are food additives permitted for the direct addition to food for human consumption (21 CFR §172.325 and §172.898, respectively). Baker's yeast extract, the concentrated or dried soluble component of mechanically ruptured cells of *S. cerevisiae*, is GRAS for use as a flavoring agent and adjuvant at a level not to exceed 5% in food (21 CFR §184.1983 – U.S. FDA, 2017a). Food enzymes produced by *S. cerevisiae* (e.g., invertase, GRN No. 88) (U.S. FDA 2002) as well as several *S. cerevisiae* strains genetically-modified to alter expression of specific endogenous enzymes or pathways (GRN No. 120, 175, 350, 422, 604) (U.S. FDA, 2002, 2003, 2006, 2011b, 2012, 2016c) have GRAS status with no objection from the U.S. FDA. The taxonomy of the *S. cerevisiae* species is described in Table 4.

S. cerevisiae has also been granted Qualified Presumption of Safety (QPS) status in the European Union by the European Food Safety Authority (EFSA) and is therefore considered safe for the derivation of genetically modified strain lineages intended for use in the production of food additives and enzymes. While new reports of *S. cerevisiae* suggest that consumption by patients with fragile health may be the possible origin of an infection in such patients when it's intended use of the microorganism is to be used as a probiotic for humans, these reports do not change the QPS status of *S. cerevisiae* as long as the following qualification is met in the safety assessment: "Absence of resistance to antimycotics used for medical treatment of yeast infections in cases where viable cells are added to the food or feed chain. In the case of *S. cerevisiae,* this qualification applies for yeast strains able to grow above 37 °C" (EFSA, 2017).

The production strain contains no known pathogenicity-related proteins, toxins, allergens, or pyrogens. The genes used to create the production strain are found in Table 4. Despite the extensive history of safe use of S. cerevisiae in the food industry, rare reports of S. cerevisiae infections in humans indicate that S. cerevisiae can also be regarded as an opportunistic pathogen. A comprehensive review conducted by Enache-Angoulvant and Hennequin (2005) reported 92 cases of *Saccharomyces* invasive infection, with the most common predisposing factors being antibiotic therapy and intravascular catheter. *S. cerevisiae* strain YJM789, for example, was isolated from the lung of an AIDS patient with polymicrobial pneumonia (Twafik et al., 1989; Wei et al., 2007) and de Llanos et al. (2006) reported 4 clinical cases of S. cerevisiae in the blood. Amyris's 2'-FL produced by fermentation does not include any detectable residual protein (<0.004%, Appendix B) or residual DNA (<0.001ng DNA / 1g 2'-FL, Appendix C) from the production organism in the final product.

Table 3. Summary of enzymes and their respective functions in Amyris' 2'-FL	
Enzyme	**Function**
Lactose permease	Lactose import
Guanosine 5'-diphospho-(GDP)-mannose-4,6-dehydratase	Converts GDP-mannose to GDP-4-dehydro-alpha-D-rhamnose
GDP-L-fucose synthase	Converts GDP-4-dehydro-alpha-D-rhamnose to GDP-fucose
Alpha 1,2 fucosyltransferase	Converts GDP-fucose and lactose to 2'-fucosyllactose and difucosyllactose
Dihydrofolate reductase	Reduction of dihydrofolic acid to tetrahydrofolic acid

Table 4: Taxonomic Information on Saccharomyces cerevisiae	
Domain	Eukarya
Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Saccharomycotina
Class	Saccharomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	*Saccharomyces*
Species	*cerevisiae*
Strain	CEN.PK113-7D

Process Description: Amyris' 2'-FL is manufactured by Amyris in accordance with current good manufacturing practice (cGMP) 21 CFR 117. The flow chart for the 2'-FL manufacturing process is shown in Figure 2, with corresponding steps described below.

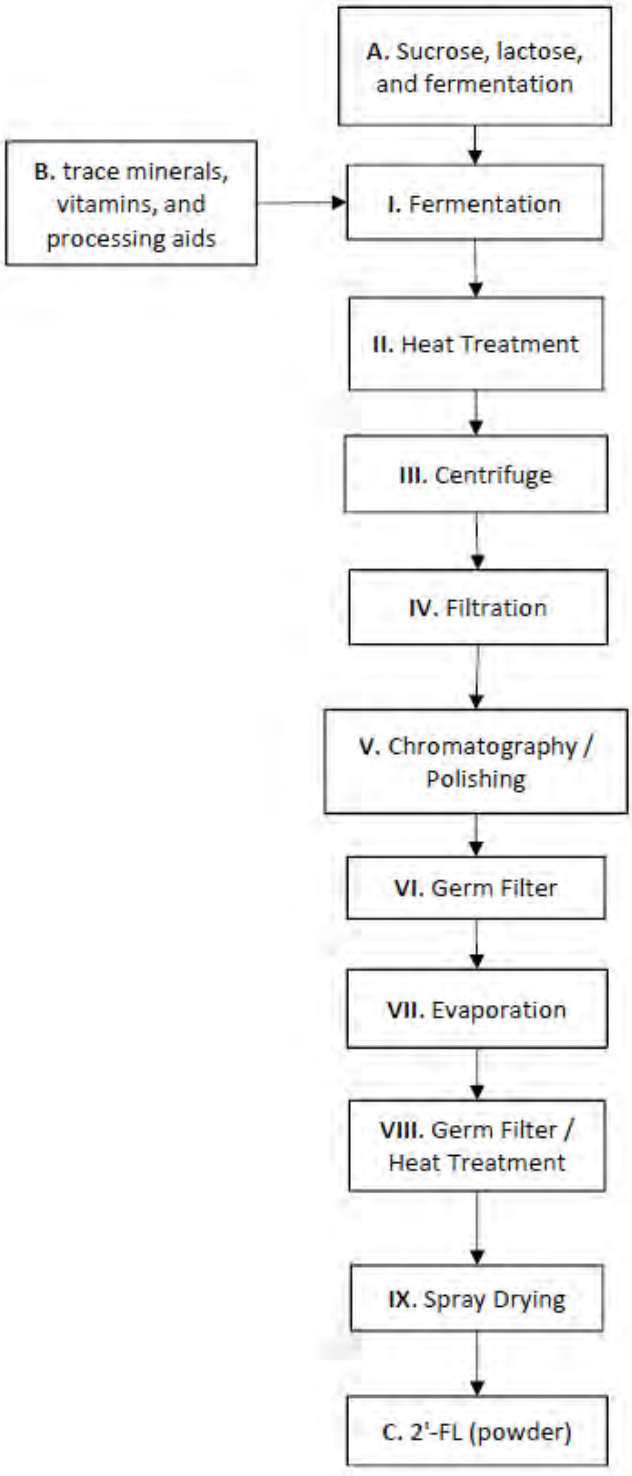

Figure 2: Manufacturing process for Amyris' 2'-FL

In the fermentation process (I), the parental strain *S. cerevisiae* was genetically engineered to contain the biosynthetic pathway for 2'-FL. In the first stage of the manufacturing process, food-grade sugar, lactose, and fermentation nutrients (A) are fed to a culture of the production strain

and fermented to produce 2'-FL and other carbohydrates (listed in Table 5). The lactose-sucrose based fermentation medium is supplemented with trace minerals and vitamins (B) (biotin [21 CFR §582.5159, 21 CFR §182.8159], calcium pantothenate [21 CFR §582.5212, 21 CFR §184.1212], nicotinic acid [21 CFR §184.1530], *myo*-inositol [21 CFR §582.5370, 21 CFR §184.1370], thiamine HCL [21 CFR §582.5875, 21 CFR §184.1875], pyridoxol HCL [21 CFR §582.5676, 21 CFR §184.1676], p-aminobenzoic acid [EAFUS listed]). Food grade processing aids such as antifoam and pH control agents are used in the process in accordance with cGMP and within applicable limitations as specified in the corresponding FDA CFR citations listed (B). Fermentation and production chemicals may contain potassium phosphate (21 CFR §160.110), ammonium sulfate (21 CFR § 582.1143, 21 CFR § 184.1143), magnesium sulfate (21 CFR § 582.5443, 21 CFR § 184.1443), succinate buffer (21 CFR §582.1091, 21 CFR §184.1091), monoammonium phosphate (21 CFR §184.1141a, 21 CFR §582.1141), potassium phosphate (21 CFR §160.110), ethylenediaminetetraacetic acid (21 CFR §172.135), zinc sulfate (21 CFR §582.5997, 21 CFR §182.8997), copper sulfate (21 CFR §184.1261), magnesium chloride (21 CFR §582.5446, 21 CFR §184.1446), sodium molybdate (40 CFR §180.920), iron (II) sulfate (21 CFR §184.1315), calcium chloride (21 CFR §582.1193, 21 CFR §582.6193, 21 CFR §184.1193), and antifoaming agents. The fermentation process is conducted under strictly controlled temperature and pH conditions. The fermentation broth is subjected to a heat treatment step (II) to kill the yeast cells prior to the purification/concentration steps wherein the production strain is removed. The *S. cerevisiae* production strain is not present in the finished product.

After the fermentation process, the 2'-FL fermentation broth is harvested, and most of the biomass is then separated from the aqueous phase by centrifugation (III). Any residual yeast is removed during the separation and purification steps. The clarified centrifuge supernatant is subjected to various filtration steps to remove residual solids, proteins, DNA, salts and organic acids, and to concentrate the solution (IV).

The filtered product is then processed through chromatographic and polishing steps to remove additional salts, metals, proteins, organic acids, and colorants (V) (Appendix D). All processing aids used in the post-fermentation process stage also are also approved for use in food processing. The solution is sterile filtered (VI) to ensure the product stream is sterile and then is concentrated by evaporation (VII) followed by an additional filtering and/or heat treatment step (VIII), if further sterilization is needed. After this purification process, the concentrated product is spray-dried to reduce moisture (IX) to ≤ 5.0 % by weight (w/w). The final product is a solid powder with $\geq$ 86% 2'-FL (C).

Batch analyses indicate absence of protein (Appendix B, Table 6). Residual DNA analysis reveals no residual DNA in the final product (Appendix C). Processing aids are not expected to be present in the final product, based on analytical results found in Table 6. While cobalt chloride is not permitted for use in human food (21 CFR §189.120), Amyris has conducted analyses to confirm that that all cobalt is removed during processing, and the product does not contain cobalt salt. As shown in table 6, cobalt is not detected in the final product (ND< 0.01 mg/kg). Amyris' 2'-FL does not contain or consist of Genetically Modified Organisms (GMOs) as defined in Regulation (EC) 1829/2003 on genetically modified food and feed. With the exception of the production strain, no other genetically engineered ingredients or technology are used in the production of Amyris' 2'-FL.

2.4 Product Specifications for Amyris' 2'-FL

Amyris has established specifications for its 2'-FL to ensure production of a consistent food-grade product. The chemical, physical and microbiological specifications of the product are presented below in Table 5a. Amyris's 2'-FL specifications compared to other 2'-FL specifications from submitted GRNs that have received no-questions letters from FDA, and a 2'-FL/DFL mixture as a novel food (EFSA, 2019), are summarized in Table 5b.

Table 5a: Specifications of Amyris' 2'-Fucosyllactose

Parameter	Specification	Method
Carbohydrate content (% area)		
2'-fucosyllactose	≥ 86% area	By ion chromatography (Amyris SOP 830)
Difucosyllactose (DFL)	< 8% area	
Lactose/allo-lactose	< 7% area	
2'-fucosyllactitol	≤ 6% area	
3-Fucosyllactose (3FL)	< 7% area	
Fucosyl-galactose		
Xylitol		
Dulcitol/sorbitol		
Glucose/Galactose		
Fucose		
Glycerophosphoethanolamine (GPE)		
Fructose		
Chemical		
Water Content (KF titration)	≤5.0% w/w	Karl Fischer titration (Amyris SOP 842)
pH (20 °C, 5% solution)	3.0 – 7.5	EP 2.2.3 v9
Protein Content	≤0.01% w/w	Modified Bradford Assay (Amyris SOP 843)
Total Ash	≤0.5% w/w	FCC 11 appendix II
Arsenic	≤ 0.2 mg/kg	EP 2.2.58 v9
Cadmium	≤ 0.05 mg/kg	EP 2.2.58 v9
Lead	≤ 0.05 mg/kg	EP 2.2.58 v9
Mercury	≤ 0.1 mg/kg	EP 2.2.58 v9
GMO detection (rDNA from production strain)	Negative	PCR (Amyris SOP 844)
Microbial Specifications		
Total Aerobic Microbial Count/Standard Plate Count	≤ 1000 cfu/g	EP 2.6.12 v9
Total Yeast/Mold Count	≤ 100 cfu/g	EP 2.6.12 v9
Sulfite Reducing Bacteria	< 100 cfu/g	ISO 15213: 2003
Enterobacteriaceae	Negative in 10 g	EP 2.6.13 v9
Salmonella	Not detected in 25 g	EP 2.6.13 v9
Cronobacter sakazakii	Not detected in 10 g	ISO/TS 22964
Coliforms	Not detected in 10 g	ISO 4831: 2006

Table 5a: Specifications of Amyris' 2'-Fucosyllactose		
Parameter	**Specification**	**Method**
E. coli	Absent in 10 g	EP 2.6.13 v9
Listeria monocytogenes	Absent in 10 g	ISO 11290-1: 2017
Pseudomonas aeruginosa	Absent in 10 g	EP 2.6.13 v9
Staphylococcus aureus	Negative in 10 g	EP 2.6.13 v9
Bacillus cereus	< 100 cfu/g	ISO 7932: 2004
Source: Amyris, Inc. Abbreviations: °C = degrees Celsius; cfu = colony-forming unit; EP = European Pharmacopoeia; EU = endotoxin units; FCC = Food Chemicals Codex; g = grams; ISO = International Organization for Standardization; KF = Karl Fischer; m = milli; SOP = Standard Operating Procedure; w/w = by weight; v = version.		

Table 5b: Comparison of Amyris' 2'-FL and Other GRAS 2'-FL Specifications									
Parameter	**Units**	**Amyris**	**Glycom (546)**	**Glycom (650)**	**Jennewein (571)**	**Glycosyn (735)**	**DuPont (749)**	**BASF (852)**	**EFSA (2019)[a]**
Carbohydrate content									
2'-fucosyllactose	%	≥ 86	≥95	≥94	≥ 90	≥90	≥ 82	≥90	≥ 75
Difucosyllactose (DFL)	%	< 8	--	≤ 1	≤ 5	--	≤ 7	≤ 2	≥ 5
Other carbohydrates	%	--	--	--	--	--	< 6	--	≤ 6
2'-Fucosyl-D-lactulose	%	--	--	≤ 1	--	--	--	≤ 2	≤ 2
Lactose/allo-lactose	%	< 7	--	≤ 3	≤ 5	≤ 2	≤ 8	≤ 3	≤ 10
2'-fucosyllactitol	%	≤ 6	--	--	--	--	--	--	--
3-Fucosyllactose (3FL)	%	< 7	--	--	≤ 5	--	--	--	--
Fucosyl-galactose	%		--	--	≤ 3	--	--	--	--
Xylitol	%		--	--	--	--	--	--	--
Dulcitol/sorbitol	%		--	--	--	--	--	--	--
Glucose/Galactose	%		--	--	≤ 3	≤ 2	--	--	--
Fucose	%		--	≤ 1	≤ 3	≤ 2	--	≤ 2	≤ 1
Glycerophosphoethanolamine (GPE)	%		--	--	--	--	--	--	--
Fructose	%		--	--	--	--	--	--	--
Chemical									
Water Content (KF titration)	%	≤5.0	≤ 9.0	≤ 5.0	≤ 9	≤ 5.0	≤ 9.0	≤ 9.0	≤ 6.0
pH (20 °C, 5% solution)	--	3.0 – 7.5	3.0 – 7.5	3.2 - 5.0	--	3.0 – 7.5	--	3.2 – 7.5	4.0 - 6.0
Protein Content	% or µg/g	≤0.01%	0.01%	0.01%	≤ 100 µg/g	≤0.01%	≤ 100 µg/g	≤0.01%	≤0.01%
Total Ash	%	≤0.5	≤ 0.2	≤ 1.5	≤ 0.5	≤ 0.2	≤ 0.5	≤ 1.5	≤ 0.8
Acetic Acid	%	--	≤ 0.3	≤ 1	--	--	--	≤ 1	--
Arsenic	mg/kg	≤ 0.2	--	--	≤ 0.2	≤ 0.1	≤ 0.2	≤ 0.1	--
Cadmium	mg/kg	≤ 0.05	--	--	≤ 0.1	≤ 0.1	≤ 0.05	≤ 0.05	--
Lead	mg/kg	≤ 0.05	≤ 0.8	≤ 0.1	≤ 0.02	≤ 0.05	≤ 0.05	≤ 0.05	--
Mercury	mg/kg	≤ 0.1	--	--	≤ 0.5	≤ 0.05	≤ 0.1	≤ 0.05	--

Table 5b: Comparison of Amyris' 2'-FL and Other GRAS 2'-FL Specifications									
Parameter	**Units**	**Amyris**	**Glycom (546)**	**Glycom (650)**	**Jennewein (571)**	**Glycosyn (735)**	**DuPont (749)**	**BASF (852)**	**EFSA (2019)[a]**
GMO detection (rDNA from production strain)	--	Negative	--	--	Negative	Negative	Negative	--	--
Microbial Specifications									
Total Aerobic Microbial Count/Standard Plate Count	mg/kg or cfu/g	≤ 1000 cfu/g	--	≤ 0.1 mg/kg	--	--	≤ 1000 cfu/g	≤ 500 cfu/g	--
Aerobic mesophilic total count	cfu/g	--	≤ 500	≤ 500	≤ 10000	≤ 3000	--	--	≤ 1000
Total Yeast/Mold Count	cfu/g	≤ 100	≤ 10	≤ 10	≤ 100	≤ 10	≤ 100	≤ 100	≤ 100
Sulfite Reducing Bacteria	cfu/g	< 100	--	--	--	< 30	--	--	
Enterobacteriaceae	grams or cfu/g	Negative in 10 g	Absent in 10 g	Absent in 10 g	Absent in 11 g	Absent in 10 g	ND in 10 g	Absent in 10 g	≤ 10 cfu/g
Salmonella	grams	ND in 25	Absent in 25	Absent in 25	Absent in 100	Absent in 25	ND in 100	Absent in 25	Absent in 25
Cronobacter sakazakii	grams	ND in 10	Absent in 10	Absent in 10	Absent in 100	Absent in 25	ND in 10	Absent in 10	Absent in 10
Coliforms	grams	ND in 10	--	--	Absent in 11	--	ND in 10	--	--
E. coli	grams	Absent in 10	--	--	--	Absent in 10	--	--	--
Listeria monocytogenes	grams	Absent in 10	Absent in 25	Absent in 25	--	--	Absent in 25	Absent in 25	--
Pseudomonas aeruginosa	grams	Absent in 10	--	--	--	--	--	--	--
Staphylococcus aureus	grams	Negative in 10	--	--	--	Absent in 1	--	--	--
Bacillus cereus	cfu/g	< 100	≤ 50	≤ 50	--	≤ 100	≤ 10	--	--

Source: Amyris, Inc.

a = EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA). Safety of 2'-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283. This substance is a 2'-FL/DFL mixture.

Abbreviations: -- = not specified or not applicable; % = percent; °C = degrees Celsius; cfu = colony-forming unit; g = grams; k = kilo; KF = Karl Fischer; m = milli; ND = not detected; w/w = by weight.

2.5 Batch Analysis

Production batches of Amyris' 2'-FL were analyzed and the results demonstrate that Amyris' 2'-FL consistently meets the specifications provided in Table 6 (Appendix B, C, D, and E). Batches were analyzed for carbohydrate, water, and protein content (Appendix B); residual DNA (Appendix C); residual sugars (Appendix D); and microbiological analyses (Appendix E), and heavy metal analysis to demonstrate compliance with the current Codex general standards for contaminants and toxins in foods (CODEX STAN 193-1995) (Appendix E). All testing was completed in accordance with Amyris SOPs (843, 830, 842, 844). The results of the batch analyses are tabulated in Table 6 and the certificates of analysis are included in Appendices B and E.

Table 6. Analysis of Amyris' 2'-FL Production Batches							
Parameter	**Specification**	**Batch Number**					**Method**
		H8163*	**H8452**	**H8561**	**H8781**	**H8750**	
Batch Date		2/6/19	8/13/19	9/25/19	10/3/19	10/18/19	
Carbohydrate content (% area)							
2'-fucosyllactose	≥ 86% area	86.06	91.1	92.2	91.2	91.3	By ion chromatography (Amyris SOP 830)
Lactose/allo-lactose	< 8% area	2.18	1.18	0.08	1.43	0.44	
Difucosyllactose (DFL)	< 7% area	0.52	1.40	1.30	1.24	1.25	
2'-fucosyllactitol	≤ 6% area	4.92	3.54	3.93	3.19	3.52	
3-Fucosyllactose (3FL) & Fucosyl-galactose	< 7% area	0.92	0.33	0.38	0.23	0.18	
Sorbitol & Galactitol		0.79	0.14	0.05	0.15	0.10	
Xylitol		0.42	0.73	0.59	0.67	1.30	
Fucose		3.36	0.18	0.12	0.15	0.19	
Glucose & Galactose		0.20	0.26	0.33	0.22	0.52	
Glycerophosphoethanolamine (GPE)		0.24	0.27	0.42	0.53	0.25	
Fructose		0.24	0.40	0.43	0.31	0.46	
Sugar alcohols, other		0.15	--	--	--	--	
Sub-total, minor oligosaccharides and sugar alcohols		6.32	2.31	2.32	2.26	3.0	
Total		100	99.53	99.83	99.32	99.51	
Appearance							
Color	White to off-white/ivory	N/A	Pass	Pass	Pass	Pass	Visual
Form	Dry powder	N/A	Pass	Pass	Pass	Pass	Visual
Appearance in solution (at 5%)	Clean, colorless to slightly yellow	N/A	Pass	Pass	Pass	Pass	Visual
Chemical							
Water Content (KF titration)	≤5%	N/A	2.66	3.07	2.53	2.66	Karl Fischer titration (Amyris SOP 842)
pH (20 °C, 5% solution)	3.0 – 7.5	N/A	5.8	5.3	5.8	5.5	EP 2.2.3 v9
Protein Content (% w/w)	≤0.01% w/w	N/A	<0.004	<0.004	<0.004	<0.004	Modified Bradford Assay (Amyris SOP 843)
Total Ash (% w/w)	≤0.5% w/w	N/A	<0.3%	0.49%	0.33%	<0.3%	FCC 11 appendix II

Table 6. Analysis of Amyris' 2'-FL Production Batches

Parameter	Specification	Batch Number					Method
		H8163*	H8452	H8561	H8781	H8750	
Batch Date		2/6/19	8/13/19	9/25/19	10/3/19	10/18/19	
Arsenic (mg/kg)	≤ 0.2 mg/kg	N/A	ND <0.005	ND <0.005	ND <0.005	ND <0.005	EP 2.2.58 v9
Cadmium (mg/kg)	≤ 0.05 mg/kg	N/A	ND <0.01	ND <0.01	ND <0.01	ND <0.009	EP 2.2.58 v9
Cobalt (mg/kg)	--	N/A	ND <0.01	ND <0.01	ND <0.01	ND <0.01	EP 2.2.58 v9
Lead (mg/kg)	≤ 0.05 mg/kg	N/A	0.004	0.00683	0.00795	0.0123	EP 2.2.58 v9
Mercury (mg/kg)	≤ 0.1 mg/kg	N/A	ND <0.002	ND <0.006	ND <0.002	ND <0.002	EP 2.2.58 v9
Endotoxins (total, EU/g)	--	N/A	ND <100	ND <100	ND <100	ND <100	EP 2.6.14 v9
GMO detection (rDNA from production strain)	Negative	N/A	Negative	Negative	Negative	Negative	PCR (Amyris SOP 844)
Microbial Specifications							
Total Aerobic Microbial Count/Standard Plate Count (cfu/g)	≤ 1000 cfu/g	N/A	240	710	35	45	EP 2.6.12 v9
Total Yeast/Mold Count (cfu/g)	≤ 100 cfu/g	N/A	ND <10	ND <10	ND <10	ND <10	EP 2.6.12 v9
Sulfite Reducing Bacteria (cfu/g)	< 100 cfu/g	N/A	ND <10	ND <10	ND <10	ND <10	ISO 15213: 2003
Bacillus cereus (cfu/g)	<100 cfu/g	N/A	ND <10	ND <10	ND <10	ND <10	ISO 7932: 2004
Enterobacteriaceae	Negative in 10 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9
Salmonella	ND in 25 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9
Cronobacter sakazakii	ND in 10 g	N/A	ND	ND	ND	ND	ISO/TS 22964
Coliforms	ND in 10 g	N/A	ND	ND	ND	ND	ISO 4831: 2006
E. coli	Absent in 10 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9
Listeria monocytogenes	Absent in 25 g	N/A	ND	ND	ND	ND	ISO 11290-1: 2017
Pseudomonas aeruginosa	Absent in 10 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9
Staphylococcus aureus	Negative in 10 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9

Source: Amyris, Inc.

*Batch results are from an initial pilot plant manufacturing lot.

Abbreviations: °C = degrees Celsius; cfu = colony-forming unit; EU = endotoxin units; FCC = Federal Communications Commission Food Chemicals Codex; g = grams; KF = Karl Fischer; k = kilo; m = milli; ND = not detected; w/w = by weight.

N/A = Not analyzed.

2.6 Stability Data

Stability data for batches were developed in accelerated mode (13 weeks, 40 °C, 75% Relative Humidity (RH)) and demonstrate product stability. Carbohydrate and water content were measured at 0, 1, 4, 8, and 13 weeks for the accelerated mode (Table 7). The results of the accelerated stability study equate to 1.5 years shelf life (Appendix F). A description of the assay with the protocol is provided in Appendix F. Study results are provided in Table 7. The results indicate that the product maintains its composition and is stable under accelerated test conditions.

Table 7: Stability of Amyris' 2'-FL Under Accelerated Storage Conditions

2'-FL spray-dried product accelerated stability study (40 °C, 75% RH)

	Specification	0 wk	1 wk	4 wk	8 wk	13 wk
H8452						
2'-fucosyllactose (area %)	≥ 86	91.10	90.70	90.95	90.92	90.55
Allo-Lactose/Lactose (area %)	< 7	1.18	1.48	1.64	1.31	1.15
DFL (area %)	< 8	1.40	0.97	0.91	1.17	0.98
2'-fucosyllactitol (area %)	≤ 6	3.54	3.77	3.69	3.94	3.90
3'-FL & Fucosylgalactose (area %)	< 7	0.33	0.63	0.48	0.34	0.55
Fructose (area %)		0.40	0.37	0.29	0.39	0.55
Glucose & Galactose (area %)		0.26	0.31	0.32	0.24	0.35
Glycerophosphoethanolamine (GPE) (area %)		0.27	0.43	0.47	0.37	0.46
Fucose & Trehalose (area %)		0.18	0.19	0.18	0.20	0.26
Sorbitol & Galactitol (area %)		0.14	0.17	0.12	0.17	0.31
Xylitol (area %)		0.73	0.76	0.76	0.82	0.82
Sub-total		2.31	2.86	2.60	2.53	3.30
Water Content (%)	< 5	2.66	3.00	2.99	2.84	2.74
H8561						
2'-fucosyllactose (area %)	≥ 86	92.20	92.10	92.45	92.39	91.96
Allo-Lactose/Lactose (area %)	< 7	0.08	0.18	0.24	0.14	0.07
DFL (area %)	< 8	1.30	1.04	1.0	1.21	1.02
2'-fucosyllactitol (area %)	≤ 6	3.93	4.13	4.01	4.19	4.22
3'-FL & Fucosylgalactose (area %)	< 7	0.38	0.54	0.40	0.30	0.40
Fructose (area %)		0.43	0.43	0.32	0.48	0.61
Glucose & Galactose (area %)		0.33	0.29	0.29	0.23	0.32
Glycerophosphoethanolamine (GPE) (area %)		0.42	0.44	0.47	0.40	0.48
Fucose & Trehalose (area %)		0.12	0.13	0.12	0.12	0.16
Sorbitol & Galactitol (area %)		0.05	0.03	0.05	0.06	0.105
Xylitol (area %)		0.6	0.59	0.58	0.63	0.62
Sub-total		2.33	2.45	2.23	2.22	2.695
Water Content (%)	< 5	3.07	3.39	3.23	3.10	3.12
H8781						
2'-fucosyllactose (area %)	≥ 86	91.2	91.0	91.15	90.89	90.94
Allo-Lactose/Lactose (area %)	< 7	1.43	1.87	1.85	1.57	1.5

Table 7: Stability of Amyris' 2'-FL Under Accelerated Storage Conditions						
2'-FL spray-dried product accelerated stability study (40 °C, 75% RH)						
	Specification	**0 wk**	**1 wk**	**4 wk**	**8 wk**	**13 wk**
DFL (area %)	< 8	1.24	0.82	0.83	1.07	0.84
2'-fucosyllactitol (area %)	≤ 6	3.19	3.30	3.23	3.29	3.37
3'-FL & Fucosylgalactose (area %)	< 7	0.23	0.55	0.45	0.31	0.49
Fructose (area %)		0.31	0.37	0.38	0.53	0.49
Glucose & Galactose (area %)		0.22	0.15	0.20	0.16	0.22
Glycerophosphoethanolamine (GPE) (area %)		0.53	0.47	0.31	0.53	0.58
Fucose & Trehalose (area %)		0.15	0.15	0.14	0.17	0.17
Sorbitol & Galactitol (area %)		0.15	0.17	0.15	0.20	0.21
Xylitol (area %)		0.67	0.67	0.66	0.73	0.78
Sub-total		2.26	2.53	2.29	2.63	2.94
Water Content (%)	< 5	2.53	2.81	2.69	2.64	2.77
H8750						
2'-fucosyllactose (area %)	≥ 86	91.30	90.70	91.31	90.76	90.47
Allo-Lactose/Lactose (area %)	< 7	0.44	0.62	0.61	1.38	0.44
DFL (area %)	< 8	1.25	0.91	0.87	1.10	0.92
2'-fucosyllactitol (area %)	≤ 6	3.52	3.75	3.65	3.82	3.84
3'-FL & Fucosylgalactose (area %)	< 7	0.18	0.55	0.45	0.33	0.46
Fructose (area %)		0.46	0.47	0.39	0.63	0.68
Glucose & Galactose (area %)		0.52	0.46	0.42	0.24	0.51
Glycerophosphoethanolamine (GPE) (area %)		0.25	0.35	0.34	0.40	0.36
Fucose & Trehalose (area %)		0.19	0.21	0.20	0.20	0.26
Sorbitol & Galactitol (area %)		0.10	0.11	0.11	0.16	0.24
Xylitol (area %)		1.30	1.36	1.32	0.81	1.44
Sub-total		3.0	3.51	3.23	2.77	3.95
Water Content (%)	< 5	2.66	2.83	2.70	2.68	6.31

Source: Amyris, Inc.

Abbreviations: RH = relative humidity; wk = week; wt = weight.

2.7 Production of 2'-flol

Amyris' 2'-FL contains about 4% w/w 2'-fucosyllactitol. 2'-fucosyllacitol, 2'-flol, is the reduced form of 2'-fucosyllactose to glucose alcohol (sorbitol). Previous studies have evaluated 2'-flol reduced from 2'-FL in artificial lab conditions (Egge et al., 1983; Yamashita et al., 1985). A comparison of the chemical structures of 2'-FL and 2'-flol is in figure 3, below.

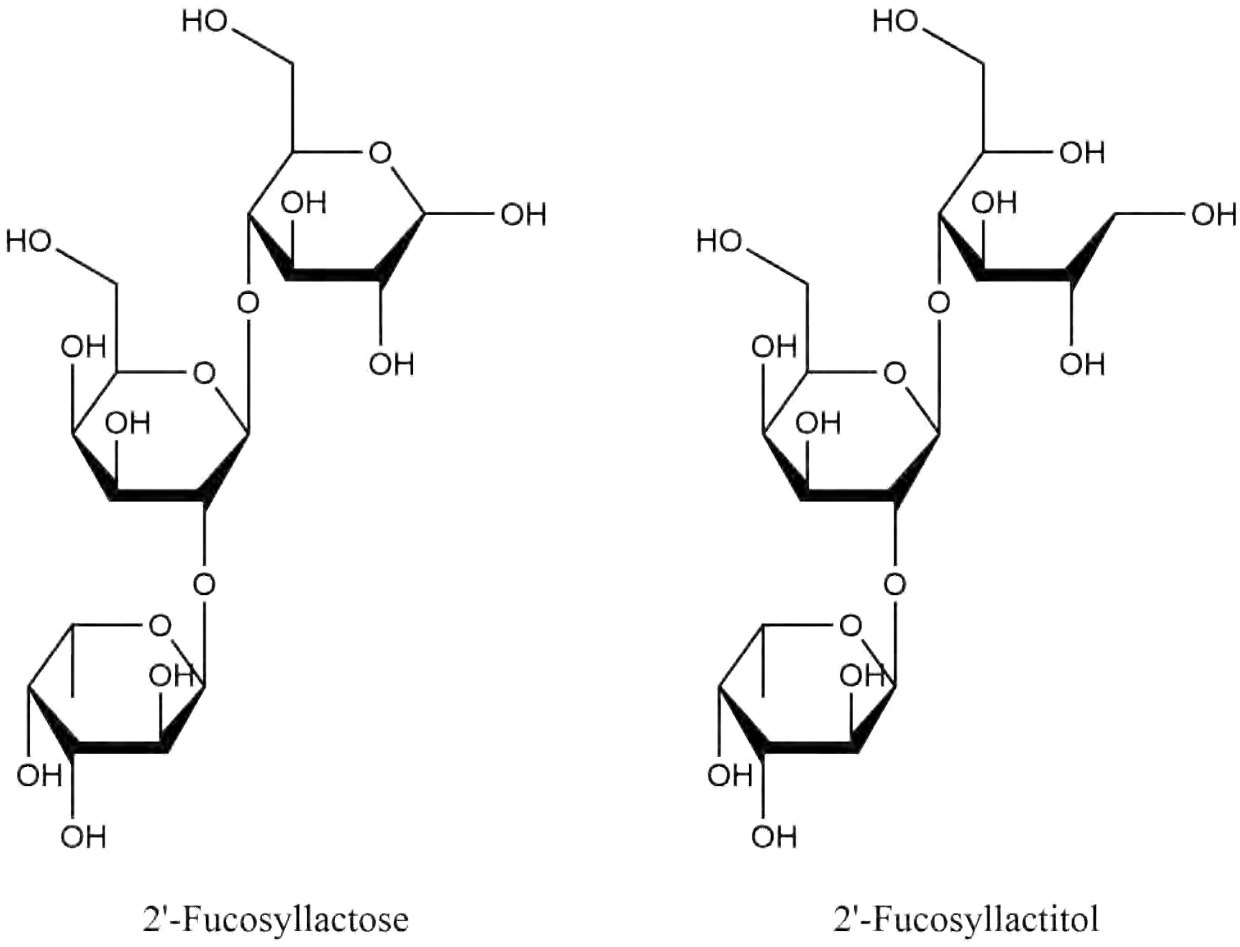

Figure 3: Chemical Structure of 2'-FL Compared to 2'-flol

2.8 Biogenic Amines

Amino acid analysis of several batches demonstrated that amino acids were not present at detectable levels in Amyris's 2'-FL (Appendix G). Results from additional analyses for secondary metabolites and impurities indicated that other amino acids and biogenic amines were not present at detectable levels in the final 2'-FL ingredient (Table 8).

Table 8: Biogenic Amines Analysis				
	Bath Number			
Biogenic amines	**Units**	**H8452**	**H8781**	**H8750**
Cadaverine	mg/kg	ND <1.0	ND <1.0	ND <1.0
Histamine	mg/kg	ND <10	ND <10	ND <10
Phenylethylamine	mg/kg	ND <1.0	ND <1.0	ND <1.0
Putrescine	mg/kg	ND <1.0	ND <1.0	ND <1.0
Spermidine	mg/kg	ND <1.0	ND <1.0	ND <1.0
Spermine	mg/kg	ND <1.0	ND <1.0	ND <1.0
Tyramine	mg/kg	ND <1.0	ND <1.0	ND <1.0
Source: Amyris, Inc. Abbreviations: kg = kilogram; mg = milligram; ND = Not detected.				

2.9 GMO Status

Other than the *S. cerevisiae* production strain, no other genetically modified ingredients or genetic modification technology were used in the production of the Amyris 2'-FL (Appendix C).

2.10 Allergens

The methodology outlined by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), show that engineered constructs used for Amyris 2'-FL production have a low risk for potential allergenicity. The two-part assessment returned 68 6-mer matches with 100% similarity, and 135 80-mer matches with >35% similarity when aligned to the AllergenOnline database. Total protein sequences queried for >35% similarity against the entire allergen database returned zero hits. Although the FAO/WHO guidelines published in 2001 continue to be standard practice, the EFSA provides detailed interpretation guidelines based on empirical data published between 2001 and 2010. Specifically, the EFSA and FARRP note that the use of a 6-mer amino acid identity search generates too many false positives and does not imply similar IgE binding in the absence of more extensive identity alignments. Both EFSA and FARRP also concur that sequences sharing less than 50% identity over their full-lengths are rarely cross-reactive, making 35% similarity a stringent threshold. Zero hits with full-length identity/similarity of >50% were found, suggesting the engineered constructs used for Amyris 2'-FL production have low potential for allergenicity. (Allergenicity report summary in Appendix H).

PART 3: DIETARY EXPOSURE. (21 CFR § 170.235)

3.1 Current Dietary Exposure: Background Intake

2'-FL is the most abundant HMO occurring naturally in human breast milk. Mean concentrations of 2'-FL vary based on populations, and secretor status of the lactating mother (Goehring et al., 2014, McGuire et al., 2017). Studies report mean concentrations of 2'-FL in human breast milk ranging from 1.1 g/L (McGuire et al. 2017; Bao et al 2013) to 4.26 g/L (Galeotti et al 2014), with levels up to 7.3 g/L reported (Gabrielli et al. 2011). Part 5 of this dossier describes exposure to naturally-occurring 2'-FL in the context of the proposed uses of Amyris 2'-FL.

3.2 Intended Human Food Uses

Table 1 provides a summary of the proposed food ingredient uses and use levels for Amyris 2'-FL in the U.S. One such use is as an ingredient in infant formula, which is defined by the FFDCA as "a food which purports to be or is human milk or its suitability as a complete or partial substitute for human milk" (FFDCA §201(z)). Amyris intends to use its 2'-FL as a food ingredient in term infant formulas (non-exempt), toddler formulas and "growing-up" milks (12-36 months) at a maximum level of 2.4 g 2'-FL per liter consumed. Amyris also intends to use its 2'-FL in baby foods for infants and young children (children older than one year of age) and beverages for young children. Other uses in infant and toddler food and beverage products include processed cereals, infant meal replacement products, ready-to-eat hot cereals, "baby" yogurts and drinks, "junior" desserts such as fruit desserts and cobblers, baby snack crackers and cookies, milk modifiers, and milk-based drinks for young children. Amyris is proposing to use its 2'-FL in conventional foods and beverages intended for children and adults such as in milk substitutes, flavoring in milk-based beverages such as coffees and smoothies, frozen dairy desserts such as ice cream and frozen yogurt, fruit pie fillings, fruit preserve products, meal replacement beverages and meal replacement bars, breakfast bars, cereal products (hot and ready-to-eat), energy drinks, sports drinks, and fruit drinks/juices. Amyris also proposes to use its 2'-FL as an ingredient in oral nutritional supplements for enteral feeding for ages 11 years and older.

3.3 Estimate of Dietary Exposures

Using food consumption data reported in the United States Department of Health and Human Service's 2013-2016 National Health and Nutrition Examination Surveys (NHANES), estimates of potential intakes of ingredients from the intended uses of Amyris 2'-FL were calculated. The estimated mean and 95th percentile of average intake of 2'-FL and 2'-FL by body weight for each target consumer population were calculated. Summaries of these estimated intakes are presented in Tables 9a and 9b. The proposed uses of Amyris's 2'-FL do not increase the cumulative EDI already reviewed in previous GRNs of chemically equivalent 2'-FL.

Table 9a: Summary of the Estimated Daily Intake of 2'-FL from All Proposed Food and Beverage Uses in the U.S. by Population Group (2013-2016 NHANES Data)

Population Group	Age Group	All-Users Consumption (g/day)			
		% Users	N	Mean	95th Percentile
Infants	0-6 mo.	100	241	2.73	5.87
Infants	7-12 mo.	99.66	228	3.82	8.43
Toddlers	1 to 3 yr.	98.77	1117	2.30	5.49
Children	4 to 10 yr.	99.34	2315	2.61	7.08
Male Teenager	11-18 yr.	99.49	1213	3.12	8.99
Female Teenager	11-18 yr.	99.13	1216	2.36	7.28
Female Adults of childbearing age	19-40 yr.	99.30	1807	1.78	5.79
Female Adults	19-64 yr.	99.49	3767	1.72	5.45
Male Adults	19-64 yr.	99.30	3313	2.38	7.84
Elderly Adults	65 yr. and up	98.95	1215	2.25	6.30

Abbreviations: 2'-FL = 2'-fucosyllactose; g = grams; mo. = months; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; yr. = years

Table 9b: Summary of the Estimated Daily Intakes of 2'-FL per Kilogram Body Weight from All Proposed Food and Beverage Uses in the U.S. by Population Group (2013-2016 NHANES Data)

Population Group	Age Group	Body Weight (kg)			All-Users Consumption (g/kg·bw/day)		
		Mean	95th Percentile	% Users	N	Mean	95th Percentile
Infants	0-6 mo.	6.8	8.9	100	241	0.40	0.82
Infants	7-12 mo.	9.3	11.3	99.6	227	0.42	0.88
Toddlers	1 to 3 yr.	13.8	18.5	98.77	1103	0.18	0.45
Children	4 to 10 yr.	28.9	48.5	99.34	2303	0.10	0.29
Male Teenager	11-18 yr.	60.4	94.5	99.49	1210	0.054	0.18
Female Teenager	11-18 yr.	65.6	106.0	99.13	1205	0.043	0.15
Female Adults of childbearing age	19-40 yr.	76.5	120.2	99.30	1791	0.025	0.082
Female Adults	19-64 yr.	78.2	120.3	99.49	3739	0.024	0.076
Male Adults	19-64 yr.	89.6	130.3	99.30	3295	0.028	0.093
Elderly Adults	65 yr. and up	79.5	113.9	98.95	2283	0.029	0.086

Abbreviations: 2'-FL = 2'-fucosyllactose; bw = body weight; g = grams; mo. = months; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; yr. = years

PART 4: SELF-LIMITING LEVELS OF USE. (21 CFR § 170.240)

4.1 Self-limiting

Based on a preliminary study, Elison et al. 2016 report a tolerance limit for 2'-FL of approximately 20 g/day for adults due to participants reports of softer stools as compared to baseline. Participants receiving the highest dose of 20 g/day 2'-FL and LNnT (2:1 mass ratio) reported significantly higher occurrence of bloating and gas compared to baseline (Elison et al., 2016; Table 11d).

PART 5: EXPERIENCE BASED ON COMMON USE IN FOOD. (21 CFR § 170.245)

This assessment of the GRAS status of the proposed uses of 2'-FL is based on scientific procedures. The information in this part is intended to present the natural background exposure to human milk oligosaccharides in general and 2'-FL in particular.

5.1 Naturally-Occurring Human Milk Oligosaccharides

Human breast milk provides immunity and nutrition benefits important for infant growth and health. Human breast milk is recommended as the first food for infants, and breastfeeding is the preferred method for infant nutrition supported and promoted by professional pediatric organizations and federal health agencies. The American Academy of Pediatrics recommends exclusive breastfeeding for the first six months of life with breastfeeding to support other foods for at least another year (AAP, 2012). The Surgeon General of the United States supports breastfeeding and notes that it is "vitally important to mothers' and infants' health" (US HHS, 2011). Human breast milk contains hundreds of compounds including oligosaccharides. Many of the oligosaccharides are non-digestible carbohydrates that serve as prebiotics; that is, substrates for commensal bacteria in the human gut.

Over 200 different HMOs have been identified and the structures of at least 85 have been characterized (Goehring et al., 2014; Thurl et al., 2010). HMOs are saccharide-based polymers consisting of the following monomers: D-glucose (Glu), D-galactose (Glc), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc) and the sialic acid *N*-acetylneuraminic acid (Neu5Ac) with lactose (Galβ1-4Glc) at the reducing end of the HMO. When this lactose is sialylated at the terminal Gal, the α2-3 linkage generates 3'-sialyllactose while the α2-6 linkage generates 6'-sialyllactose (6'-SL). When lactose is fucosylated at the terminal Gal with α1-2 linkage, 2'-FL is formed.

5.2 Presence of 2'-FL in Human Milk

Approximately 85% of the world's population is exposed to 2'-FL from human milk. Even infants fed breast milk from non-secretor mothers excrete 2'-FL in the urine and in the stool (Kunz and Rudloff 2017). This is because the secretor genotype (*Se*) is a dominant allele, while the non-secretor type (*se*) is the recessive allele. Non-secretor mothers (genotype *sese*) can therefore deliver secretor infants (*Sese*) if the father has the secretor genotype (*SeSe* or *Sese*). This would result in an infant with the heterozygous secretor genotype (*SeSe*) that exhibits the dominant genotype. Therefore, the infant itself can produce alpha-1,2-epitope containing glycans.

Because 2'-FL is one of the most abundant HMOs in human milk, most infants have a history of exposure to naturally-occurring 2'-FL. Naturally occurring 2'-FL accounts for approximately 20 to 30% of total HMOs. The average concentration of 2'-FL in human breast milk has been estimated in several studies. These concentrations of 2'-FL in human breast milk range from 1.1 g/L (McGuire et al. 2017; Bao et al 2013) to 4.26 g/L (Galeotti et al 2014), with levels up to 7.3 g/L reported (Gabrielli et al. 2011). These reported average 2'-FL concentrations vary slightly based on differences in populations and regions. Levels of 2'-FL in human milk also can vary from individual to individual mainly due to the secretor status of the lactating mother (Goehring et al., 2014, McGuire et al. 2017).

5.3 Dietary Exposure to Naturally Occurring 2'-FL

Most infants have been exposed to 2'-FL because it is a naturally occurring component of human breast milk. HMOs are the third largest component of breast milk solids after lactose and lipids and 2-'FL is the most abundant HMO in human breast milk (Castanys-Munoz et al., 2013; Coppa et al., 2004). In a study of milk sampled from women from 10 different countries, 2'-FL was the most abundant HMO at a mean concentration of 2.38 g/L, and was identified in 85% of the samples. (Erney et al., 2000). A subsequent study found that the average concentration of 2'FL over a lactation period of 50 weeks was 2.43 ± 0.26 g 2'-FL/L of breast milk (Chaturvedi et al., 2001).

Not all women produce breast milk containing 2'-FL, however infants of these women are still exposed to 2'-FL. The fucosylation of glycans depends on the mother's blood group status: Lewis (+)/(-) and Secretor/non-Secretor. The Secretor can synthesize 2'-FL in the mammary gland (Castanys-Munoz et al., 2013). About 70% of women are Secretors due to the presence of 1-2 fucosyltransferases (FUT2) in their milk (Kunz et al., 1999). The breast milk of non-Secretor women does not contain FUT2 but another fucosyltransferase, FUT3, which links Fuc to subterminal GlcNAc in α1-4 linkages (Bode & Jantscher-Krenn, 2012). Secretor Lewis (+) women have the most complex HMO composition while non-Secretor Lewis (-) women have the least complex (Bode & Jantscher-Krenn, 2012). Because the majority of women are Secretors, infants receiving milk from donor human milk programs are likely to be ingesting 2'-FL. Therefore, infants born to Secretor and non-Secretor mothers are routinely exposed to 2'-FL.

Thurl et al. (2010) reported a decrease in 2'-FL concentration in women's breast milk from day 3 (4.1 g/L) to day 90 (2.6 g/L) of lactation. While the concentration of 2'-FL declines through the progression of lactation, the volume of breast milk consumed increases as the infant develops, making the amount of 2'-FL consumed by infants fairly constant throughout the nursing period (Asakuma et al., 2008; Thurl et al., 2010).

Human breast milk is not the only mammalian milk with 2'-FL; domestic goat, sheep, and pig milk contains very small amounts (Albrecht et al., 2014), as well as mammals in the Hominidae family including chimpanzee, bonobo, and orangutans (Castanys-Munoz et al., 2013). Cow's milk, which does not contain 2'-FL, is commonly used in infant formula (Bode 2012).

5.4 The Role of HMOs and Gut Microflora in Infants

Compared to formula-fed infants, breastfed infants have a higher concentration of gut bifidobacteria, likely due to the oligosaccharide content in human breast milk (Coppa et al., 2004; Donovan et al., 2012). For the initial acquisition of microflora, a natural birth provides vaginal and feces microflora from the mother host with some influence of the surrounding environment, whereas infants born via Cesarean section tend to have microflora related to the hospital and attending hospital personnel. Infants that are delivered vaginally have higher concentrations of bifidobacteria as compared to C-section delivered infants (Penders et al., 2006). This eventually leads the flora of formula-fed infants to resemble an adult gut containing bacteroides, clostridia, bifidobacteria and a few others early on (Rodricks et al., 2007). After two years of age, the gut microflora is generally comparable to that of an adult, it may continue to develop as an individual continues through life (Hopkins et al., 2002).

A critical window for the colonization of the newborn's gastrointestinal (GI) tract by microbiota is right after birth. This early colonization involves the translocation of maternal microbiota from mouth, skin, vagina, GI tract, and breastmilk (Mackie et al., 1999). Breastfeeding transfers maternal microbiota into the offspring and the gut microflora require nutritional substrate for

propagation (Mackie et al., 1999). The substrates are usually oligosaccharides that are not digestible by the endogenous enzymes of the upper GI tract of the infants.

While oligosaccharides are a large component of human breast milk, they occur only at very low concentrations in cow's milk, the most common milk used for infant formula in the United States (USDA, 2009). HMO concentrations vary from over 20 g/L in colostrum to 12 g/L in mature milk (Thurl et al., 2010). Because breastfed infants consume at least 0.44 L of breast milk daily, they ingest at minimum several grams of HMO per day (Kent et al., 2006). In contrast, bovine milk used in infant formulas contains less than 1 g/L of oligosaccharides (Coppa et al., 2004).

Intestinal microbiota are responsible for the complex metabolism of HMOs into short-chain fatty acids that are used as an energy source by colonocytes, and stimulate sodium and water absorption (Rodricks et al., 2007, Engfer et al., 2000). The composition of fecal oligosaccharides varies based on the HMOs profile of maternal milk and lactation stage (Albrecht et al, 2011a;) Kunz & Rudloff, 2008). When breast milk serves as the primary food for newborns, neutral and acidic HMOs are present in significant amounts in the feces of these infants. One of the most common prebiotics in human breast milk is the neutral trisaccharide 2'-FL which helps encourage growth of beneficial bacteria (e.g., bifidobacteria) in the infant's intestine (Engfer et al., 2000; Marcobal & Sonnenburg, 2012). The HMO concentration varies based on milk stage and the individual mother (Kunz & Rudloff, 2008). The composition of intestinal microflora and fecal HMOs change as the infant transitions to formula or solid foods (Albrecht et al, 2011a; Albrecht et al., 2011b Mackie et al., 1999). In comparing the fermentation of HMOs of breastfed and formula-fed infants through fecal inoculum, *in vitro* data suggests that 2'-FL and another oligosaccharide, lacto-*N*-*neo*tetraose are fermented rapidly (Vester Boler et al., 2013).

5.5 Summary of Regulatory History

FDA and Health Canada have both reviewed 2'-FL as an ingredient in infant formula in various GRNs and NFNs, respectively. 2'-FL (Jennewein Biotechnologie, GmbH) is listed on Health Canada's list of Completed Novel Food Safety Assessments with a decision date of December, 5, 2018. In the United States, GRNs have been submitted for 2'-FL for intended use in infant formulas, toddler formulas/foods, and conventional foods and beverages (Glycom A/S, 2014, 2016; Jennewein Biotechnologie, 2015; Glycosyn, LLC, 2017; DuPont Nutrition and Health, 2017; BASF Corporation, 2019). These submissions all received letters of no objection from the FDA (U.S. FDA 2014, 2015, 2016, 2018a, 2018b, 2019). Amyris is not requesting any new uses for 2'-FL: All of Amyris's intended uses and maximum use levels of 2'-FL have been presented in one or more 2'-FL GRNs that have received no-questions letters from FDA. The intended food uses and use levels presented in these GRNs are summarized in Table 10.

Table 10: GRAS Notifications of 2'-FL					
GRN	**Year**	**Substance**	**Intended Uses**	**Proposed Maximum Use Level (g/kg or g/L)**	**Notification**
546	2014	2'-FL chemically synthesized from benzyl-2-fucosyllactose; >95% 2'-FL	Infant formula (0-12 months)	2.4	Glycom (Glycom A/S, 2014) (U.S. FDA, 2014)
			Toddlers (12-35 months)	2.4	
			Various other uses in conventional foods intended for children and adults	Ranging from 5.4 – 48	
			Various other uses in beverages intended for children and adults	Ranging from 1.2 - 10	
571	2015	2'-FL derived from fermentation with E. coli BL21; >90% 2'-FL	Infant formula (0-12 months)	2.0	Jennewein (Jennewein Biotechnologie, 2015) (U.S. FDA, 2015)
			Toddlers (12-35 months)		
650	2016	2'-FL derived from fermentation with E. coli K-12; >94% 2'-FL	Term infant formula	2.4	Glycom (Glycom A/S, 2016) (U.S. FDA, 2016)
			Toddler formula	2.4	
			Other baby foods for infants	12	
			Other drinks for young children	1.2	
			Various other uses in conventional foods intended for children and adults	Ranging from 1.2 - 5.3	
735	2017	2'-FL derived from fermentation with E. coli K-12; >90% 2'-FL	Term infant formula (0 to 6 months)	2.4	Glycosyn (Glycosyn, LLC, 2017) (U.S. FDA, 2018)
			Follow-on formula (6-12 months)	2.4	
			Toddler formula (12-36 months)	2.4	
			Other various baby foods and beverages	Ranging from 2.4 – 57	
			Other drinks for young children	12	
			Various other uses in conventional foods intended for children and adults	Ranging from 4.8 – 80	
			Various other uses in conventional beverages intended for children and adults	Ranging from 0.8 – 7.0	
			Oral nutritional supplements and enteral tube feeding for 11 years and older	20	
749	2017	2'-FL derived from fermentation with E. coli K12; >82% 2'-FL	Infant formulas	2.4	DuPont (DuPont Nutrition and Health, 2017) (U.S. FDA, 2018)
			Toddler formulas	2.4	
			Other baby foods for infants and young children	12	

Table 10: GRAS Notifications of 2'-FL					
GRN	**Year**	**Substance**	**Intended Uses**	**Proposed Maximum Use Level (g/kg or g/L)**	**Notification**
			Other drinks for young children	1.2	
852	2019	2'-FL derived from fermentation with E. coli K12; >90% 2'-FL	Term infant formula (0 to 6 months)	2.4	BASF Corporation
			Follow-on formula (6-12 months)	2.4	
			Toddler formula (12-36 months)	2.4	
			Other various baby foods and beverages	Ranging from 2.4 – 57	
			Other drinks for young children	12	
			Various other uses in conventional foods intended for children and adults	Ranging from 4.8 – 80	
			Various other uses in conventional beverages intended for children and adults	Ranging from 0.8 – 7.0	
			Oral nutritional supplements and enteral tube feeding for 11 years and older	20	

PART 6: NARRATIVE. (21 CFR § 170.250)

6.1 Introduction

As discussed in Section 5.1, human breast milk is the preferred food for infants (AAP, 2012; US HHS, 2011), and is a contributor to healthy growth and development for infants. However, human breast milk is not always available or attainable. To alleviate this problem, the production of an infant formula with a nutritional content and chemical profile close to human breast milk is needed. The addition of Amyris' 2'-FL in infant formula will enable the infant formula to more closely approximate the composition of human milk. Specific information regarding the manufacturing and analytical testing of Amyris 2'-FL is found in Part 2. Here, in Part 6, the safety data from the toxicological and clinical testing for other synthesized 2'-FL are presented and discussed.

6.2 Absorption, distribution, metabolism, and excretion of 2'-FL and other HMOS

2'-FL isolated from human milk can be useful in studying HMOs from non-maternal sources. Evaluation of absorption, distribution, metabolism and excretion (ADME) of oligosaccharides in previous studies have shown that HMOs can be absorbed into the systemic circulation to a limited extent. In studies specifically evaluating ADME of infant formula oligosaccharides, it has been consistently demonstrated that HMOs are not readily absorbed by infants and arrive intact in the colon where they are metabolized by resident microbiota and/or excreted in the feces (Engfer et al., 2000; Gnoth et al., 2000). In the neonate intestine, HMOs provide a substrate for bacteria including *Bifidobacterium* and *Bacteroides* spp. that are capable of metabolizing the HMOs using glycoside hydrolases and other specific enzymes (Marcobal and Sonnenburg, 2012).

Rudloff et al. 2012 administered a single oral bolus of ^{13}C-labeled galactose to breastfeeding mothers and reported HMOs present in the urine of their infants for a period of 36 hours after the bolus. 1-2% of the total ^{13}C-labeled HMOs ingested by the infants were excreted unchanged or only slightly metabolized in the urine. The authors concluded that since infants consumed 50 to 150 mg of individual oligosaccharides per suckling, and 1-2% of the total ^{13}C-labeled HMOs was detected in infant's urine, then the remainder HMOs could be absorbed intact and enter the circulatory system.

Goehring et al. (2014) reported on the absorption of 2'-FL and other HMOs in the circulatory system of breastfed infants. The study found that less than 5% of ingested 2'-FL was absorbed intact into the circulatory system, with 0.1% in plasma and 4% in urine. Other HMOs were also detected intact in urine including (3-FL, lacto-*N*-neotetraose [LNnT], lacto-N-fucopentaose [LNFP] I, II, and III, 6'-SL and 6'-sialyl-N-acetyllactosamine [6'-SLN]) (Goehring et al., 2014).

These studies show that less than 5% 2'-FL and other HMOs are absorbed from the GI tract and most of 2'-FL consumed by infants will be transported intact to the large intestine where it is then subjected to partial fermentation by the indigenous microbiota (Brand-Miller *et al.*, 1995, 1998). This is further supported by the detection of unchanged 2'-FL in fecal samples of infants at levels amounting to 40 to 50% of the ingested amount following consumption of breast milk (Chaturvedi *et al.,* 2001; Coppa *et al.*, 2001; Albrecht *et al.*, 2011b).

Marriage et al., 2015 compared the mean plasma concentrations and relative absorption of infants fed chemically synthesized 2'-FL, and infants fed a human milk control. At day of life 42, the mean 2'-FL concentrations in plasma were significantly different for each treatment group, and at day of life 119, the mean 2'-FL concentrations in plasma between treatment groups were not significantly different. This demonstrates a difference in 2'-FL found in the circulatory system

at 42 days of life, but not at 119 days between those fed 2'-FL formula and a human milk control. The plasma concentrations in all study groups decreased significantly from day 49 to 119 of life (0.2 g 2'-FL/L formula group [p = 0.017], 1.0 g 2'-FL/L formula group [p= 0.008] and the human milk groups [p= 0.015]).

The relative absorption of 2'-FL between the two groups was comparable at 0.7% among infants fed formula containing 0.2 g 2'-FL/L, 0.05% among infants fed formula containing 1.0 and 0.05 g 2'-FL/L among infants fed human milk. The urine concentrations decreased significantly for the human milk-fed group (p=0.018) but did not change significantly for the groups fed formula containing 2'-FL. Mean urine concentrations were significantly different among the groups, but relative excretion was similar among the groups fed human milk or formula containing 2'-FL: 1.35% (human milk), 1.50% (formula containing 0.2 g 2'-FL/L) and 1.26% (formula containing 1.0 g 2'-FL/L). While the mean plasma and urine concentrations varied between treatment groups of the study, the relative absorption and excretion observed from day 42 of life to day 119 of life were similar, without a statistically significant difference among treatment groups (Marriage et al., 2015).

The above studies show that at least 95% of ingested 2'-FL is directly available to gut microbiota and of this 95%, less than 5% is absorbed intact by infants before being excreted intact or minimally metabolized in the urine. The unabsorbed 2'-FL is then metabolized by gut microbiota into short-chain fatty acids with 40 to 50% of the ingested amount excreted unchanged in the feces.

6.3 Toxicological and Clinical Studies Regarding the Safety of 2'-FL

Studies demonstrate that chemically synthesized 2'-FL or 2'-FL produced by microbial fermentation is safe and suitable for its proposed uses in term Infant and toddler formulas. The toxicology studies were performed on 2'-FL from various sources. Although Amyris 2'-FL was not the test substance evaluated, the other 2'-FLs that have been evaluated are appropriate for evaluating Amyris's 2'-FL because the profiles of 2'-FL and the associated substances, i.e., non-2'-FL carbohydrates (Table 5b), were sufficiently similar to base safety interpretations on the results of these studies. This substantial chemical equivalency supports bridging to the published studies.

6.3.1 A Review of Mutagenicity and Genotoxicity Studies

Several in vitro micronucleus tests have been performed to confirm that 2'-FL produced by either chemical synthesis or microbial fermentation is not mutagenic of cytotoxic. Each study is described below and in Table 11a.

Four studies (Coulet et al. 2014, Verspeek 2015 as cited in GRN 650, Phipps et al. 2018, and van Berlo et al. 2018) of multiple bacterial reverse mutation in *Salmonella typhimurium* (strains TA 98, TA 100, TA 102, TA 1535, and TA 1537) and *E. coli* strain WP2 *uvrA* showed no evidence of cytotoxicity nor mutagenicity of 2'-FL produced by chemical synthesis. Concentrations up to 5000 µg/plate of chemically synthesized 2'-FL were used and compared to control counts. When pre-incubation tests and plate incorporation tests were compared to the control counts, there were no signs of cytotoxicity reported and no increase in revertant colony numbers in any of the test strains. These results were consistent in two independent experiments with and without metabolic activation. The positive control tests showed a significant increase in the number of revertant colonies for each of the corresponding test strains and confirmed the validity of the test conditions and the sensitivity of the test system.

Four mammalian cell micronucleus tests were conducted with cultured human peripheral lymphocytes showing that 2'-FL produced by either chemical synthesis or microbial fermentation had no evidence of mutagenicity or cytotoxicity (Verbaan 2015a and Verbaan 2015b as cited in GRN 650, Phipps et al. 2018, and van Berlo et al. 2018). 2'-FL concentration was tested up to 2000 or 5000 µg/mL with and without metabolic activation.

The thymidine kinase (TK) test was used to evaluate the potential of 2'-FL (concentrations up to 5000 µg/mL) to induce gene mutations at the TK-locus of mouse lymphoma cells in both the absence and presence of S9 metabolic mix (Coulet, et al, 2014). Results showed that 2'-FL did not induce any biologically relevant increases in mutant frequency in the absence or presence of S9-mix, and no signs of cytotoxicity were reported at any of the concentrations tested.

Under the conditions of the published studies described above, 2'-FL that is either chemically synthesized or produced by microbial fermentation was not cytotoxic or mutagenic.

Table 11a. Summary of Genotoxicity / Mutagenicity Studies of 2'-Fucosyllactose					
Reference	**Study Type**	**Test Substance**	**Species**	**Dose Duration**	**Conclusions Under Test Conditions**
Coulet et al. 2014	Organisation for Economic Co-operation and Development (OECD) 471	Chemically synthesized 2'-FL (purity = 99%)	S. typhimurium (strains: TA98, TA100, TA102, TA1535 and TA1537)	Up to 5000 µg/plate with and without metabolic activation	2'-FL is not mutagenic.
Verspeek-Rip et al. 2015 (as cited in GRN 650 and GRN 735)	Mutagenicity OECD 471	2'-FL produced by fermentation (purity = 97.6%)	S. typhimurium (strains: TA1535, TA1537, TA98, and TA100); E. coli strain WP2 uvrA	Up to 5000 µg/plate with and without metabolic activation	2'-FL is not mutagenic.
Phipps et al. 2018	Mutagenicity OECD 471	2'-FL (82.5%) and DFL (9.7%) mixture at an 8:1 ratio produced by microbial fermentation, using lactose as a substrate	S. typhimurium (strains: TA98, TA100, TA1535, and TA1537); E. coli WP2 uvrA	Up to 5000 µg/plate with and without metabolic activation	2'-FL/DFL is not mutagenic.
Van Berlo et al. 2018	Mutagenicity OECD 471	2'-FL produced through fermentation by genetically modified E. coli K12 GI724/ATCC 55151 bacteria (purity = 94%)	S. typhimurium (strains: TA1535, TA1537, TA98, and TA100); E. coli strain WP2 uvrA	Up to 5000 µg/plate with and without metabolic activation	2'-FL is not mutagenic.
Coulet et al. 2014	Mutagenicity OECD 476	Chemically synthesized 2'-FL (purity = 99%)	Mouse lymphoma cells (TK-locus)	Up to 5000 µg/mL; with metabolic activation for 4 hours, and without for 4 hours and 8 hours	2'-FL is not mutagenic.
Verbaan et al. 2015a (as cited in GRN 650 and 735)	Genotoxicity OECD 487	Chemically synthesized 2'-FL	Peripheral human lymphocytes	Up to 2000 µg/mL with and without metabolic activation	2'-FL is not mutagenic.
Verbaan et al. 2015b (as cited in GRN 650 and 735)	Genotoxicity OECD 487	2'-FL produced by fermentation (purity 97.6%)	Peripheral human lymphocytes	Up to 2000 µg/mL with and without metabolic activation for 3 hours with a 27-hour harvest time or for 24 hours with a 24-hour harvest time	2'-FL is not genotoxic.

Table 11a. Summary of Genotoxicity/Mutagenicity Studies of 2'-Fucosyllactose					
Reference	**Study Type**	**Test Substance**	**Species**	**Dose Duration**	**Conclusions Under Test Conditions**
Phipps et al. 2018	Genotoxicity OECD 487	2'-FL (82.5%) and DFL (9.7%) mixture at an 8:1 ratio produced by microbial fermentation, using lactose as a substrate	Human peripheral blood lymphocytes	Up to 2000 ug/mL, 3 hours with and without metabolic activation or 20 hours without metabolic activation	2'-FL/DFL is not genotoxic.
Van Berlo et al. 2018	Genotoxicity OECD 487	2'-FL produced through fermentation by genetically modified E. coli K12 GI724/ATCC 55151 bacteria (purity = 94%)	Cultured binucleated human lymphocytes	Up to 2000 µg/mL with and without metabolic activation, 4 hours treatment/20 hours recovery or 20 hours treatment and no recovery	2'-FL is not genotoxic.

6.3.2 Oral Toxicity Studies

6.3.2.1 Repeated Dose Toxicity Studies of 2'-FL Ingestion by Rats

Four repeated dose toxicity studies showed that 2'-FL does not induce toxic effects after repeated ingestion for 90 days. The studies administered evaluated the safety of 2'-FL produced via microbial fermentation or chemical synthesis administered by gavage or as a dietary admixture to rats (Phipps *et al.,* 2018, Van Berlo *et al.,* 2018, Penard *et al.,* 2015 as cited in GRN 650, Coulet *et al.,* 2014).

Van Berlo et al. (2018) fed male juvenile rats (strain Crl:WI(Han)) mean intake levels of 0, 2.17, 4.27, and 7.25 g/kg·bw/day 2'-FL, and female juvenile rats mean intake levels of 0, 2.45, 5.22, and 7.76 g/kg·bw/day 2'-FL produced via microbial fermentation by E. Coli K12 for 90 days in an oral toxicity study (OECD 408). Van Berlo et al. reported that 2'-FL "did not induce adverse changes in any test group." Relative liver weight was significantly increased in males in the high-dose group, and absolute and relative filled and empty cecum weights were significantly increased in the mid-and high-dose males and females; the authors did not consider this an adverse effect. No mortalities and no significant or exposure-related changes were reported (Table 11b). Van Berlo et al. also reported "Thus, the NOAEL is placed at the highest concentration tested, corresponding to ≥7.25 g/kg·bw/day for males and ≥7.76 g/kg·bw/day for females."

Coulet et al. (2014) also conducted a subchronic oral toxicity study that administered chemically synthesized 2'-FL via oral gavage doses at 0, 2000, 5000, or 6000 mg 2'-FL/kg· bw/day in juvenile rats (strain Crl:WI(Han)). There were some incidences of diarrhea and urogenital erythema reported in the mid and high-dose exposure. There were no significant changes in body weight, clinical signs, organ weights or histopathology observed that were related to exposure and toxicologically relevant. Two mortalities were observed in the highest dose group during treatment, and one mortality occurred during recovery. Upon investigation, it was determined that all mortalities were unrelated to treatment (Coulet *et al.,* 2014).

Phipps et al., 2018 and Penard et al., 2015 (as cited in GRN 650), reported on a 90-day toxicity studies with 2'-FL administered 2'-FL by gavage at doses of 1000 to 5000 mg/kg·bw/day in neonatal rats (strain Crl:CD(SD) and Crl:WI(Han), respectively). Phipps et al. (2018) evaluated the safety of a mixture of the human-identical milk oligosaccharides 2′-fucosyllactose and difucosyllactose; Penard (2015) evaluated a 2'-FL produced by microbial fermentation. Both tests reported a NOAEL of 5,000 mg/kg·bw/day. There were no exposure-related mortalities, body weight, body weight gain, food consumption, clinical chemistry, hematology, urinalysis, organ weight gross and histopathological findings. These studies demonstrated that no treatment-related adverse effects were observed.

6.3.2.2 Pre-Clinical Study of 2'-FL in Neonatal Pigs

Neonatal pigs are a useful and appropriate model frequently used in evaluating the safety of dietary compounds [e.g., infant formulas] on the development of infants because the digestive enzymes, nutrient absorption, gut closure, gut transit time, dietary requirements, and microbial population in the first three weeks of the piglets' lives are similar to those of human infants in the first three months of development (Guilloteau et al., 2010; Flamm et al., 2012).

Hanlon & Thorsrud (2014) reported on the health and developmental effects of orally administered (as a dietary admixture) 2'-FL produced by fermentation to 48 male and 48 female neonatal farm piglets. The authors did not report any toxicity after repeated ingestion of 2'-FL at concentrations of 0, 200, 500, and 2000 mg 2'-FL/L/day for 21 days and dose levels of 29.37,

72.22 and 291.74 mg/kg/day in males and 29.30, 74.31, and 298.99 mg/kg/day in females. There were no 2'-FL-related adverse effects reported on clinical pathology findings, body weight, food efficiency, growth and development, gastrointestinal pH, and macroscopic and microscopic findings (Hanlon & Thorsrud, 2014).

Table 11b. Summary of 2'-Fucosyllactose Repeated Dose Toxicity Studies to Support Safety						
Reference	**Study Type**	**Test Substance**	**Study Population**	**Route of Exposure**	**Significant Exposure-Related Outcomes**	**Conclusions Under Test Conditions**
Phipps *et al.* 2018	90-day Oral Toxicity OECD 408	2'-FL (82.5%) and DFL (9.7%) mixture at an 8:1 ratio produced by microbial fermentation, using lactose as a substrate	Two groups of neonatal rats randomly allocated at PND 4; 10 male and 10 female per group dosed at PND 7	0, 1000, 3000, or 5000 mg 2'-FL/DFL in an 8:1 ratio)/kg·bw/day via oral gavage for 90 days with 28-day recovery	**Body weight**: None **Clinical signs**: None **Hematology and clinical chemistry**: None **Histopathology:** None **Mortalities**: None **Neurotoxicity**: None **Organ weights:** Relative kidney and seminal vesicle weight were significantly increased in males in the low-dose group only. Thymus weight was significantly increased for all male treatment groups, but no dose-response observed. Relative pituitary weights were significantly increased in females in the high-dose group at the end of the recovery period. **Sexual maturation and development:** The mean age for balano-preputial skinfold separation was slightly higher in highest-exposed males compared with vehicle controls. **Urinalysis:** None 2'-FL did **not induce toxic effects** after repeated administration to rats.	**NOAEL:** Highest level tested of 5000 mg/kg·bw/day

Table 11b. Summary of 2'-Fucosyllactose Repeated Dose Toxicity Studies to Support Safety						
Reference	**Study Type**	**Test Substance**	**Study Population**	**Route of Exposure**	**Significant Exposure-Related Outcomes**	**Conclusions Under Test Conditions**
Van Berlo *et al. 2018*	90-day Oral Toxicity OECD 408	2'-Fl produced through fermentation by genetically modified E. coli K12 GI724/ATCC 55151 bacteria (purity = 94%).	40 male and 40 female rats (four experimental groups, 10 rats per group) administered 2'-FL produced through fermentation by E. Coli K12. Rats were dosed at 25 days of age. Exposure ended on PND 115.	0, 3, 6, or 10% (w/w) 2'-FL added to food with mean intake levels as follows: 0, 2.17, 4.27, and 7.25 g/kg·bw/day for males; 0, 2.45, 5.22, 7.76 g/kg·bw/day in females) consumed in feed for 13 weeks	**Body weight**: None **Clinical signs**: None **Hematology and Clinical chemistry**: None **Histopathology:** None **Mortalities**: None **Neurotoxicity**: None **Organ weights:** Relative liver weight was significantly increased in males in the high-dose group. Absolute and relative filled and empty cecum weights significantly increased in the mid- and high-dose males and females 2'-FL did **not induce toxic effects** after repeated ingestion by rats.	**NOAEL:** Highest level tested of ≥7.25 g/kg·bw/day in males and ≥7.76 g/kg·body weight/day in females.
Penard *et al*. 2015 (as cited in GRN 650)	90-day Oral Toxicity OECD 408	2'-FL produced by fermentation (purity = 97.6%)	Neonatal rats receiving dose of 2'-FL at PND 7. Four groups, consisting of 10 male and 10 female rats each.	0, 2000, 4000, or 5000 mg 2'-FL/kg·bw/day & FOS at 5000 mg/kg·bw/day via oral gavage; 90 to 91 days with 28-day recovery	**Body weights and organ weights**: None **Clinical Signs:** Liquid feces in mid- and high-dose groups and reference groups; soiled urogenital areas in mid- and high-dose groups; hypersalivation, abnormal foraging and/or pedaling in mid- and high dose group and reference group. **Hematology and clinical chemistry**: There were some hematological changes observed, but within the control ranges and therefore deemed not significant.	**NOAEL:** The highest dose tested of 5000 mg/kg/day.

Table 11b. Summary of 2'-Fucosyllactose Repeated Dose Toxicity Studies to Support Safety						
Reference	**Study Type**	**Test Substance**	**Study Population**	**Route of Exposure**	**Significant Exposure-Related Outcomes**	**Conclusions Under Test Conditions**
					Macroscopic and histopathological evaluation: None **Mortalities:** None **Sexual maturation and development:** None **Urinalysis:** None 2'-FL did **not induce toxic effects** after repeated ingestion by rats.	
Coulet *et al.* 2014	Oral Toxicity, Repeated Dose OECD 408	Chemically synthesized 2'-FL (purity = 97.6%)	Four groups of juvenile rats, consisting of 10 male and 10 female rats per group. Receiving first dose at PND 21 and end of study period at PND 63.	0, 2000, 5000, 6000 mg/kg·bw/day & FOS at 6000 mg/kg·bw/day via oral gavage; 90 days	**Body weight:** Transient lower mean body weight gains were observed in mid- dose, high- dose, and FOS groups from 0 to 10 days compared to controls, resulting in lower mean body weights in male high-dose (days 3-10), female mid- and high-dose groups (days 3), and in the FOS group (days 3-7 for males; 3-10 for females). At the end of the 90-day treatment period, body weights between groups were not significantly different. **Clinical signs:** Diarrhea in all high-, mid-dose 2'-FL, & FOS animals, & several low-dose 2'-FL. Erythema in urogenital area high dose & FOS groups. Hyper-salivation in most high-dose 2'-FL & FOS groups, half of mid-dose 2'-FL group. **Histopathology:** Higher incidence of minimal cortical tubular epithelial cytoplasmic vacuolation in kidneys of the	**NOAEL:** 5000 mg/kg·bw/day

Table 11b. Summary of 2'-Fucosyllactose Repeated Dose Toxicity Studies to Support Safety						
Reference	**Study Type**	**Test Substance**	**Study Population**	**Route of Exposure**	**Significant Exposure-Related Outcomes**	**Conclusions Under Test Conditions**
					mid- & high-dose 2'-FL and FOS groups. This feature was also seen in control animals, and not associated with any relevant clinical pathology changes or histological evidence of degeneration, and therefore considered by the authors as "non-adverse, of unclear origin and unrelated to treatment." **Mortalities:** 1 male and 1 female rat of high dose 2'-FL on day 2. 2 males of FOS group on days 12 & 13; 1 female of FOS group on day 108. Authors report that since the cause of these deaths could not be determined by histopathological evaluation, that a relationship between mortality and the treatment could not be demonstrated. The test substance did **not induce toxic effects** after repeated ingestion by rats. The results are consistent with other indigestible carbohydrates.	
Hanlon & Thorsrud 2014 (as cited in GRN 571)	Oral Toxicity, Pre- clinical	2'-FL produced by fermentation (purity = 97.9%)	48 male and 48 female neonatal farm pigs	0, 200, 500, and 2000 mg/L; 21 days oral dietary exposure	**Body weight:** None **Clinical signs:** None **Mortalities**: None. **Necropsy:** 1 male and 1 female in high-dose group, 1 female in mid-dose group exhibited mild to moderate inflammation within the keratinized portion of the squamous epithelium of the non-glandular part of the stomach. Another male of high-dose group exhibits focal loss/thinning of this area, but no ulceration.	2'-FL did not induce toxic effects after repeated ingestion by piglets at dose levels of 29.37, 72.22 and 291.74 mg/kg/day in males and 29.30, 74.31, and 298.99

Table 11b. Summary of 2'-Fucosyllactose Repeated Dose Toxicity Studies to Support Safety						
Reference	**Study Type**	**Test Substance**	**Study Population**	**Route of Exposure**	**Significant Exposure-Related Outcomes**	**Conclusions Under Test Conditions**
					The authors considered these effects incidental, typical to this study, and not related to exposure as these findings are occasionally observed in swine of this age and strain 2'-FL did **not induce toxic effects** after repeated ingestion by piglets.	mg/kg/day in females.

6.3.3 Clinical Studies of the Safety and Tolerance of Synthesized 2'-FL

Additional clinical studies evaluate the growth and tolerance of 2'-FL in infants (Marriage *et al.* 2015, Elison *et al*. 2016; Kajzer *et al.* 2016 via Reverri *et al*. 2018; Puccio *et al.* 2017; Storm *et al*. 2019). The four studies summarized below evaluate the tolerance of 2'-FL in concentrations of 0.2 g 2'-FL/L formula to 1.2 g 2'-FL/L formula administered to infants. No adverse effects or alterations in growth were reported. These studies evaluate the combined effects of 2'-FL with GOS (Marriage *et al.,* 2015), short-chain fructooligosaccharides (scFOS) (Kajzer *et al*., 2016 via Reverri *et al*., 2018), LNnT (Puccio *et al.,* 2017) or whey (Storm *et al.,* 2019). These studies are summarized in the sections below and in Table 11c.

6.3.3.1 Clinical Studies Regarding 2'-FL Tolerance in Infants

Marriage et al. (2015) reported on a randomized, 119-day study with 2'-FL and GOS to examine growth and tolerance by infants fed formulas of chemically-synthesized 2'-FL (one group at a concentration of 0.2 g 2'-FL/L/day and a group at concentrations of 1.0 g 2'-FL/L) supplemented with a caloric density approximating human milk, compared to subjects fed formula without 2'-FL or fed human milk. All subjects enrolled were healthy, full-term infants of singleton birth, enrolled within five days of birth from 28 sites throughout the United States. Galactooligosaccharides (GOS) were included in the formulas in order to increase the prebiotic concentrations to 2.4 g/L. Growth was assessed through weight, length and head circumference measurements; tolerance was assessed through measurement of average stool consistency, number of stools per day, and percent of feedings associated with spit-up or vomit; and uptake was assessed through measurement of 2'-FL levels in infant plasma and urine in a subset of infants at day of life 42 and 119, and from the human milk of the breast-feeding mothers at day of life 42.

There were no significant differences reported between groups for weight, length or head circumference. The concentrations of 2'-FL present in the plasma and urine of infants were not significantly different between the 2'-FL uptake groups, and the human milk-fed infants. The only significant tolerance parameter was the percent of feedings with spit-up or vomit within 1 hour of feeding. This difference was only reported in the first month of life. The group fed formula without 2'-FL had a significantly higher spit-up frequency (17.5%, $p \leq 0.05$) compared to the groups fed formula containing 2'-FL (0.2 g/L: 21.5%; 1.0 g/L: 18%) and compared to the breastfeeding group (10.5%). Spit-up frequency was not dose dependent. There were no significant differences in number of stools and consistency of stool among the infant groups fed 2'-FL containing formula or human milk. In the first month of life, the breastfeeding group exhibited a significantly higher mean number of stools per day than the formula groups. After the first month of life, the mean rank stool consistency (1=watery, 5=hard) was not significantly different among the three formula groups but was significantly greater ($p<0.05$) compared to the breastfed group.

The authors reported no safety concerns with any of the formulas containing 2'-FL, and no significant differences in adverse events were reported between the experimental groups and the control group. An analysis of adverse events was conducted, and each subject's adverse events was reviewed and evaluated. The types of adverse events reported included upper respiratory tract symptoms, otitis media, viral infections, and oral candidiasis. The group fed formula containing 0.2 g 2'-FL/L had fewer reported adverse events with respect to "infections and infestations" compared to the other formula-fed groups. The group fed formula without 2'-FL also reported five incidents of eczema, while the groups fed formula containing 2'-FL reported none. Author's concluded that "formulas supplemented with 2'-FL are well tolerated, and 2'-FL absorption profiles are similar to those of breast-fed infants."

Goehring et al. 2016 reported on a sub-study nested within the above described (Marriage et al., 2015) study to examine the effect of 2'-FL on circulating inflammatory biomarkers such as cytokines interleukin receptor antagonist (IL-Ira), IL-1α, IL-1β, IL-6, and tumor necrosis factor (TNF-α) in blood samples. Blood samples were drawn from infants at 6 weeks of age (n = 31-42/group) that were fed formula containing 2'-FL and infants that were breastfed. Results indicated that infants fed formula containing 2'-FL were not different from breastfed infants and had 29-83% lower concentrations of plasma inflammatory cytokines than did infants fed the control formula with only GOS. Authors concluded that "2-FL fortification supports aspects of immune development and regulation similar to that in a BF reference group of infants."

Two prospective, randomized, multi-center, double blinded, controlled trials assessed the gastrointestinal tolerance of a test formula consisting of 0.2 g/L 2'-FL and 0.2 g/L scFOS (Kajzer et al., 2016 via Reverri et al., 2018) in healthy term infants. The infant formula was determined to be safe and well tolerated in infants. The infants were given the test formula for 35 days compared to infants fed breast milk. There were no statistically significant differences in demographic characteristics of sex, ethnicity, and race, nor age of enrollment or gestational age between the two groups. There were no statistically significant differences in weight, length, or anthropometric measurements between study groups. At 35 days of age, there were no significant differences in tolerance parameters of mean rank stool consistency, formula intake, anthropometric measures, and percent feedings with spit-up or vomit associated with feedings across the test groups. Author's concluded that "2'-FL and scFOS containing formula was safe and well tolerated."

Puccio et al. (2017) (cited in GRN 735) reported on a double-blind, randomized, controlled clinical trial in 175 healthy, full-term infants recruited from two hospitals in Italy and Belgium at age 0 to 14 days old between October 2012 and July 2013. The study aimed to evaluate infant growth, tolerance, and morbidity from exposure to an infant formula supplemented with two HMOs (2'-FL and LNnT). The treatment group received a mean daily intake of 908 mL formula containing a 2'-FL (1 – 1.2 g/L) and LNnT (0.5 – 0.6 g/L) (n = 88), and the control group received a mean daily intake of 929 mL standard formula that did not contain either oligosaccharide (n = 86). Each group received the assigned formula for up to 6 months and received standard follow-up formula without HMOs from six to 12 months of age. The study evaluated weight gain through four months as the primary endpoint, and anthropometric measures, gastrointestinal tolerance, behavioral patterns, and morbidity through 12 months of age as the secondary endpoints. Weight gain, digestive symptoms, and behavioral patterns were similar for both the control and test groups. At two months, there were a greater number of softer stools and fewer night- time wake-ups in the test group compared to the control group. The test group fed formula containing 2'-FL and LNnT had significantly fewer parental reports of bronchitis through four and 12 months, antipyretics use through four months, lower respiratory tract infection through 12 months, and antibiotics use through six and 12 months. Puccio et al. (2017) demonstrated that infant formula containing synthesized 2'-FL and LNnT is safe, well-tolerated and supports age-appropriate growth. In the evaluation of secondary outcome, there were associations between consuming 2'-FL and LNnT-supplemented formula and lower parent-reported morbidity (particularly bronchitis) and medication use (antipyretics and antibiotics).

Storm *et al.* (2019) reported on a randomized, controlled multicenter study to evaluate the safety and tolerability of a 100% whey, partially hydrolyzed infant formula with or without the addition of 2'-FL. Healthy infants 14 days of age were recruited from seven sites in the United States from September 2017 through February 2018, and randomized into two groups: one group received formula made from partially hydrolyzed, 100% whey protein with the addition of 0.25 2'-FL g/L (test group), and the second group (control group) received the 100% whey-

partially hydrolyzed formula without the addition of 0.25 2'-FL g/L. All infants were fed their assigned formula for six weeks. The Infant Gastrointestinal Symptom Questionnaire (IGSQ) was administered and anthropometric measurements were taken at baseline, this data was then compared to IGSQ and anthropometric measurements taken after the six weeks of formula exposure. At the end of the six weeks of formula intake, the subject and caregivers returned for a second visit after completing a formula intake, stooling, spit-up, and vomit for two days prior to the visit. This form also recorded any adverse events to be assessed by the site investigator.

IGSQ scores, stool frequency and consistency, crying, fussing duration, and vomiting frequencies from baseline to week six were similar between study groups. Average formula intake, body weight, and body lengths did not differ between the control and test groups. Differences in parameters between the two groups included: More stools were reported to be difficult to pass in the control subjects compared to the test group ($p = 0.04$); however, the number of infants with difficulty passing stools did not differ between groups. There were more subjects with a higher spit-up frequency in the test group compared to controls, and more subjects with reported infections in the control group compared to the test group. This study demonstrated that 100% whey, partially hydrolyzed infant formula with or without the addition of 0.25 g 2'-FL/L formula is safe and well tolerated.

Although no tolerance data are available from clinical studies, the levels of intake are not expected to result in any tolerance issues.

Table 11c. 2'-Fucosyllactose Clinical Trials in Infants					
Reference	**Study Type**	**Study Population**	**Route of Exposure**	**Significant Exposure-Related Outcomes**	**Conclusions Under Test Conditions**
Storm *et al.* 2019	Randomized, controlled, double-blind multicenter study	63 infants age 14 ± 5 days	Ingestion of formula containing 0 or 0.25 g/L 2'-FL for 42 days	Outcomes based on questionnaires completed by caregivers Primary Outcome **IGSQ score:** None Secondary Outcomes **Stool frequency, consistency, and ease of passing:** No significant differences in stool frequency or consistency between the control and test group. Significantly more stools reported difficult to pass in the control groups compared to the test group. However, the number of infants with stool difficult to pass did not differ significantly between groups. **Spit up, Vomiting, Crying, and Fussing:** No differences in the occurrences of crying and fussing and vomiting frequency between groups. Proportion of infants to have any spit up did not differ between groups; however, in the infants reported to spit up, significantly more were reported to spit up > 5 times per day in the test groups compared to the control. **Formula intake**: None **Adverse Events:** None. Spit up as an adverse event occurred in more test subjects compared to controls. Significantly more infections and infestations reported in the control group than in the treated groups.	Formula containing 2'-FL was **tolerated well** based on a comprehensive tolerance assessment tool.
Puccio et al. (2017)	Multi-center, randomized, double-blind trial of two parallel groups	175 healthy full-term infants 0 to 14 days old	Ingestion of formula containing 0 or 1 to 1.2 g/L 2'-FL for 6 months	**Weight:** None **Length, head circumference and body mass index (BMI):** None **Stool endpoints:** None **Other:** Significantly lower incidences of bronchitis and antibiotic use in treated infants compared to infants in the control group.	Formula containing 2'-FL was **tolerated well**

Table 11c. 2'-Fucosyllactose Clinical Trials in Infants					
Reference	**Study Type**	**Study Population**	**Route of Exposure**	**Significant Exposure-Related Outcomes**	**Conclusions Under Test Conditions**
Kajzer *et al.* (2016) (as cited in Reverri *et al.* (2018))	Prospective, randomized, multi-center, double-blinded, controlled tolerance trial	131 healthy term infants	Infant formula containing 0 (n = 30) or 0.2 g/L 2'-FL and 2 g/L scFOS (n = 35) or human breast milk (n = 36) for 35 days	**Stool endpoints:** None. Breast milk fed infants had a greater number of stools/day than formula fed infants. **Formula intake:** None **Anthropometric measure:** None **Spit up and vomiting:** None	**No significant differences in gastrointestinal tolerance** between infants fed formula containing 2'-FL and infants fed human breast milk were reported.
Marriage *et al.* (2015)	Randomized, double-blind and controlled study.	424 Healthy full-term singleton infants enrolled by five days of age; 304 infants completed the study (control formula: 79; experimental formula 1: 70; experimental formula 2: 72; and human milk: 83)	Ingestion of formula containing 0, 0.2, 1.0 g/L 2'-FL or human breast milk for 119 days	**Weight, length or head circumference:** None **Formula uptake:** None **Other:** 2'-FL was present in the plasma and urine of infants fed 2'-FL; growth and 2'-FL uptakes were similar to those of breast-fed infants.	Formula containing 2'-FL was **tolerated well.**
Goehring et al. (2016)	Sub-study nested within Marriage *et al.* (2015)	Healthy full-term singleton infants enrolled by 5 days of age, 315 of the 424 originally enrolled in Marriage *et al*. (2015). • 39 Control formula • 37 Experimental formula 1 • 37 Experimental formula 2 • 42 Human milk	Ingestion of formula containing 0, 0.2, 1.0 g/L 2'-FL or human breast milk for 119 days	**Inflammatory endpoints**: Infants fed formula supplemented with 2'-FL exhibited lower plasma and inflammatory cytokine profiles, similar to those of the breastfed reference group.	Formula containing 2'-FL was t**olerated well.**

6.3.3.2 Safety and Tolerance of 2'-FL in the Diets of Adults

The safety and tolerability of 2'-FL at doses of 5, 10, and 20 g per day for 14 days were evaluated in 100 healthy adults ages 19 to 57 years old in a double-blind, parallel, randomized, placebo-controlled study (Elison *et al.* 2016). Study participants (51 males and 49 females) received 5, 10 or 20 grams of either 2'-FL, LNnT or 2'-FL with LNnT (2:1 mass ratio), as well as a placebo group that received 2 g of glucose at breakfast each day.

Gastrointestinal endpoints (abdominal pain, indigestion, reflux, diarrhea, and constipation) were self-reported via questionnaire, and fecal samples collected prior to study entry and at the end of the intervention were analyzed for calprotectin, secretory IgA, and short-chain fatty acid levels as well as fecal microbiota composition. Blood samples were also collected at screening and the end of the intervention, and were analyzed for hemoglobin, erythrocytes, hematocrit, leucocytes, thrombocytes, creatinine, sodium, potassium, alanine aminotransferase, alkaline phosphatases, coagulation factor II, VII and X, bilirubin, albumin, C-reactive protein and glucose as well as HbA1c, apoA1, apoB, transferrin, progesterone, cortisol, estradiol, interleukin-10, interleukin-6, tumor necrosis factor-α, blood urea nitrogen, iron, high density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, total free fatty acids, insulin, lysozyme, testosterone and glucagon.

All participants completed the study. Any adverse events reported were considered to be mild, and all participants tolerated the investigational products throughout the trial period. The most commonly reported adverse events include flatulence, bloating, constipation, stomach pain, diarrhea, loose stool, and "rumbling" mostly reported by those in the group receiving the highest doses of 2'-FL and LNnT. These individuals did not have statistically significant changes on the gastrointestinal symptom rating scale. Changes in the average number of daily bowel movements were slightly increased in treatment groups compared to the baseline group (increase of 0.3 movements per day), and participants receiving 20 g 2'-FL reported softer stools as compared to baseline. The authors determined these effects to be clinically irrelevant. The authors note that it is difficult to discern whether the common gastrointestinal symptoms reported by study participants were due to treatment or due to normal variation and increased awareness of symptoms during the study period.

All measured clinical chemistry and hematology parameters remained within normal ranges. Blood safety assessments and physical examinations revealed no treatment-related irregularities.

Analyzing results of LNnT with 2'-FL can confound the study results specifically related to 2'-FL. While this study evaluates results for a 2'-FL group only, LnNT group only, and a combination group, results pertaining to the 2'-FL group only are most relevant and accurate in the evaluation of safety and tolerability of 2'-FL in adults. Further details can be found below in Table 11d.

Table 11d. 2'-Fucosyllactose Clinical Studies in Adults					
Reference	**Study Type**	**Population**	**Route of Exposure**	**Outcomes**	**Conclusions Under Test Conditions**
Elison *et al.* (2016)	Double-blind, parallel, randomized, placebo-controlled study	100 health adults age 19 to 57 years (51 males and 49 females).	Diets supplemented with 5, 10 or 20 g of either: (1) 2'-FL (2) LNnT (3) 2'-FL+LNnT (2:1 mass ratio) (4) Placebo: 2 g of glucose each day at breakfast	**Flatulence, bloating, and constipation:** 56 reported total from 44 participants. The most adverse events were reported by participants receiving the highest doses of 2'-FL and LNnT with flatulence being the most commonly reported adverse event followed by stomach pain, diarrhea or loose stool, and "rumbling" Reports of bloating and gas were significantly higher in the 20 g 2'-FL and LNnT groups **Mean GSRS scores:** Scores were low and participants receiving the highest dosages did not have statistically significant changes in their GSRS; **Stool consistency**: participants receiving 20 g 2'-FL reported softer stools as compared to baseline **Clinical chemistry and hematology parameters:** None	**No adverse health effects** were noted in adults consuming diets supplemented with up to 20 g 2'-FL per day.

6.4 Safety of 2'-flol

6.4.1 Safety of 2'-flol in Silico Testing

While there is no published literature describing the toxicological information of 2'-flol, testing of lactitol oligosaccharides (LO) in rats showed resistance to metabolism in the small intestines (Yanahira et al 1997) and found LO not affecting the well-being, growth, feed intake and feed efficiency in rats receiving LO as a 5% dietary admixture for 3 weeks (Yanashira et al 1995, Yanashira et al 1997). Amyris commisioned multiple in silico testing to confirm the safety of 2-flol using Nexus software (version 2.2.1): ICH M7 assessment using Lhasa software and in addition Derek Nexus (version 6.0.1) for other toxicological endpoints. The ICH M7 evaluation for the endpoint mutagenicity showed negative prediction using the statistical tool Sarah (version 3.0.0). The expert rule-based tool Derek prediction was negative without misclassified or unclassified features. For the other toxicological endpoints, the following were determined: 2-flol was negative for skin sensitization and equivocal for nephrotoxicity. No other alerts were fired. Toxtree software (version 3.1.0) characterized 2-flol as Cramer Class I compound. Thus, 2-flol is a substance of simple chemical structure with known metabolic pathways and innocuous end products. This suggest a low order of oral toxicity, with a Threshold of Toxicological Concern (TTC) of 1,800 µg/d (30 µg/kg bw/d). Due to 2-flol's high molecular weight of 490.45 g/mol, absorption from the gastrointestinal tract is expected to be negligible. Several fucosidases are described in the literature and these would be able to hydrolyze 2-flol, and some of these activities are expected to be present in the human gut (Schopohl et al., 1992; Ogata-Arakawa et al., 1977).

6.4.2 Amyris 2'-flol in vitro Fermentation

Amyris conducted an in vitro fermentation test using a 2'-FL prototype with 4% 2'-flol. The aim of the study was to evaluate the hydrolysis of 2'-flol into fucose and lactitol. Methods and results are described in Appendix I.

Results show that the fucosidases which can hydrolyze 2'-FL are also able to hydrolyze 2'-flol. This study showed that 2'-flol is hydrolyzed to fucose and lactitol, as 2'-FL is hydrolyzed to fucose and lactose. Fucose, lactose, and lactitol are all safe for use as food ingredients. Lactose is the largest macronutrient component in mature milk, and lactitol, is a non-naturally occurring sugar alcohol derived from the reduction of lactose. Lactose is not directly absorbed in the small intestine, and is hydrolyzed into galactose and glucose. Its low sweetness levels and relatively slow fermentation in the oral cavity, compared to simple sugars make it an appealing carbohydrate for use in infant formula. Lactitol is not digested in the upper gastrointestinal tract. Lactitol has been reported to act as a prebiotic by promoting the growth of bifidobacteria and lactobacilli (O'Donnell and Kearsley 2012; Finney et al 2007; Drakoularakou et al 2007). Lactitol is not fermented by oral bacteria, is considered to be non-cariogenic, and has a mild sweetness, which makes it particularly advantageous for use in infant formula and other milk, food and beverage products (Ackerman et al 2017). It has been reported that lactitol and lactose are not mutagenic, and not readily metabolized by humans or animals. Animal and human studies show that single and repeated large doses of lactitol is of very low toxicity (Joint FAO/WHO Committee on Food Additives, 2003).

6.5 Summary of Safety Data and Conclusion

2'-FL serves as a prebiotic for commensal gut bacteria which metabolize prebiotics into short-chain fatty acids used for energy by colonocytes, and to stimulate sodium and water absorption. Toxicological and clinical study data were evaluated for several existing 2'-FL formulations. The clinical and toxicological studies evaluating the safety and tolerance of 2'-FL support the determination that 2'-FL is safe for its intended food uses and proposed use levels.

As discussed in Section 6.3.1 and summarized in Table 11a, *in vitro* assays for genotoxicity and mutagenicity tests demonstrate that 2'-FL is not mutagenic and is not genotoxic.

As discussed in Section 6.3.2 and summarized in Table 11b, a pre-clinical study evaluating the safety of 2'-FL in neonatal pigs reported no observed adverse effects after repeated ingestion of 2'-FL at concentrations of 0, 200, 500, and 2000 mg 2'-FL/L/day for 21 days and dose levels of 29.37, 72.22 and 291.74 mg/kg/day in males and 29.30, 74.31, and 298.99 mg/kg/day in females.

Results of several repeated dose toxicity studies in rats demonstrate that 2'-FL is not toxic with a reported NOAEL of 5,000 mg/kg·bw/day. A 90-day oral toxicity study in juvenile rats reported a NOAEL of 7,760 mg/kg·bw/day.

Based on Amyris's proposed uses of 2'-FL in infant formula at up to 2.4 g/L, the mean EDI for an infant 0 to 6 months of age (6.8 kg average weight; approximately 1 L/day) consumption is 400 mg/kg·bw/day and an infant 7 to 12 months of age (9.3 kg average weight; approximately 1.5 L/day) consumption is 420 mg/kg·bw/day. Background levels of intake of 2'-FL as consumed in human milk based on human secretor mothers' milk ranges from 1.1 to 4.26 g/L (Bao et al., 2013; Galeotti et al., 2014). At an intake of 1.1 to 4.26 g/L for a 6.8 kg weight infant (0 to 6 months of age), this results in consumption of 162 to 627 mg/kg·bw/day. At an intake of 1.1 to 4.26 g/L for an 9.3 kg weight infant (7 to 12 months of age), this results in consumption of 118 to 458 mg/kg·bw/day. Thus, the highest consumption of 2'-FL for the proposed infant formula use does not exceed the range of consumption of 2'-FL in human breastmilk for breastfed infants. These proposed uses and intake levels are consistent with other safety assessments by authoritative bodies: as presented in other 2'-FL GRNs which received no-questions letters from FDA; as presented in EFSA opinions for 2'-FL as a novel food (EFSA, 2019).

All toddler, children, and adult intakes based on the proposed uses are well within the range of background intakes for infants described above. As presented in Table 3b, the 95th percentile EDI for toddlers is 450 mg/kg·bw/day, for children is 290 mg/kg·bw/day, for male teenagers is 180 mg/kg·bw/day, for female teenagers is 150 mg/kg·bw/day, for female adults of child-bearing age is 82 mg/kg·bw/day, for female adults is 76 mg/kg·bw/day, for male adults is 93 mg/kg·bw/day, and elderly adults is 86 mg/kg·bw/day (age ranges, body weights, and other information are provided in Table 3b). These EDIs for children, toddlers, and adults do not exceed the range of intakes on a per kg bw basis in breastfed infants. Thus, the intake of 2'-FL from Amyris's intended uses at the proposed use levels do not exceed the intake level of naturally occurring 2'-FL in breastfed infants per kilogram body weight, therefore, the specified uses and use levels of Amyris's 2'-FL are suitable, safe, and generally recognized as safe.

7. LIST OF SUPPORTING DATA AND INFORMATION. (21 CFR § 170.255)

7.1 List of Abbreviations

°C	Degrees Celsius
21 CFR	Part 21 of the Code of Federal Regulations
2'-FL	2'-fucosyllactose
2'-flol	2'-fucosyllacitol
3-FL	3-fucosyllactose
6'-SL	6'-sialyllactose
6'-SLN	6'-sialyl-*N*-acetyllactosamine
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, excretion
AMU	atomic mass unit
Amyris	Amyris, Inc.
bw	body weight
CASRN	Chemical Abstracts Service Registry Number
cfu	colony-forming units
cGMP	current good manufacturing practice
cm	centimeter
d	day(s)
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
EDI	estimated daily intake
EFSA	European Food Safety Authority
EU	endotoxin unit
FAO	Food and Agriculture Organization of the United Nations
FDA	United States Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FSSC	Food Safety System Certification
FOIA	Freedom of Information Act
g	gram(s)
GDP	guanosine 5'-diphospho-
GFSI	Global Food Safety Initiative
GOS	galactooligosaccharide
GRAS	Generally Recognized as Safe
GRN	Generally Recognized as Safe notice
HARPC	Hazard Analysis and Risk-Based Prevention Control

HDL	high-density lipoprotein
HMO	human milk oligosaccharide
HPAEC/PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
IGSQ	Infant Gastrointestinal Symptom Questionnaire
ISO	International Organization for Standardization
kg	kilogram(s)
L	liter(s)
LC-MS	liquid chromatography – mass spectrometry
LDL	low-density lipoprotein
LNFP	lacto-*N*-fucopentaose
LNnT	lacto-*N*-neotetraose
M	Molar
mg	millgram(s)
mL	milliliter(s)
mm	millimeter(s)
mol	mole
MW	molecular weight
ng	nanogram(s)
NHANES	National Health and Nutrition Examination Survey
NMR	nuclear magnetic resonance
No.	number
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
qPCR	quantitative real-time polymerase chain reaction
QPS	Qualified Presumption of Safety
RH	relative humidity
µg	microgram(s)
scFOS	short-chain fructooligosaccharides
S. cerevisiae	Saccharomyces cerevisiae
SOP	Standard Operating Procedure
TK	thymidine kinase
TNF	tumor necrosis factor
µg	microgram(s)
UV	ultraviolet
Vol.	volume
w/w	by weight
WHO	World Health Organization

7.2 References

All references listed below are generally available in accordance with 21 CFR §170.255:

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7.3 Appendices

Appendix A: Amyris and Carbosynth 2'-FL Proton NMR Spectra

Appendix B: Batch Analysis – Carbohydrate, KF titration, Protein, and DNA Results

Appendix C: Residual DNA Analysis

Appendix D: Chromatogram Results

Appendix E: Microbiological Specifications and Results

Appendix F: Real-time and Accelerated Stability Study of 2'-FL

Appendix G: Amino Acid Analysis

Appendix H: Allergenicity Evaluation

Appendix I: Enzymatic Hydrolysis of 2'-flol Report

Appendix J: Consensus Report of the GRAS Panel

Consensus Statement of the GRAS Panel on the Generally Recognized as Safe Status of the Proposed Uses of Amyris's 2'-Fucosyllactose

INTRODUCTION

The undersigned, an independent panel of experts, qualified by their scientific training and national and international experience to evaluate the safety of food and food ingredients (the GRAS Panel), was specially convened by Amyris, Inc., to conduct a critical and comprehensive evaluation of the available pertinent data and information, and to determine whether under the conditions of intended use as a food ingredient Amyris's 2'-Fucosyllactose (2'-FL), produced using a genetically engineered strain of *Saccharomyces cerevisiae* is safe and "generally recognized as safe" (GRAS) based on scientific procedures. For purposes of this evaluation, "safe" or "safety" as it relates to GRAS within the terms of the Federal Food, Drug, and Cosmetic Act means that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2012a).

Amyris, Inc. performed a comprehensive search of the literature relating to the safety of 2'-Fucosyllactose (2'-FL). Amyris summarized the results of the literature search and prepared a dossier, "**Safety Assessment and Generally Recognized as Safe (GRAS) Notification of 2'-Fucosyllactose (2'-FL) for Use as an Ingredient in Foods**" for independent consideration and review by the GRAS Panel.

The GRAS Panel consisted of the following individuals: Joseph F. Borzelleca, PhD, Emeritus Professor Virginia Commonwealth University School of Medicine; Roger A. Clemens, DrPH, PolyScience Consulting and Adjunct Professor, University of Southern California School of Pharmacy; and Stanley M. Tarka, Jr., PhD, ATS, (The Pennsylvania State University College of Medicine, Tarka Group, Inc). The GRAS Panel critically evaluated the safety documentation (the dossier), and other available data and information the members of the GRAS Panel believed to be pertinent to the safety of Amyris's 2-FL and its intended use as an ingredient in specified foods.

Following its independent and collective critical evaluation of the available information, the GRAS panel, convened on April 24 and May 5, 2020. Following its deliberations, the GRAS panel unanimously agreed to the conclusions described herein. A summary of the basis for these conclusions follows.

AMYRIS'S 2'-FL: MANUFACTURING PROCESS AND PRODUCT SPECIFICATIONS

The substance in this GRAS determination is 2'-Fucosyllactose (2'-FL) produced using a genetically engineered strain of *Saccharomyces cerevisiae* (strain CEN.PK113-7D). *S. cerevisiae*, also known as brewer's yeast or baker's yeast, has an extensive history of safe use in the food industry (21 CFR §172.896, 21 CFR §172.325 21 CFR §172.898, 21 CFR §184.1983). *S. cerevisiae* also has been granted Qualified Presumption of Safety (QPS) status in the European Union by the European Food Safety Authority (EFSA)[1] and, therefore, is considered safe for the derivation of genetically modified strain lineages intended for use in the production of food additives and enzymes. In Amyris's production strain, the DNA construct was inserted by homologous recombination, and the introduced genetic elements are stable. The production strain is not toxigenic or pathogenic, and does not contain or produce any known pathogenicity-related proteins, toxins, allergens, pyrogens, or antibiotic resistance markers. The manufacturing process consists of a fermentation process where food-grade sugar, lactose, and fermentation nutrients are fed to a culture of the production strain and fermented to produce 2'-FL and other carbohydrates. The fermentation process is conducted under strictly controlled temperature and pH conditions with appropriate heat treatment and purification steps. After fermentation, the 2'-FL

[1] Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Girones, R., ... & Robertson, L. (2017). Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. *EFSA Journal*, *15*(3). https://doi.org/10.2903/j.efsa.2017.4664

fermentation broth is separated from the aqueous phase by centrifugation. Any residual yeast is removed though separation and purification steps, and residual solids, proteins, DNA, salts, and organic acids are removed through various filtration steps. Chromatographic and polishing steps then remove any additional salts, metals, proteins, organic acids, and colorants. The 2'-FL then goes through additional filtration, evaporation, heat treatment, and sterilization steps before the concentrated product is spray-dried to reduce moisture to ≤ 5.0 % by weight (w/w). All processing aids in the post-fermentation process are approved for use in food processing as noted in the dossier.

Amyris's 2'-FL has a molecular weight of 488.44 AMU and conforms to 2'-FL structure (CAS# 41263-94-9). A comparison of the proton NMR spectra for Carbosynth 2'-FL (reference standard) and the Amyris 2'-FL demonstrate structural equivalence (Appendix A of dossier). Amyris's 2'-FL was not evaluated in any toxicology studies; however, numerous other sources of 2'-FL (Tables 11a and 11b in the dossier) were test materials for toxicity studies. These studies are evaluated and summarized in the dossier. To ensure that the toxicology studies are appropriate for evaluating Amyris's 2'-FL, the profiles of the 2'-FL substances (i.e., the distribution of non-2'-FL carbohydrates) were compared and found to be sufficiently similar to base safety conclusions on the results of these studies.

To ensure that a consistent food-grade ingredient is produced, Amyris has established specifications for their 2'-FL. The chemical, physical and microbiological specifications are presented in **Table 1**. Batches were analyzed for conformity to the established specifications and all batches meet specifications.

Table 1: Specifications of Amyris's 2'-Fucosyllactose

Parameter	Specification	Method
Carbohydrate content (% area)		
2'-fucosyllactose	≥ 86% area	Ion chromatography (Amyris SOP 830)
Difucosyllactose (DFL)	< 8% area	
Lactose/allo-lactose	< 7% area	
2'-fucosyllactitol	≤ 6% area	
3-Fucosyllactose (3FL)	< 7% area	
Fucosyl-galactose		
Xylitol		
Dulcitol/sorbitol		
Glucose/Galactose		
Fucose		
Glycerophosphoethanolamine (GPE)		
Fructose		
Chemical		
Water Content (KF titration)	≤5.0% w/w	Karl Fischer titration (Amyris SOP 842)
pH (20 °C, 5% solution)	3.0 – 7.5	EP 2.2.3 v9
Protein Content	≤0.01% w/w	Modified Bradford Assay (Amyris SOP 843)
Total Ash	≤0.5% w/w	FCC 11 appendix II
Arsenic	≤ 0.2 mg/kg	EP 2.2.58 v9
Cadmium	≤ 0.05 mg/kg	EP 2.2.58 v9
Lead	≤ 0.05 mg/kg	EP 2.2.58 v9
Mercury	≤ 0.1 mg/kg	EP 2.2.58 v9
GMO detection (rDNA from production strain)	Negative	PCR (Amyris SOP 844)

Table 1: Specifications of Amyris's 2'-Fucosyllactose		
Parameter	**Specification**	**Method**
Microbial Specifications		
Total Aerobic Microbial Count/Standard Plate Count	≤ 1000 cfu/g	EP 2.6.12 v9
Total Yeast/Mold Count	≤ 100 cfu/g	EP 2.6.12 v9
Sulfite Reducing Bacteria	< 100 cfu/g	ISO 15213: 2003
Enterobacteriaceae	Negative in 10 g	EP 2.6.13 v9
Salmonella	Not detected in 25 g	EP 2.6.13 v9
Cronobacter sakazakii	Not detected in 10 g	ISO/TS 22964
Coliforms	Not detected in 10 g	ISO 4831: 2006
E. coli	Absent in 10 g	EP 2.6.13 v9
Listeria monocytogenes	Absent in 10 g	ISO 11290-1: 2017
Pseudomonas aeruginosa	Absent in 10 g	EP 2.6.13 v9
Staphylococcus aureus	Negative in 10 g	EP 2.6.13 v9
Bacillus cereus	< 100 cfu/g	ISO 7932: 2004
Abbreviations: °C = degrees Celsius; cfu = colony-forming unit; EP = European Pharmacopoeia; EU = endotoxin units; FCC = Food Chemicals Codex; g = grams; ISO = International Organization for Standardization; KF = Karl Fischer; m = milli; SOP = Standard Operating Procedure; w/w = by weight; v = version.		

In addition to batch testing, batches were analyzed in an accelerated mode stability study (13 weeks, 40°C, 75% Relative Humidity (RH)). Data from this study confirmed that Amyris 2'-FL powder is stable for 1.5 years (Appendix F in the dossier).

USES AND EXPOSURES

History of Use and Exposure

Approximately 85% of the world's population is exposed to 2'-FL from human milk. Human milk oligosaccharides (HMOs) are the third largest component of breast milk solids after lactose and lipids and 2-'FL is the most abundant HMO in human breast milk.[2] Most infants have been exposed to 2'-FL because it is a naturally occurring component of human breast milk, synthesized in the mammary glands of secretor mothers.[3] Even infants fed breast milk from non-secretor mothers excrete 2'-FL in the urine and in the stool, indicating the infant can produce alpha--1,2-epitope containing glycans.[4] The mean

[2] Coppa GV, Bruni S, Morelli L, Soldi S, Gabrielli O (2004) The first prebiotics in humans: human milk oligosaccharides. J Clin Gastroenterol 38(6 Suppl) S80-83. https://doi.org/10.1097/01.mcg.0000128926.14285.25.

[3] Castanys-Munoz E, Martin MJ, Prieto PA (2013) 2'-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. Nutr Rev 71(12) 773-789. https://doi.org/10.1111/nure.12079

[4] Kunz C, Rudloff S (2017) Compositional analysis and metabolism of human milk oligosaccharides in infants. Intestinal microbiome: functional aspects in health and disease 88 137-148. Karger Publishers. https://doi.org/10.1159/000455398.

concentration of 2'-FL in human breast milk ranges from 1.1 g/L[5,6] to 4.26 g/L,[7] with levels up to 7.3 g/L reported.[8]

Proposed Uses and Estimated Daily Intakes

The intended uses and maximum use levels of Amyris's 2'-FL were presented in one or more 2'-FL GRNs. Amyris intends to use its 2'-FL as a food ingredient in term infant formulas (non-exempt), toddler formulas (12-36 months) at a maximum level of 2.4 g 2'-FL per liter. Amyris also intends to use its 2'-FL in baby foods for infants and young children (children older than one year of age) and beverages for young children. Other uses in infant and toddler food and beverage products include processed cereals, infant meal replacement products, ready-to-eat hot cereals, yogurts and drinks, desserts such as fruit desserts and cobblers, snack crackers and cookies, milk modifiers, and milk-based drinks. Amyris is proposing to use its 2'-FL in conventional foods and beverages intended for children and adults such as in milk substitutes, flavoring in milk-based beverages such as coffees and smoothies, frozen dairy desserts such as ice cream and frozen yogurt, fruit pie fillings, fruit preserve products, meal replacement beverages and meal replacement bars, breakfast bars, cereal products (hot and ready-to-eat), energy drinks, sports drinks, and fruit drinks/juices. Amyris also proposes to use its 2'-FL as an ingredient of oral nutritional supplements for enteral feeding for ages 11 years and older. Specific food uses and use levels are presented in **Table 2.**

Table 2. Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the U.S.

Food Category	Proposed Food Use	RACC[a]	Proposed Use Level (g/RACC or g/serving)	Proposed Maximum Use level (g/kg or g/L)[b]
Dairy	Unflavored Pasteurized and Sterilized milk	240 mL	0.28	1.2
	Buttermilk	240 mL	0.28	1.2
	Yogurt	225 g	1.2	5.3
	All acidophilus or fortified milks, non-fat and low-fat milk fluids, including fluid milk and reconstituted milk powder	240 mL	0.28	1.2
	Flavored milks, including chocolate milk, coffee drinks, cocoa, smoothies (dairy and fruit based), other fruit and dairy combinations, yogurt drinks and fermented milk drinks including kefir	240 mL	0.28	1.2
	Frozen dairy desserts including ice cream and frozen yogurts, frozen novelties	~70 g	1.2	17
	Milk product for pregnant women ("mum formulas") -9 to 0 months	200 mL	1.2	6.0
Dairy analogs	Milk substitutes such as soymilk and imitation milks	240 mL	0.28	1.4
	Non-dairy yogurt	225 g	1.2	5.3

[5] McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, Kamau-Mbuthia EW, Kamundia EW, Mbugua S, Moore SE, Prentice AM (2017) What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. The American journal of clinical nutrition 105(5):1086-100. https://doi.org/10.3945/ajcn.116.139980.

[6] Bao, Y C. Chen, D. S. Newburg, *Anal. Biochem.* 2013, *433* (1), 28-35. *Quantification of neutral human milk* oligosaccharides *by graphitic carbon HPLC with tandem mass spectrometry.* https://doi.org/10.1016/j.ab.2012.10.003.

[7] Galeotti, F., Coppa, G. V., Zampini, L., Maccari, F., Galeazzi, T., Padella, L., ... & Volpi, N. (2014). Capillary electrophoresis separation of human milk neutral and acidic oligosaccharides derivatized with 2-aminoacridone. *Electrophoresis*, *35*(6), 811-818. https://doi.org/10.1002/elps.201300490.

[8] Gabrielli, O., Zampini, L., Galeazzi, T., Padella, L., Santoro, L., Peila, C., ... & Coppa, G. V. (2011). Preterm milk oligosaccharides during the first month of lactation. *Pediatrics*, *128*(6), e1520-e1531. https://doi.org/10.1542/peds.2011-1206.

Table 2. Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the U.S.				
Food Category	**Proposed Food Use**	**RACC[a]**	**Proposed Use Level (g/RACC or g/serving)**	**Proposed Maximum Use level (g/kg or g/L)[b]**
Infant formulas, Follow-on formula, and baby foods	Infant formula (non-exempt formula)	100 mL	0.24	2.4
	Toddler formulas, growing-up milks (12-36 months)	100 mL	0.24	2.4
	Processed cereal-based food and baby food for infants and young children	7 to 170 g	0.084 to 2.04	12
	Other Foods for Infants and Young Children	7 to 170 g	0.084 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2
	Infant meal replacement products	100 mL	0.24	2.4 (0.4 g/100kcal)
	Ready-to-eat, ready-to-serve, hot cereals	15 g (dry) 110 g (ready-to-serve)	1.2	10.9 (as consumed)
	Yogurt and juice beverages ("baby drinks")	120 mL	1.2	10
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type dessert")	110 g or mL	1.2	10.9
	Baby crackers, pretzels, cookies and snacks items	7 g	0.4	57
	Milk-based drinks and similar products intended for young children	120 mL	0.14	1.2
	Milk modifiers (i.e. powder for addition in milk such as cacao-based powders, etc.)	120 mL (ready to serve)	0.14	1.2
	Syrups used to flavor milk beverages	40 g	0.28	7.0
	Dairy based pudding custards and mousses	~70 g	1.2	17
	Fruit pie filling	85 g	1.2	14.1
	Fruit preparation such as fruit filing in bars, cookies, yogurt and cakes	~40 g	1.2	30
	Jellies and jams, fruit preserved and fruit butters	~20 g	1.2	60
	Non-dairy yogurt	225 g	1.2	5.3
Meal substitutes	Oral nutritional supplements and enteral tube feeding (11 years and older)	200 g or mL	4.0	20
	Milk-based meal replacement beverages or diet beverages / meal replacement drinks for weight reduction (milk-based and non-milk-based)	240 mL	1.2	5.0
	Meal replacement bars for weight reduction	30g	1.2	40
Grain products	Grain bars, including snack bars, meal replacement bars, and breakfast bars	40g	0.48	12
Breakfast cereals	Ready-to-eat breakfast cereals for adults and children - puffed	15g	1.2	80
	Ready-to-eat breakfast cereals for adults and children – high-fiber	40g	1.2	30
	Ready-to-eat breakfast cereals for adults and children – biscuit types	60g	1.2	20

Table 2. Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the U.S.				
Food Category	**Proposed Food Use**	**RACC[a]**	**Proposed Use Level (g/RACC or g/serving)**	**Proposed Maximum Use level (g/kg or g/L)[b]**
	Hot cereals for adults and children	40g (dry) (~240g prepared)	1.2	4.8 (as consumed)
Beverage	Flavored drinks	360 mL	0.28	0.8
	Energy drinks	360 mL	0.28	0.8
	Fitness and thirst quenchers, sport and isotonic drinks / sport, isotonic drinks	360 mL	0.28	0.8
	Fruit drink, including vitamin and mineral-fortified products	240 mL	0.28	1.2
	Fruit juices / fruit juices and nectar	240 mL	0.28	1.2

[a] RACC = Reference Amounts Customarily Consumed per Eating Occasion in the U.S. Code of Federal Regulations, 2018 (21 CFR 101.12).

[b] Proposed maximum use level is presented on g/kg basis for solids, and g/L basis for liquids and forms. The basis for the calculation of Estimated Daily Intake is presented in Tables 3a and 3b.

The estimated daily intake (EDI) of Amyris's 2'-FL was estimated using food consumption data reported in the United States Department of Health and Human Service's 2013-2016 National Health and Nutrition Examination Surveys (NHANES), is presented in **Tables 3a and 3b**.

Table 3a: Summary of the Estimated Daily Intake of 2'-FL from All Proposed Food and Beverage Uses in the U.S. by Population Group (2013-2016 NHANES Data)					
Population Group	**Age Group**	**All-Users Consumption (g/day)**			
		% Users	**N**	**Mean**	**95th Percentile**
Infants	0-6 mo.	100	241	2.73	5.87
Infants	7-12 mo.	99.66	228	3.82	8.43
Toddlers	1 to 3 yr.	98.77	1117	2.30	5.49
Children	4 to 10 yr.	99.34	2315	2.61	7.08
Male Teenager	11-18 yr.	99.49	1213	3.12	8.99
Female Teenager	11-18 yr.	99.13	1216	2.36	7.28
Female Adults of childbearing age	19-40 yr.	99.30	1807	1.78	5.79
Female Adults	19-64 yr.	99.49	3767	1.72	5.45
Male Adults	19-64 yr.	99.30	3313	2.38	7.84
Elderly Adults	65 yr. and up	98.95	1215	2.25	6.30
Abbreviations: 2'-FL = 2'-fucosyllactose; g = grams; mo. = months; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; yr. = years					

Table 3b: Summary of the Estimated Daily Intakes of 2'-FL per Kilogram Body Weight from All Proposed Food and Beverage Uses in the U.S. by Population Group (2013-2016 NHANES Data)							
		Mean	95th Percentile	% Users	N	Mean	95th Percentile
Infants	0-6 mo.	6.8	8.9	100	241	0.40	0.82
Infants	7-12 mo.	9.3	11.3	99.6	227	0.42	0.88
Toddlers	1 to 3 yr.	13.8	18.5	98.77	1103	0.18	0.45
Children	4 to 10 yr.	28.9	48.5	99.34	2303	0.10	0.29
Male Teenager	11-18 yr.	60.4	94.5	99.49	1210	0.054	0.18
Female Teenager	11-18 yr.	65.6	106.0	99.13	1205	0.043	0.15
Female Adults of childbearing age	19-40 yr.	76.5	120.2	99.30	1791	0.025	0.082
Female Adults	19-64 yr.	78.2	120.3	99.49	3739	0.024	0.076
Male Adults	19-64 yr.	89.6	130.3	99.30	3295	0.028	0.093
Elderly Adults	65 yr. and up	79.5	113.9	98.95	2283	0.029	0.086
Abbreviations: 2'-FL = 2'-fucosyllactose; bw = body weight; g = grams; k = kilo; mo. = months; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; yr. = years							

SAFETY ASSESSMENT

The addition of Amyris's 2'-FL in infant formula will enable the infant formula to more closely approximate the composition of human milk. 2'-FL as an ingredient in infant formula and general population uses have been reviewed by the FDA in at least six GRNs that all received letters of no objection from FDA.[9]

Absorption, distribution, metabolism and excretion of 2'-FL

It has been consistently demonstrated in studies specifically evaluating ADME of infant formula oligosaccharides that HMOs are not readily absorbed by infants and arrive intact in the colon where they are metabolized by resident microbiota and/or excreted in the feces.[10,11] Studies report that 40 to 50% of the 2'-FL in breast milk consumed by infants was reported unchanged in fecal samples.[12,13,14] Less than 5% of 2′-FL and other HMOs consumed by infants were absorbed from the GI tract and most were

[9] GRNs 546, 571, 650, 735, 749, 852

[10] Engfer MB, Stahl B, Finke B, Sawatzki G, Daniel H (2000) Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr 71(6) 1589-1596. https://doi.org/10.1093/ajcn/71.6.1589.

[11] Gnoth MJ, Kunz C, Kinne-Saffran E, Rudloff S (2000) Human milk oligosaccharides are minimally digested in vitro. J Nutr 130(12) 3014-3020. https://doi.org/10.1093/jn/130.12.3014.

[12] Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, Newburg DS (2001) Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology 11(5) 365-372. https://doi.org/10.1093/glycob/11.5.365.

[13] Coppa, G. V., Pierani, P., Zampini, L., Bruni, S., Carloni, I., & Gabrielli, O. (2001). Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. In *Bioactive components of human milk* (pp. 307-314). Springer, Boston, MA. https://doi.org/10.1007/978-1-4615-1371-1_38.

[14] Albrecht S, Schols HA, van Zoeren D, van Lingen RA, Groot Jebbink LJ, van den Heuvel EG, Voragen AG, Gruppen H (2011) Oligosaccharides in feces of breast- and formula-fed babies. Carbohydr Res 346(14) 2173-2181. https://doi.org/10.1016/j.carres.2011.06.034.

detected intact in the large intestine where they are subjected to partial fermentation by indigenous microbiota.[15] The unabsorbed 2'-FL is metabolized by gut microbiota to short-chain fatty acids.

Toxicological and Clinical Studies

The toxicological and clinical studies evaluating the safety and tolerance of 2'-FL support the determination that 2'-FL is safe for its intended food uses and proposed use levels. The toxicological studies (Tables 11a and 11b in the dossier) and clinical studies (Tables 11c and 11d in the dossier) are summarized and discussed below.

The toxicology studies were performed on 2'-FL produced either by microbial fermentation or chemical synthesis by various technologies. Although Amyris's 2'-FL was not the test substance evaluated, the other 2'-FLs that were evaluated are appropriate for evaluating Amyris's 2'-FL because the profiles of 2'-FL and the associated substances, i.e., the non-2'-FL carbohydrates (Table 5b in the dossier), were sufficiently similar to base safety interpretations on the results of these studies. This substantial chemical equivalency supports bridging to the published studies.

In vitro assays for genotoxicity and mutagenicity tests on other sources of 2'-FL suggest that 2'-FL produced either by chemical synthesis or microbial fermentation is not mutagenic and is not genotoxic. Using concentrations up to 5000 µg/plate of 2'-FL, with and without metabolic activation, there were no signs of cytotoxicity and no increase in revertant colony numbers reported in any of the test strains when compared to control counts.[16,17,18,19,20,21]

Repeated dose toxicity studies demonstrated that 2'-FL does not induce toxic effects after ingestion by rats for 90 days. The studies evaluated the safety of 2'-FL produced via microbial fermentation or chemical synthesis administered by gavage or as a dietary admixture to rats. Subchronic oral toxicity studies in which 2'-FL was administered by gavage at doses of 0, 1000, 2000, and 5000 mg

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[15] Brand-Miller , J. C., McVeagh, P., McNeil, Y. & Messer, M. 1998. Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. *J. Pediatr.,* 133, 95-98. https://doi.org/10.1016/s0022-3476(98)70185-4.

[16] Verspeek-rip, M. (2015). Evaluation of the Mutagenic Activity of 2'FL in the Salmonella Typhimurium Reverse Mutation Assay and the Eshcerichia Coli Reverse Mutation Assay. (Laboratory Project Identification: Project 507432; Substance 206374/B). Prepared by DD 's Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S. (discussed in GRN 650)

[17] Verbaan, A.J. (2015a). An In Vitro Micronucleus Assay with 2'-0-Fucosyllactose In Cultured Peripheral Human Lymphocytes: Confidential. (Laboratory Project Identification: Project 507398; Substance 206096/A). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom *Al* S (discussed in GRN 650)

[18] Verbaan, A.J. (2015b). An In Vitro Micronucleus Assay with 2'FL in Cultured Peripheral Human Lymphocytes. (Laboratory Project Identification: Project 507433; Substance 206374/B). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S (discussed in GRN 650).

[19] Coulet M, Phothirath P, Allais L, Schilter B (2014) Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-Fucosyllactose (2'-FL). Regulatory Toxicology and Pharmacology 68(1) 59-69. https://doi.org/10.1016/j.yrtph.2013.11.005.

[20] Phipps KR, Baldwin N, Lynch B, Flaxmer J, Šoltésová A, Gilby B, Mikš MH, Röhrig CH (2018). Safety evaluation of a mixture of the human-identical milk oligosaccharides 2′-fucosyllactose and difucosyllactose. Food and chemical toxicology. 120:552-65. https://doi.org/10.1016/j.fct.2018.07.054.

[21] van Berlo D, Wallinga AE, van Acker FA, Delsing DJ (2018) Safety assessment of biotechnologically produced 2′-Fucosyllactose, a novel food additive. Food and Chemical Toxicology. 118:84-93. https://doi.org/10.1016/j.fct.2018.04.049.

2'-FL/kg·bw/day in juvenile rats (strain Crl:WI(Han) or Crl:CD(SD)) reported a NOAEL of 5000 mg/kg·bw/day, the highest dose tested.[22,23,24]

In a 90-day oral (feeding) toxicity study in juvenile rats (strain Crl:WI(Han)), the mean intakes of 2'-FL were 0, 2.17, 4.27, and 7.25 g/kg·bw/day 2'-FL, and 0, 2.45, 5.22, and 7.76 g/kg·bw/day in male and female juvenile rats respectively. A NOAEL of 7.76 g/kg·bw/day 2'-FL was reported by the authors.[25] The safety of 2'-FL was evaluated in a 21-day repeated dosing study in neonatal pigs and no observed adverse effects were reported at 2'-FL concentrations of 0, 200, 500, and 2000 mg 2'-FL/L/day for 21 days. These doses are equivalent to 29.37, 72.22 and 291.74 mg/kg/day in males and 29.30, 74.31, and 298.99 mg/kg/day in females.[26]

Four clinical studies evaluated the growth and tolerance of infants fed formulas containing 2'-FL and other oligosaccharides. The studies evaluated concentrations at 0.2 to 1.2 g 2'-FL/L formula and the combined effects of 2'-FL with GOS,[27] short-chain fructo-oligosaccharides (scFOS),[28] LNnT,[29] or whey.[30] No adverse effects or alterations in growth were reported.

A clinical study evaluated the tolerability of 2'-FL administered at doses of 5, 10, and 20 g per day 2'-FL, LNnT, or 2'-FL with LNnT (2:1 mass ratio) for 14 days in adults ages 19 to 57 years.[31] Participants receiving the highest dose of 20 g/day 2'-FL and LNnT (2:1 mass ratio) reported significantly higher occurrence of bloating and gas compared to baseline, and those receiving 20 g/day 2'-FL reported softer stools as compared to baseline. The authors determined these effects were clinically irrelevant, however, these observations may suggest there is a tolerance limit of 20 g 2'-FL/day.

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[22] Penard, L. (2015). 2'-FL – 13-Week Oral (Gavage) Juvenile Toxicity Study in the Rat Followed by a 4-Week Treatment-Free Period. (Study Number AB20757; Sponsor Reference Number GSN037). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S. (discussed in GRN 650)

[23] Coulet M, Phothirath P, Allais L, Schilter B (2014) Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-Fucosyllactose (2'-FL). Regulatory Toxicology and Pharmacology 68(1) 59-69. https://doi.org/10.1016/j.yrtph.2013.11.005.

[24] Phipps KR, Baldwin N, Lynch B, Flaxmer J, Šoltésová A, Gilby B, Mikš MH, Röhrig CH (2018). Safety evaluation of a mixture of the human-identical milk oligosaccharides 2′-fucosyllactose and difucosyllactose. Food and chemical toxicology. 120:552-65. https://doi.org/10.1016/j.fct.2018.07.054.

[25] van Berlo D, Wallinga AE, van Acker FA, Delsing DJ (2018) Safety assessment of biotechnologically produced 2′-Fucosyllactose, a novel food additive. Food and Chemical Toxicology. 118:84-93. https://doi.org/10.1016/j.fct.2018.04.049.

[26] Hanlon, P. R., & Thorsrud, B. A. (2014). A 3-week pre-clinical study of 2′-fucosyllactose in farm piglets. Food and chemical toxicology, 74, 343-348. https://doi.org/10.1016/j.fct.2014.10.025.

[27] Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA (2015) Infants fed a lower calorie formula with 2′ FL show growth and 2′ FL uptake like breast-fed infants. Journal of pediatric gastroenterology and nutrition. 61(6):649. https://doi.org/10.1097/mpg.0000000000000889.

[28] Reverri EJ, Devitt AA, Kajzer JA, Baggs GE, Borschel MW (2018) Review of the clinical experiences of feeding infants formula containing the human milk oligosaccharide 2′-fucosyllactose. Nutrients. 10(10):1346. https://doi.org/10.3390/nu10101346.

[29] Puccio G, Alliet P, Cajozzo C, Janssens E, Corsello G, Sprenger N, Wernimont S, Egli D, Gosoniu L, Steenhout P (2017) Effects of infant formula with human milk oligosaccharides on growth and morbidity: a randomized multicenter trial. Journal of pediatric gastroenterology and nutrition. 64(4):624. https://doi.org/10.1097/mpg.0000000000001520.

[30] Storm HM, Shepard J, Czerkies LM, Kineman B, Cohen SS, Reichert H, Carvalho R (2019) 2′-Fucosyllactose Is Well Tolerated in a 100% Whey, Partially Hydrolyzed Infant Formula with Bifidobacterium lactis: A Randomized Controlled Trial. Global pediatric health. https://doi.org/10.1177/2333794x19833995.

[31] Elison E, Vigsnaes LK, Krogsgaard LR, Rasmussen J, Sørensen N, McConnell B, Hennet T, Sommer MO, Bytzer P (2016) Oral supplementation of healthy adults with 2′-Ofucosyllactose and lacto-N-neotetraose is well tolerated and shifts the intestinal microbiota. British Journal of Nutrition. 116(8):1356-68. https://doi.org/10.1017/s0007114516003354.

Comparison of Safe Intake Levels to Proposed Intake Levels

Based on Amyris's proposed uses of 2'-FL in infant formula at up to 2.4 g/L, the mean EDI for an infant 0 to 6 months of age (6.8 kg average weight; approximately 1 L/day) is 400 mg/kg·bw/day and an infant 7 to 12 months of age (9.3 kg average weight; approximately 1.5 L/day) is 420 mg/kg·bw/day. For comparison, background levels of intake of 2'-FL as consumed in human milk based on human secretor mothers' milk ranges from 1.1 to 4.26 g/L.[32,33] At an intake of 1.1 to 4.26 g/L for a 6.8 kg weight infant (0 to 6 months of age), this results in consumption of 162 to 627 mg/kg·bw/day. At an intake of 1.1 to 4.26 g/L for an 9.3 kg weight infant (7 to 12 months of age), this results in consumption of 118 to 458 mg/kg·bw/day. Thus, the highest consumption of 2'-FL for the proposed infant formula use does not exceed the range of consumption of 2'-FL in human breastmilk for breastfed infants. These proposed uses and intake levels are consistent with other safety assessments by authoritative bodies: as presented in other 2'-FL GRNs which received no-questions letters from FDA; as presented in EFSA opinions for 2'-FL as a novel food in 2019.[34]

All toddler, children, and adult intakes based on the proposed uses are well within the range of background intakes for infants described above. As presented in Table 3b, the 95th percentile EDI for toddlers is 450 mg/kg·bw/day, for children is 290 mg/kg·bw/day, for male teenagers is 180 mg/kg·bw/day, for female teenagers is 150 mg/kg·bw/day, for female adults of child-bearing age is 82 mg/kg·bw/day, for female adults is 76 mg/kg·bw/day, for male adults is 93 mg/kg·bw/day, and elderly adults is 86 mg/kg·bw/day (age ranges, body weights, and other information are provided in Table 3b). These 95th percentile EDIs for children, toddlers, and adults do not exceed the range of intakes on a per kg bw basis in breastfed infants. Because the intake of 2'-FL from Amyris's intended uses at the proposed use levels is unlikely to exceed the intake level of naturally occurring 2'-FL in breastfed infants per kilogram body weight, the GRAS Panel concluded that the specified uses and use levels of Amyris's 2'-FL are suitable, safe, and GRAS.

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[32] Bao, Y C. Chen, D. S. Newburg, *Anal. Biochem.* 2013, *433* (1), 28-35. *Quantification of neutral human milk* oligosaccharides *by graphitic carbon HPLC with tandem mass spectrometry.* https://doi.org/10.1016/j.ab.2012.10.003.

[33] Galeotti, F., Coppa, G. V., Zampini, L., Maccari, F., Galeazzi, T., Padella, L., ... & Volpi, N. (2014). Capillary electrophoresis separation of human milk neutral and acidic oligosaccharides derivatized with 2-aminoacridone. *Electrophoresis*, *35*(6), 811-818. https://doi.org/10.1002/elps.201300490.

[34] EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA), Turck, D., Castenmiller, J., De Henauw, S., Hirsch-Ernst, K. I., Kearney, J., ... & Pelaez, C. (2019). Safety of 2'-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283. *EFSA Journal*, *17*(6), e05717. https://doi.org/10.2903/j.efsa.2019.5717

CONCLUSIONS

We, the undersigned independent qualified members of the GRAS Panel, have individually and collectively, critically evaluated the data and information summarized above, and other data and information that we deemed appropriate to the safety of the intended use of Amyris's 2'-FL in specified foods. We unanimously conclude that the proposed uses and use levels of Amyris's 2'-FL produced via fermentation using a genetically engineered strain of *Saccharomyces cerevisiae* (as presented in the GRAS dossier and summarized herein and based on parental strain CEN.PK113-7D) in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate established specifications as presented in the supporting dossier *["Safety Assessment and Generally Recognized as Safe (GRAS) Notification of 2'-Fucosyllactose (2'-FL) for Use as an Ingredient in Foods"]* is safe.

We further unanimously conclude that the proposed uses and use levels as an ingredient in specified foods of Amyris's 2'-FL, produced via fermentation using a genetically engineered strain of *Saccharomyces cerevisiae* in a manner that is consistent with current cGMP and meeting the food grade specifications as presented in the supporting dossier are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other experts, qualified by scientific training and experience to evaluate the safety of food and food ingredients, and evaluating the same data and information, would concur with these conclusions.

Joseph F. Borzelleca, PhD
Professor Emeritus of Pharmacology and Toxicology
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signature: [redacted]

Date: [redacted]

Roger A Clemens, DrPH
Co-founder and Executive Vice President
PolyScience Consulting
Adjunct Professor, Pharmacology & Pharmaceutical Sciences
Professor, Regulatory and Quality Sciences
The D.K. Kim International Center for Regulatory Science
University of Southern California School of Pharmacy
Los Angeles, California

Signature: [redacted]

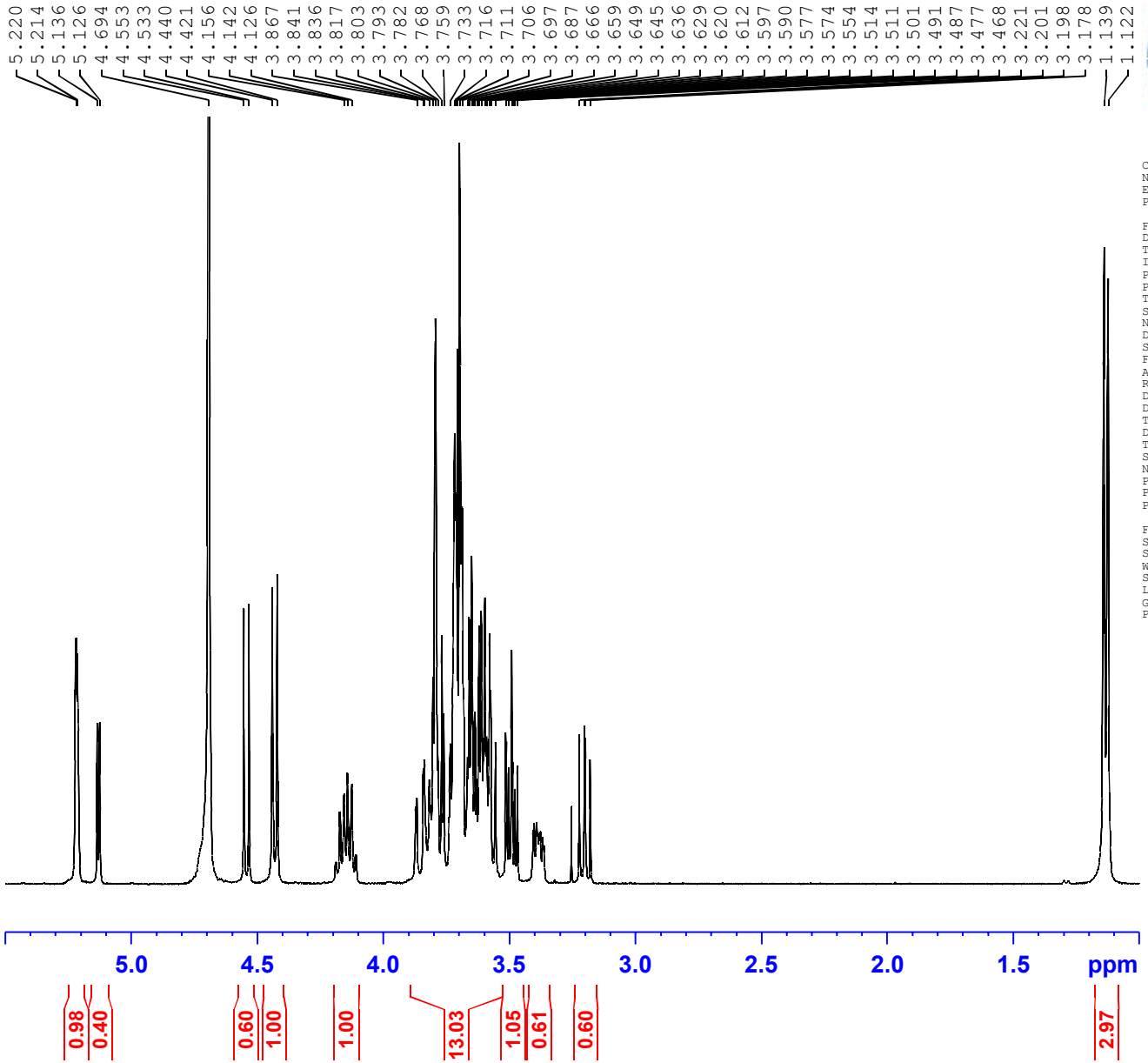
Date: [redacted]

Stanley M. Tarka, Jr. PhD, ATS
President of The Tarka Group, Inc.
Adjunct Associate Professor, The Pennsylvania State University, College of Medicine
Hershey, Pennsylvania

Signature: [redacted]

Date: [redacted]

APPENDIX A
AMYRIS AND CARBOSYNTH 2'-FL PROTON NMR SPECTRA



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EXPNO 1
PROCNO 1

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Date_ 20190116
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PULPROG zg30
TD 65536
SOLVENT D2O
NS 64
DS 2
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FIDRES 0.250967 Hz
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RG 128
DW 60.800 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
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NUC1 1H
P0 4.93 usec
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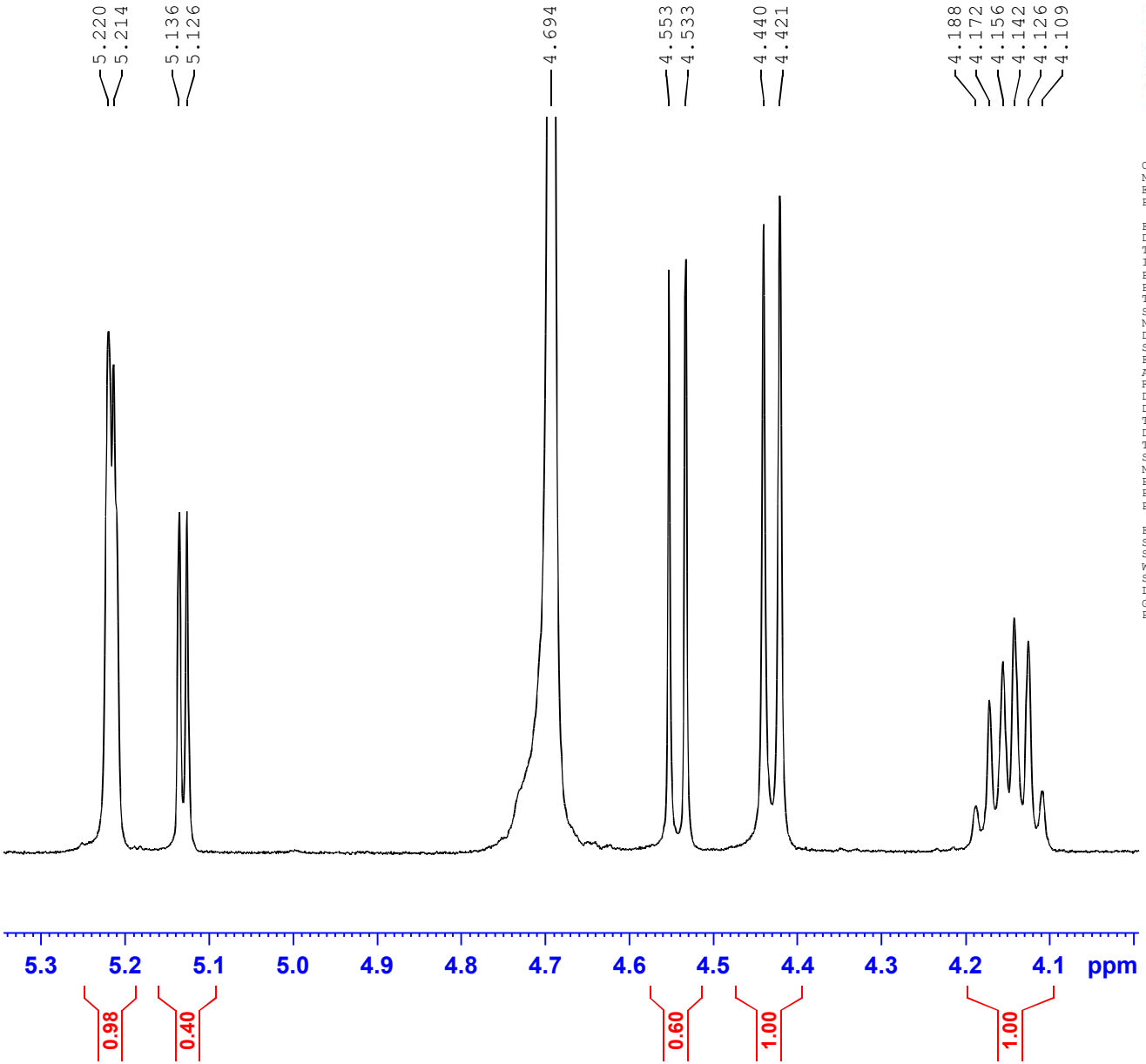
LB 0 Hz
GB 0
PC 1.00



Current Data Parameters
NAME AMY-20190116-02-2FL-standard-8mg
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190116
Time 11.44 h
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PULPROG zg30
TD 65536
SOLVENT D2O
NS 64
DS 2
SWH 8223.685 Hz
FIDRES 0.250967 Hz
AQ 3.9845889 sec
RG 128
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DE 6.50 usec
TE 300.0 K
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TD0 1
SFO1 400.1324710 MHz
NUC1 1H
P0 4.93 usec
P1 14.80 usec
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F2 - Processing parameters
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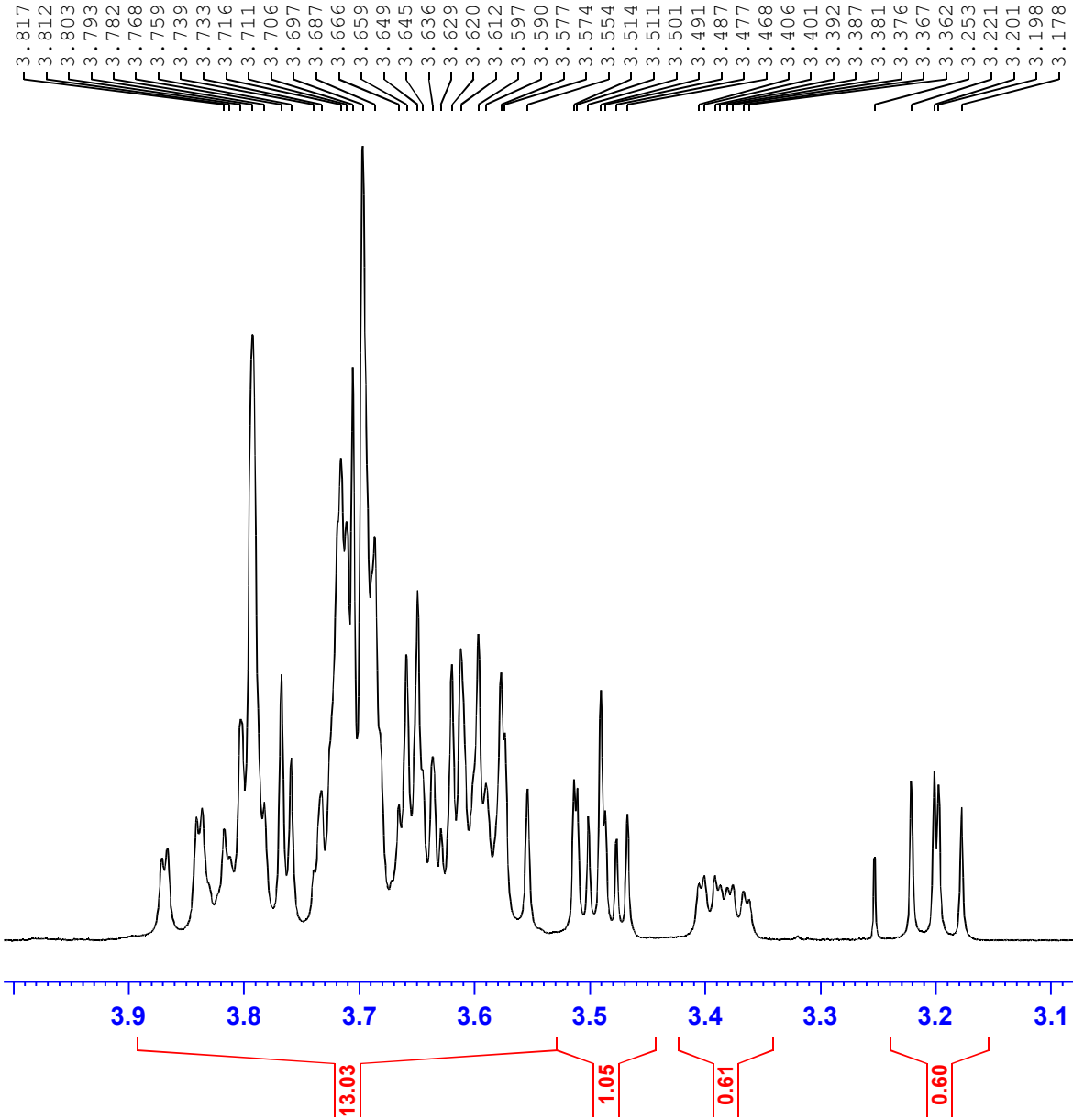
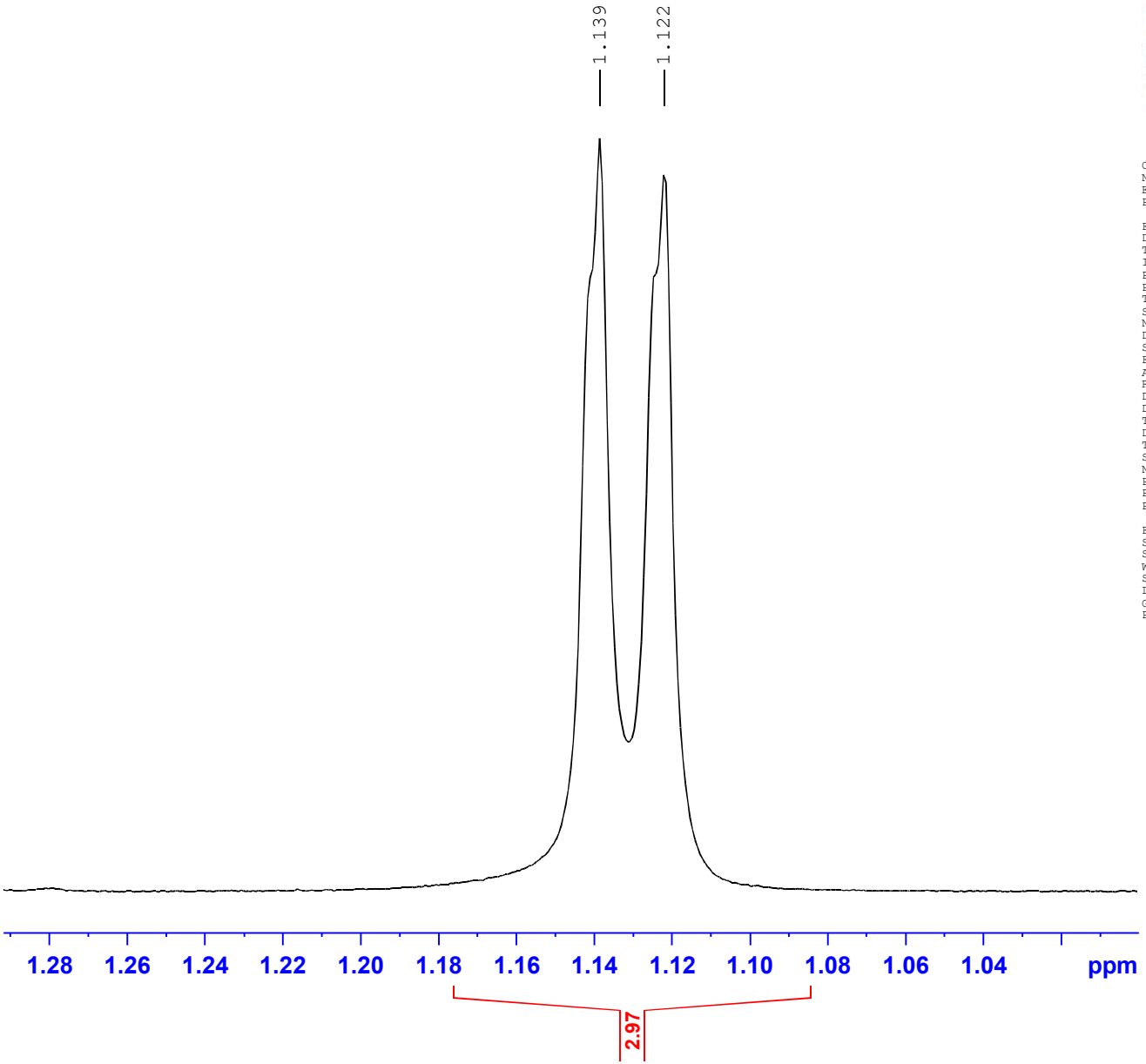


Current Data Parameters
NAME AMY-20190116-02-2FL-standard-8mg
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190116
Time 11.44 h
INSTRUM spect
PROBHD Z104450_0348 (
PULPROG zg30
TD 65536
SOLVENT D2O
NS 64
DS 2
SWH 8223.685 Hz
FIDRES 0.250967 Hz
AQ 3.9845889 sec
RG 128
DW 60.800 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1324710 MHz
NUC1 1H
P0 4.93 usec
P1 14.80 usec
PLW1 9.92000008 W

F2 - Processing parameters
SI 32768
SF 400.1300008 MHz
WDW no
SSB 0
LB 0 Hz
GB 0
PC 1.00



Current Data Parameters
NAME AMY-20190116-02-2FL-standard-8mg
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
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Time 11.44 h
INSTRUM spect
PROBHD Z104450_0348 (
PULPROG zg30
TD 65536
SOLVENT D2O
NS 64
DS 2
SWH 8223.685 Hz
FIDRES 0.250967 Hz
AQ 3.9845889 sec
RG 128
DW 60.800 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1324710 MHz
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P0 4.93 usec
P1 14.80 usec
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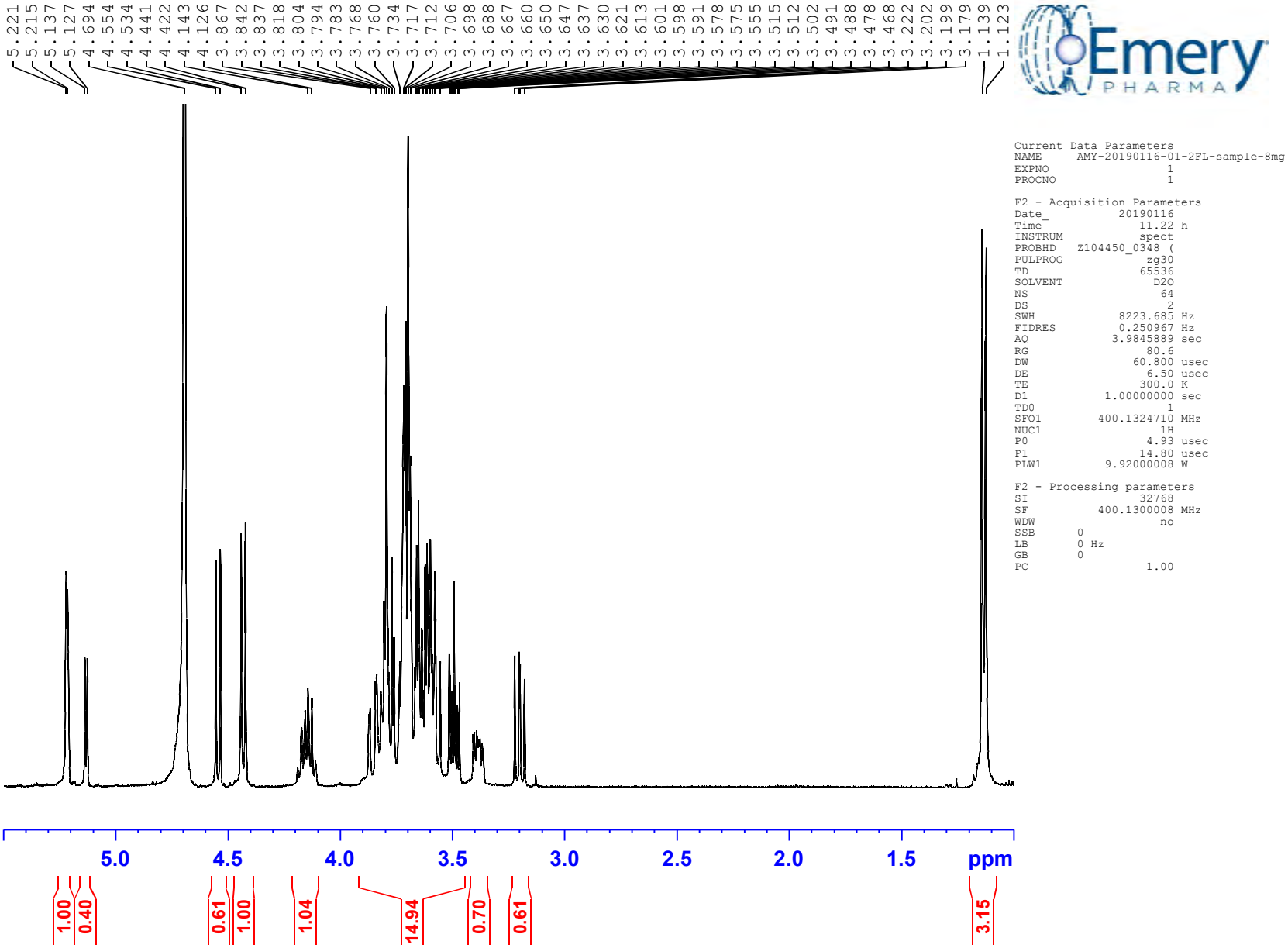

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EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
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PULPROG zg30
TD 65536
SOLVENT D2O
NS 64
DS 2
SWH 8223.685 Hz
FIDRES 0.250967 Hz
AQ 3.9845889 sec
RG 80.6
DW 60.800 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1324710 MHz
NUC1 1H
P0 4.93 usec
P1 14.80 usec
PLW1 9.92000008 W

F2 - Processing parameters
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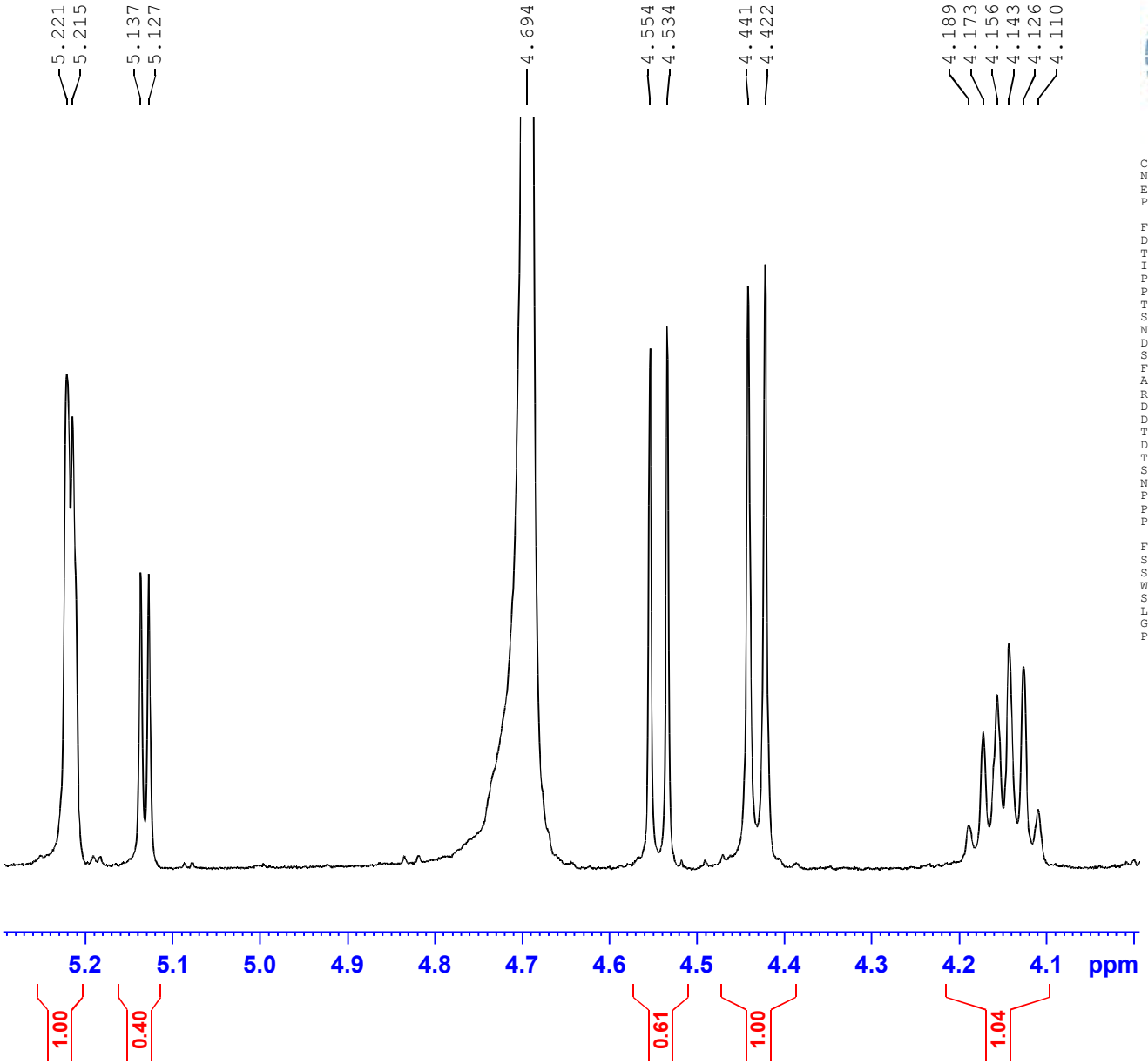
PC 1.00



Current Data Parameters
NAME AMY-20190116-01-2FL-sample-8mg
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190116
Time 11.22 h
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PROBHD Z104450_0348 (
PULPROG zg30
TD 65536
SOLVENT D2O
NS 64
DS 2
SWH 8223.685 Hz
FIDRES 0.250967 Hz
AQ 3.9845889 sec
RG 80.6
DW 60.800 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1324710 MHz
NUC1 1H
P0 4.93 usec
P1 14.80 usec
PLW1 9.92000008 W

F2 - Processing parameters
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SF 400.1300008 MHz
WDW no
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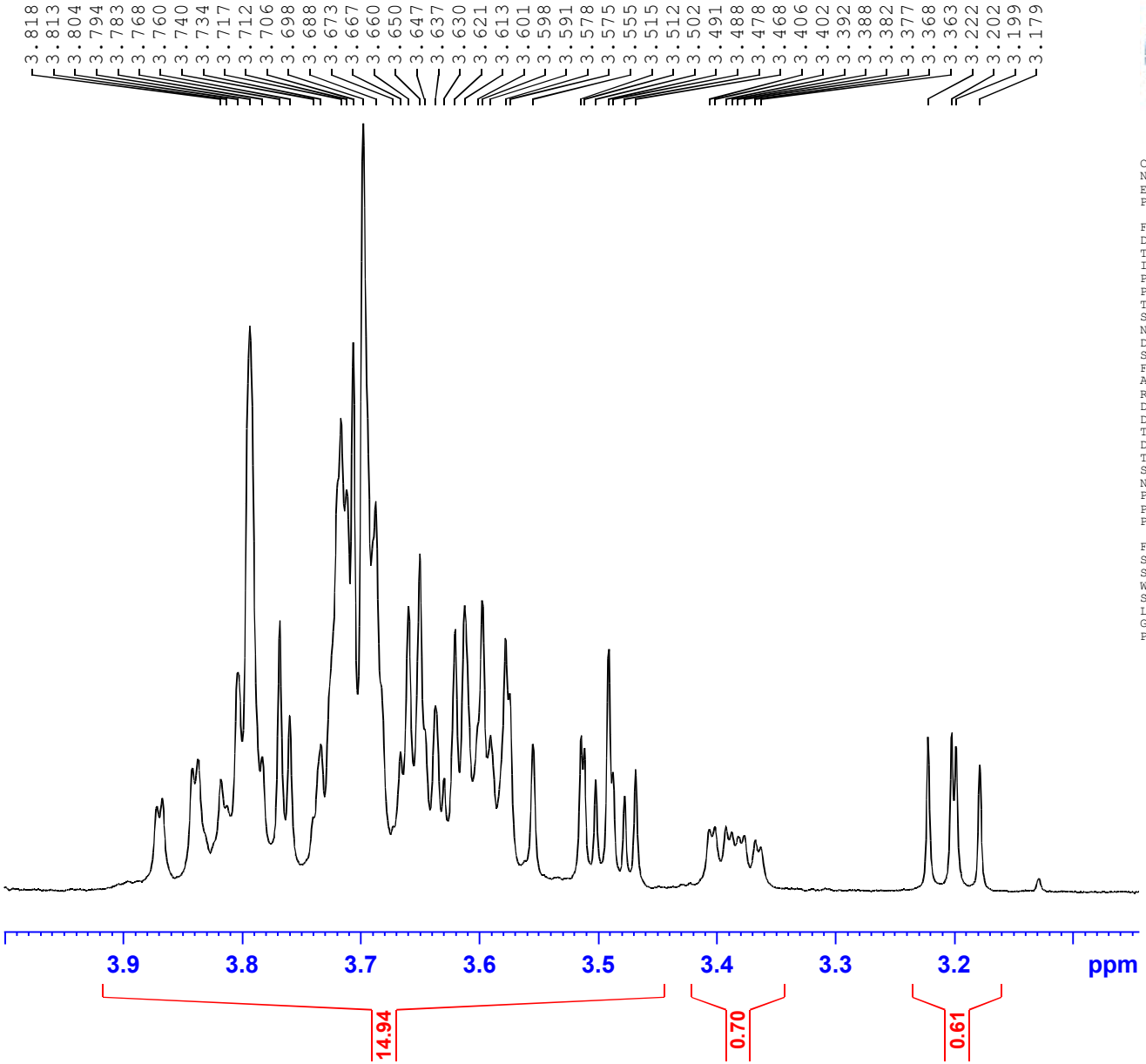
PC 1.00



Current Data Parameters
NAME AMY-20190116-01-2FL-sample-8mg
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
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Time 11.22 h
INSTRUM spect
PROBHD Z104450_0348 (
PULPROG zg30
TD 65536
SOLVENT D2O
NS 64
DS 2
SWH 8223.685 Hz
FIDRES 0.250967 Hz
AQ 3.9845889 sec
RG 80.6
DW 60.800 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1324710 MHz
NUC1 1H
P0 4.93 usec
P1 14.80 usec
PLW1 9.92000008 W

F2 - Processing parameters
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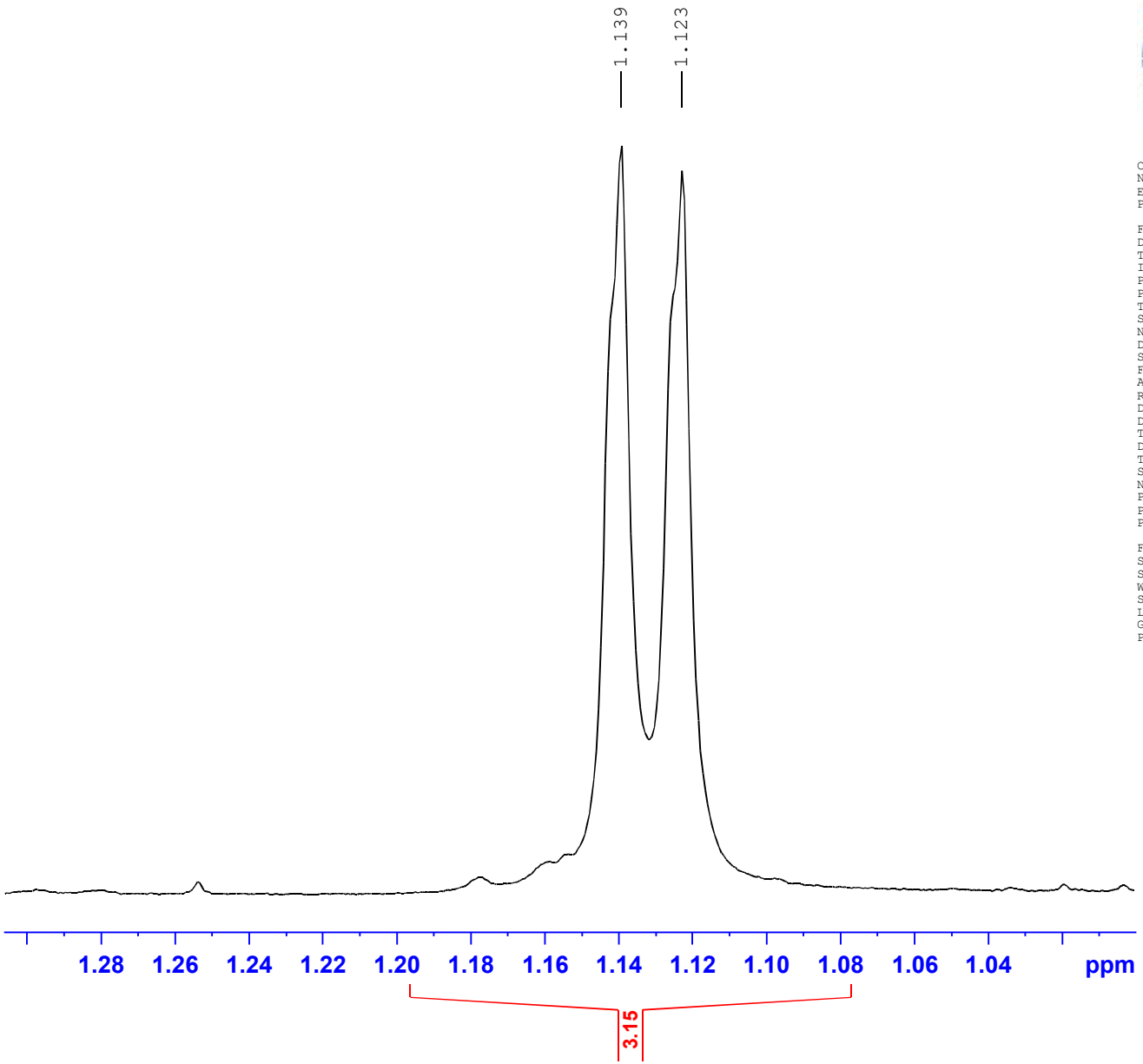
SF 400.1300008 MHz
WDW no
SSB 0
LB 0 Hz
GB 0
PC 1.00



Current Data Parameters
NAME AMY-20190116-01-2FL-sample-8mg
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190116
Time 11.22 h
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PROBHD Z104450_0348 (
PULPROG zg30
TD 65536
SOLVENT D2O
NS 64
DS 2
SWH 8223.685 Hz
FIDRES 0.250967 Hz
AQ 3.9845889 sec
RG 80.6
DW 60.800 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1324710 MHz
NUC1 1H
P0 4.93 usec
P1 14.80 usec
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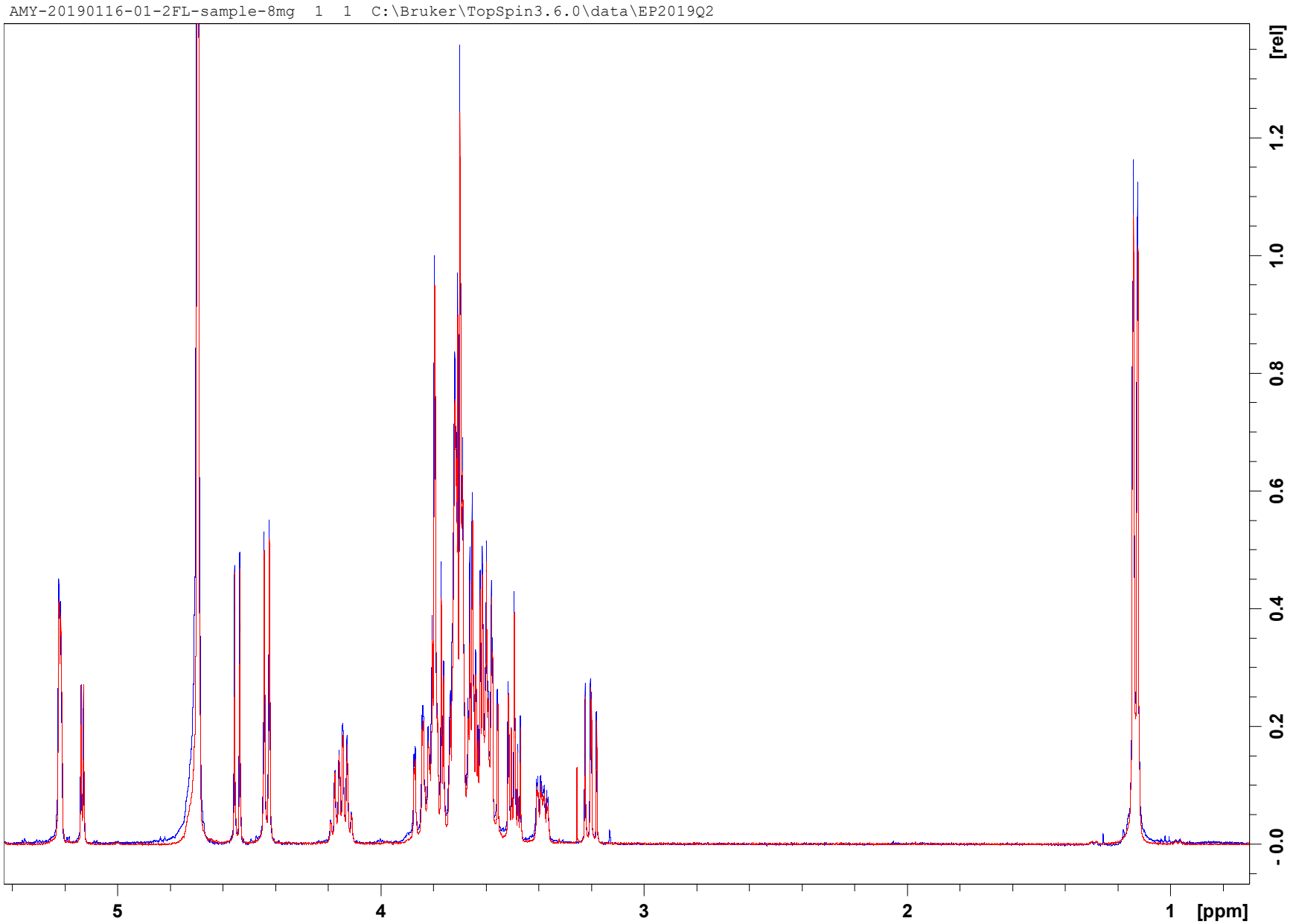
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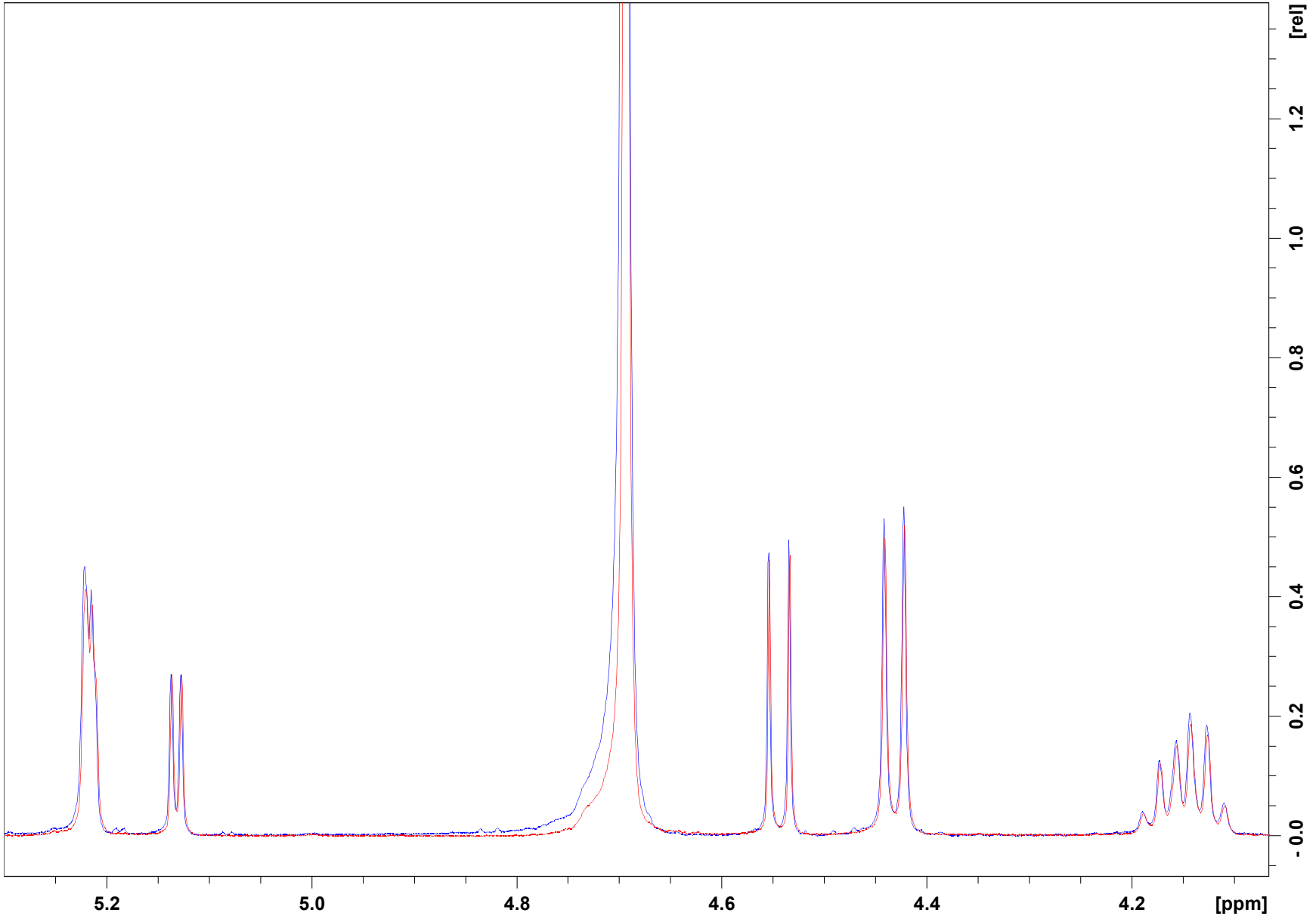
1.0
0.8
0.6
0.4
0.2
- 0.0
5
4
3
2
1
[ppm]

AMY-20190116-01-2FL-sample-8mg 1 1 C:\Bruker\TopSpin3.6.0\data\EP2019Q2



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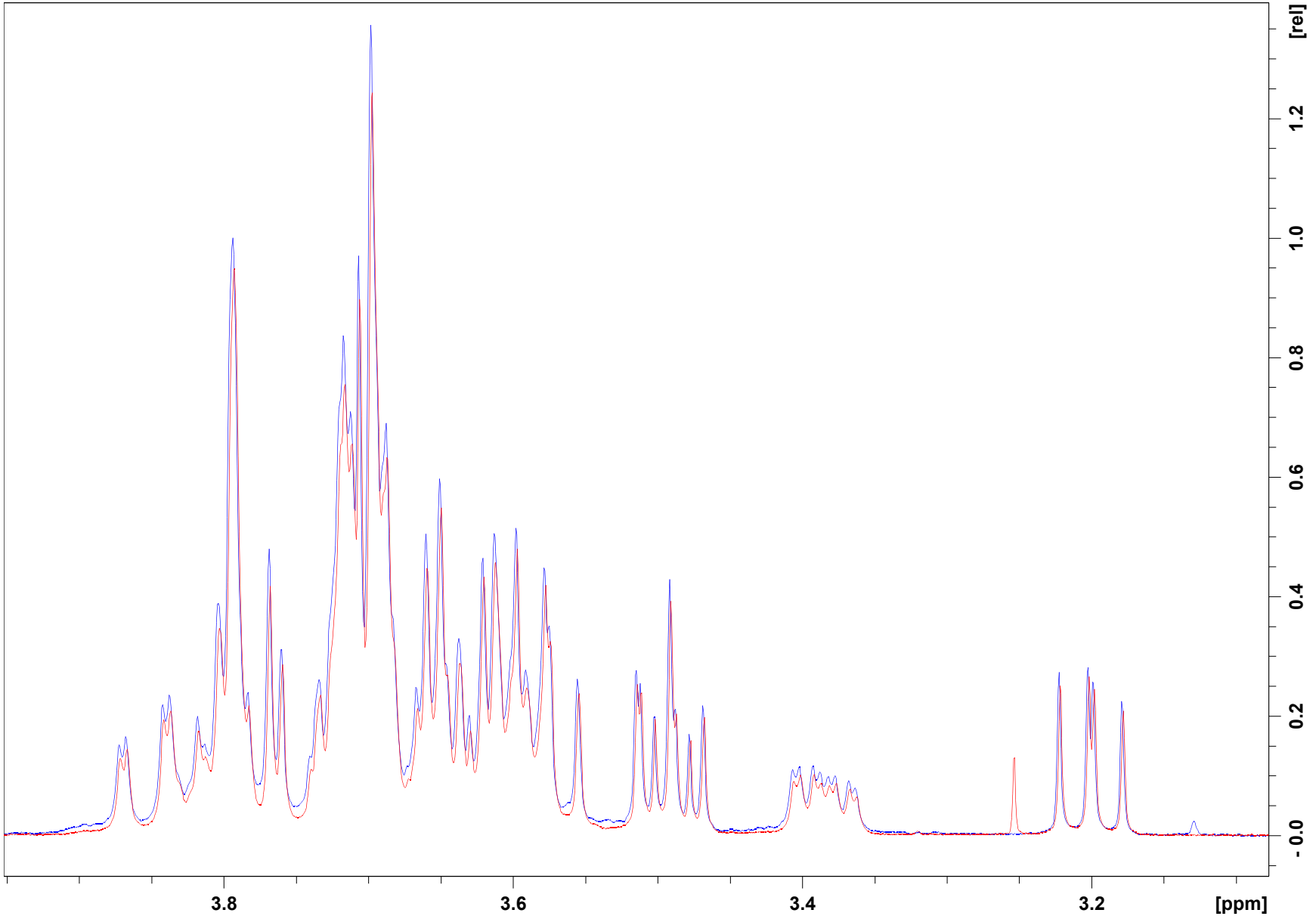

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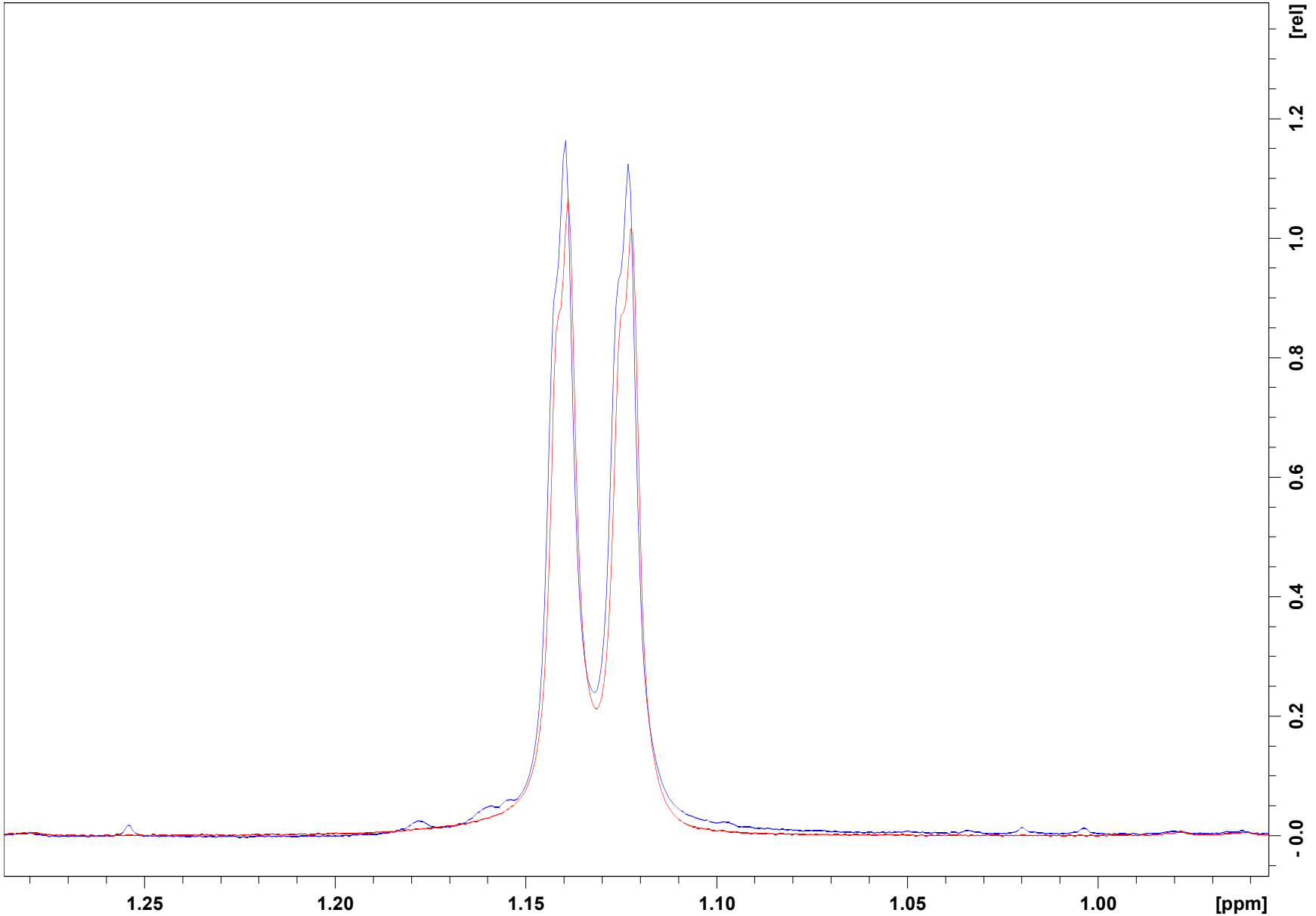

APPENDIX B

BATCH RESULTS - CARBOHYDRATE, KF TITRATION, PROTEIN, AND DNA RESULTS

amyris

Test Report for 2'Fucosyllactose

1. Product

This analytical study comprises of 2'Fucosyllactose(2'-FL) which is tested for carbohydrate profile.

2. Methods of Analysis

SOP00830 Determination of 2'-FL by Ion Chromatography (IC) area%: This method is intended for the release and quantitation of 2'-FL powder and other carbohydrate on area% basis by ion chromatography pulse amperometry detection with a Dionex Carbopac PA1 column.

3. Results of Analysis

A summary of the results is provided in the table below (Table 1):

Table 1: Summary for Carbohydrate Results

Lot #	Date of Manufacture	Assay (Carbohydrate)	Specification Limit	Result % (50 ppm concentration)	Disposition
H8163	6 February 2019	2'-Fucosyllactose	≥86 area%	86.06	Pass
		Lactose/Allo-lactose	<7 area%	2.18	Pass
		Difucosyllactose (DFL)	<8 area%	0.52	Pass
		2'-Fucosyllactitol	≤6 area%	4.92	Pass
		3-Fucosyllactose (3FL)/Fucosyl-galactose	<7 area %	0.92	Pass
		Sorbitol/Galactitol		0.79	
		Xylitol		0.42	
		Fucose		3.36	
		Glucose/Galactose		0.20	
		Glycerophosphoethanolamine (GPE)		0.24	
		Fructose		0.24	
		Sugar alcohol (Other)		0.15	

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4. Conclusion

Results for lot H8163 conforms to carbohydrate specifications.

Prepared by: [redacted] Date: 5-May-2020

Christine Erfe - Quality Control

Approved by: [redacted] Date: 5-May-2020

Beth Albino - Quality Assurance

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA

amyris

Test Report for 2'Fucosyllactose

1. Product

This analytical study comprises of 2'Fucosyllactose(2'-FL) which is tested for carbohydrate profile, Karl-Fischer titration water content, protein content and DNA presence.

2. Methods of Analysis

SOP00830 Determination of 2'-FL by Ion Chromatography (IC) area%: This method is intended for the release and quantitation of 2'-FL powder and other carbohydrate on area% basis by ion chromatography pulse amperometry detection with a Dionex Carbopac PA1 column.

SOP00842 Determination of water in purified 2'-FL samples by volumetric Karl Fischer titration: This method is intended for the absolute quantification of water by volumetric Karl Fischer titration in purified 2'-FL powder.

SOP00843 Determination of total protein for 2'FL final product by modified Bradford assay: This method is intended for quantification of total protein in 2'FL final product using a microplate reader. Bovine serum albumin (BSA) as an external calibrator. Data is reported in g/kg.

SOP00844 Determination of DNA presence in 2'Fucosyllactose Final Product by PCR: This method is intended for the detection of genomic DNA in 2-'FL final product using PCR with the motivation that the analytical method can demonstrate the absence of recombinant DNA.

3. Results of Analysis

A summary of the results is provided in the tables below (Table 1 and 2):

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Table 1: Summary for Carbohydrate Results

Lot #	Assay (Carbohydrate)	Specification limit	Result % (50 ppm concentration)	Disposition
H8452	2'-Fucosyllactose	≥86 area%	91.1	Pass
	Lactose/Allo-lactose	<7 area%	1.18	Pass
	Difucosyllactose (DFL)	<8 area%	1.40	Pass
	2'-Fucosyllactitol	≤6 area%	3.54	Pass
	Sorbitol/Galactitol	<7 area%	0.14	Pass
	Fucose		0.18	
	3-Fucosyllactose/Fucosyl-Galactose		0.33	
	Glucose/Galactose		0.26	
	Xylitol		0.73	
	Glycerophosphoethanolamine (GPE)		0.27	
	Fructose		0.40	

Table 2: Summary of KF, Protein and DNA results

Lot #	Assay	Specification Limit	Results	Disposition
H8452	KF	≤ 5%	2.66%	Pass
	Protein	≤ 0.01%	< 0.004%	Pass
	DNA	Negative	Negative	Pass

4. Conclusion

Results for carbohydrate, KF, Protein, and DNA for lot H8452 are within specifications.

Prepared by: Date: 5-May-2020

Christine Erfe - Quality Control

Approved by: Date: 5-May-2020

Beth Albino - Quality Assurance

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA

amyris

Test Report for 2'Fucosyllactose

1. Product

This analytical study comprises of 2'Fucosyllactose(2'-FL) which is tested for carbohydrate profile, Karl-Fischer titration water content, protein content and DNA presence.

2. Methods of Analysis

SOP00830 Determination of 2'-FL by Ion Chromatography (IC) area%: This method is intended for the release and quantitation of 2'-FL powder and other carbohydrate on area% basis by ion chromatography pulse amperometry detection with a Dionex Carbopac PA1 column.

SOP00842 Determination of water in purified 2'-FL samples by volumetric Karl Fischer titration: This method is intended for the absolute quantification of water by volumetric Karl Fischer titration in purified 2'-FL powder.

SOP00843 Determination of total protein for 2'FL final product by modified Bradford assay: This method is intended for quantification of total protein in 2'FL final product using a microplate reader. Bovine serum albumin (BSA) as an external calibrator. Data is reported in g/kg.

SOP00844 Determination of DNA presence in 2'Fucosyllactose Final Product by PCR: This method is intended for the detection of genomic DNA in 2-'FL final product using PCR with the motivation that the analytical method can demonstrate the absence of recombinant DNA.

3. Results of Analysis

A summary of the results is provided in the tables below (Table 1 and 2):

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA

Table 1: Summary for Carbohydrate Results

Lot #	Assay (Carbohydrate)	Specification limit	Result % (50 ppm concentration)	Disposition
H8561	2'-Fucosyllactose	≥86 area%	92.2	Pass
	Lactose/All-lactose	<7 area%	0.08	Pass
	Difucosyllactose (DFL)	<8 area%	1.30	Pass
	2'-Fucosyllactitol	≤6 area%	3.93	Pass
	Sorbitol/Galactitol	<7 area%	0.05	Pass
	Fucose		0.12	
	3-Fucosyllactose/Fucosyl-Galactose		0.38	
	Glucose/Galactose		0.33	
	Xylitol		0.59	
	Glycerophosphoethanolamine (GPE)		0.42	
	Fructose		0.43	

Table 2: Summary of KF, Protein and DNA results

Lot #	Assay	Specification Limit	Results	Disposition
H8561	KF	≤ 5%	3.07%	Pass
	Protein	≤ 0.01%	< 0.004%	Pass
	DNA	Negative	Negative	Pass

4. Conclusion

Results for carbohydrate, KF, Protein, and DNA for lot H8651 are within specifications.

Prepared by: Date: 5-May-2020

Christine Erfe - Quality Control

Approved by: Date: 5-May-2020

Beth Albino - Quality Assurance

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA

amyris

Test Report for 2'Fucosyllactose

1. Product

This analytical study comprises of 2'Fucosyllactose(2'-FL) which is tested for carbohydrate profile, Karl-Fischer titration water content, protein content and DNA presence.

2. Methods of Analysis

SOP00830 Determination of 2'-FL by Ion Chromatography (IC) area%: This method is intended for the release and quantitation of 2'-FL powder and other carbohydrate on area% basis by ion chromatography pulse amperometry detection with a Dionex Carbopac PA1 column.

SOP00842 Determination of water in purified 2'-FL samples by volumetric Karl Fischer titration: This method is intended for the absolute quantification of water by volumetric Karl Fischer titration in purified 2'-FL powder.

SOP00843 Determination of total protein for 2'FL final product by modified Bradford assay: This method is intended for quantification of total protein in 2'FL final product using a microplate reader. Bovine serum albumin (BSA) as an external calibrator. Data is reported in g/kg.

SOP00844 Determination of DNA presence in 2'Fucosyllactose Final Product by PCR: This method is intended for the detection of genomic DNA in 2-'FL final product using PCR with the motivation that the analytical method can demonstrate the absence of recombinant DNA.

3. Results of Analysis

A summary of the results is provided in the tables below (Table 1 and 2):

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA

Table 1: Summary for Carbohydrate Results

Lot #	Assay (Carbohydrate)	Specification limit	Result % (50 ppm concentration)	Disposition
H8781	2'-Fucosyllactose	≥86 area%	91.2	Pass
	Lactose/Allo-lactose	<7 area%	1.43	Pass
	Difucosyllactose (DFL)	<8 area%	1.24	Pass
	2'-Fucosyllactitol	≤6 area%	3.19	Pass
	Sorbitol/Galactitol	<7 area%	0.15	Pass
	Fucose		0.15	
	3-Fucosyllactose/Fucosyl-Galactose		0.23	
	Glucose/Galactose		0.22	
	Xylitol		0.67	
	Glycerophosphoethanolamine (GPE)		0.53	
	Fructose		0.31	

Table 2: Summary of KF, Protein and DNA results

Lot #	Assay	Specification Limit	Results	Disposition
H8781	KF	≤ 5%	2.53%	Pass
	Protein	≤ 0.01%	< 0.004%	Pass
	DNA	Negative	Negative	Pass

4. Conclusion

Results for carbohydrate, KF, protein, and DNA for lot H8781 are within specifications.

Prepared by: Date: 5-May-2020

Christine Erfe - Quality Control

Approved by: Date: 5-May-2020

Beth Albino - Quality Assurance

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA

amyris

Test Report for 2'Fucosyllactose

1. Product

This analytical study comprises of 2'Fucosyllactose(2'-FL) which is tested for carbohydrate profile, Karl-Fischer titration water content, protein content and DNA presence.

2. Methods of Analysis

SOP00830 Determination of 2'-FL by Ion Chromatography (IC) area%: This method is intended for the release and quantitation of 2'-FL powder and other carbohydrate on area% basis by ion chromatography pulse amperometry detection with a Dionex Carbopac PA1 column.

SOP00842 Determination of water in purified 2'-FL samples by volumetric Karl Fischer titration: This method is intended for the absolute quantification of water by volumetric Karl Fischer titration in purified 2'-FL powder.

SOP00843 Determination of total protein for 2'FL final product by modified Bradford assay: This method is intended for quantification of total protein in 2'FL final product using a microplate reader. Bovine serum albumin (BSA) as an external calibrator. Data is reported in g/kg.

SOP00844 Determination of DNA presence in 2'Fucosyllactose Final Product by PCR: This method is intended for the detection of genomic DNA in 2-'FL final product using PCR with the motivation that the analytical method can demonstrate the absence of recombinant DNA.

3. Results of Analysis

A summary of the results is provided in the tables below (Table 1 and 2):

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA

Table 1: Summary for Carbohydrate Results

Lot #	Assay (Carbohydrate)	Specification limit	Result % (50 ppm concentration)	Disposition
H8750	2'-Fucosyllactose	≥86 area%	91.3	Pass
	Lactose/Allo-lactose	<7 area%	0.44	Pass
	Difucosyllactose (DFL)	<8 area%	1.25	Pass
	2'-Fucosyllactitol	≤6 area%	3.52	Pass
	Sorbitol/Galactitol	<7 area%	0.10	Pass
	Fucose		0.19	
	3-Fucosyllactose/Fucosyl-Galactose		0.18	
	Glucose/Galactose		0.52	
	Xylitol		1.30	
	Glycerophosphoethanolamine (GPE)		0.25	
	Fructose		0.46	

Table 2: Summary of KF, Protein and DNA results

Lot #	Assay	Specification Limit	Results	Disposition
H8750	KF	≤ 5%	2.66	Pass
	Protein	≤ 0.01%	< 0.004%	Pass
	DNA	Negative	Negative	Pass

4. Conclusion

Results for carbohydrate, KF, Protein, and DNA for lot H8750 are within specifications.

Prepared by: Date: 5-May-2020

Christine Erfe - Quality Control

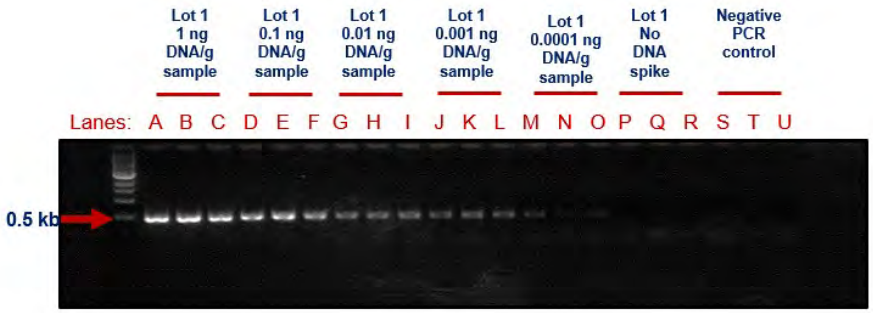
Approved by: Date: 5-May-2020

Beth Albino - Quality Assurance

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA

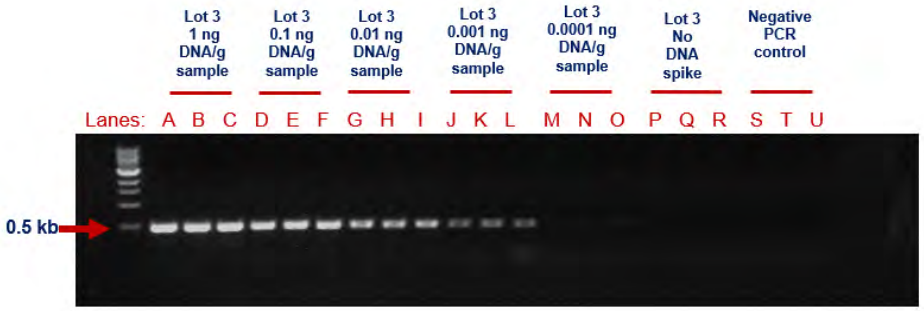
APPENDIX C
RESIDUAL DNA ANALYSIS

H8452 (Lot #1)



H8561 (Lot #2)

H8595 (Lot #3)



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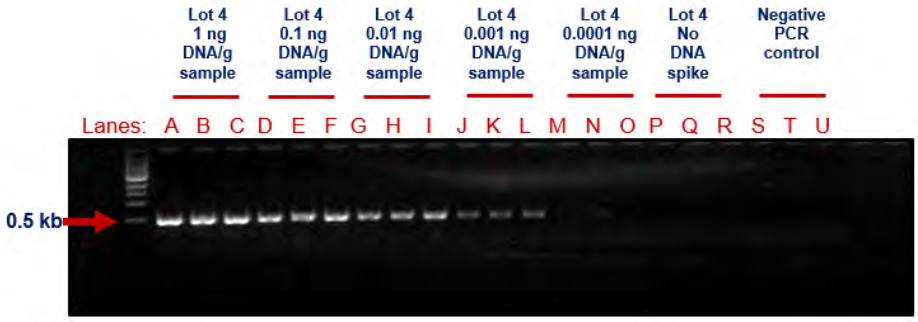


H8750 (Lot #5)

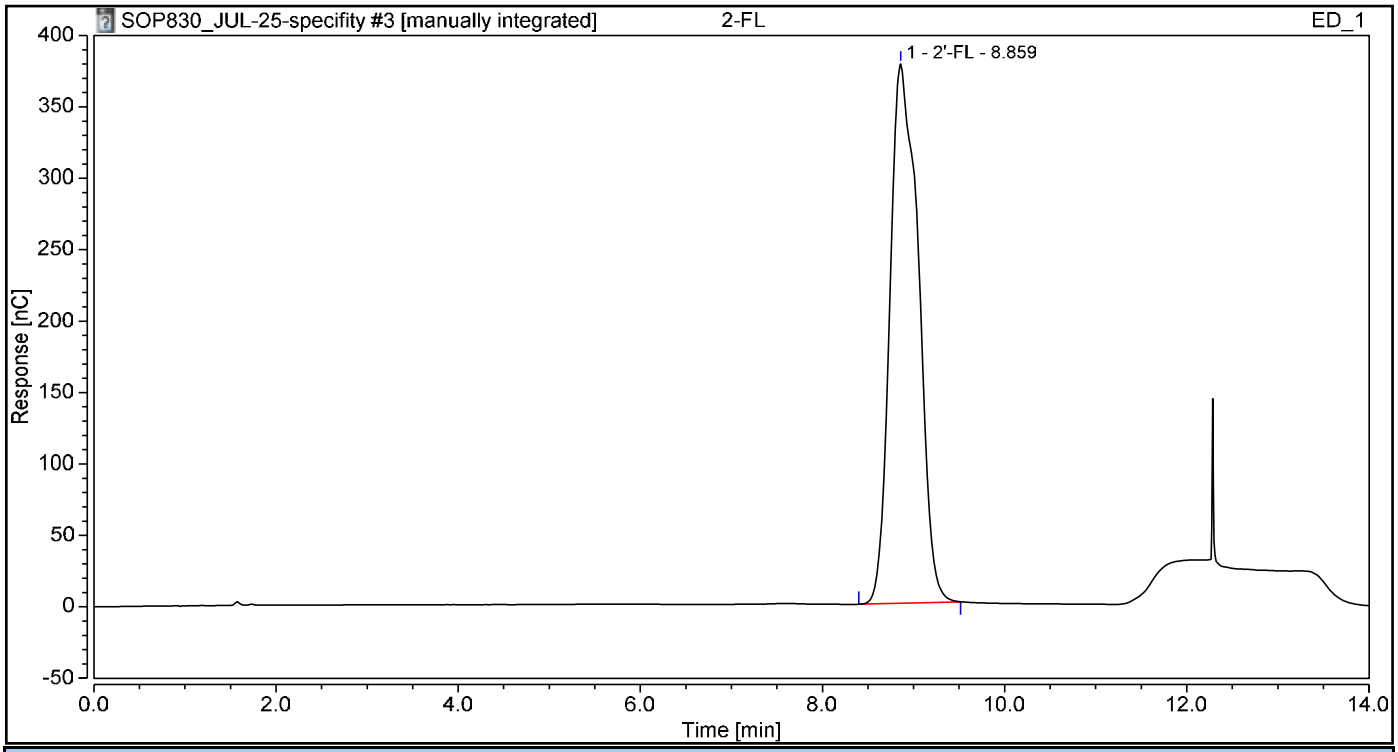
APPENDIX D
CHROMATOGRAM RESULTS

Chromatogram and Results

Injection Details

Injection Name:	**2-FL**	*Run Time (min):*	**14.00**
Vial Number:	**BD1**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 16:54**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

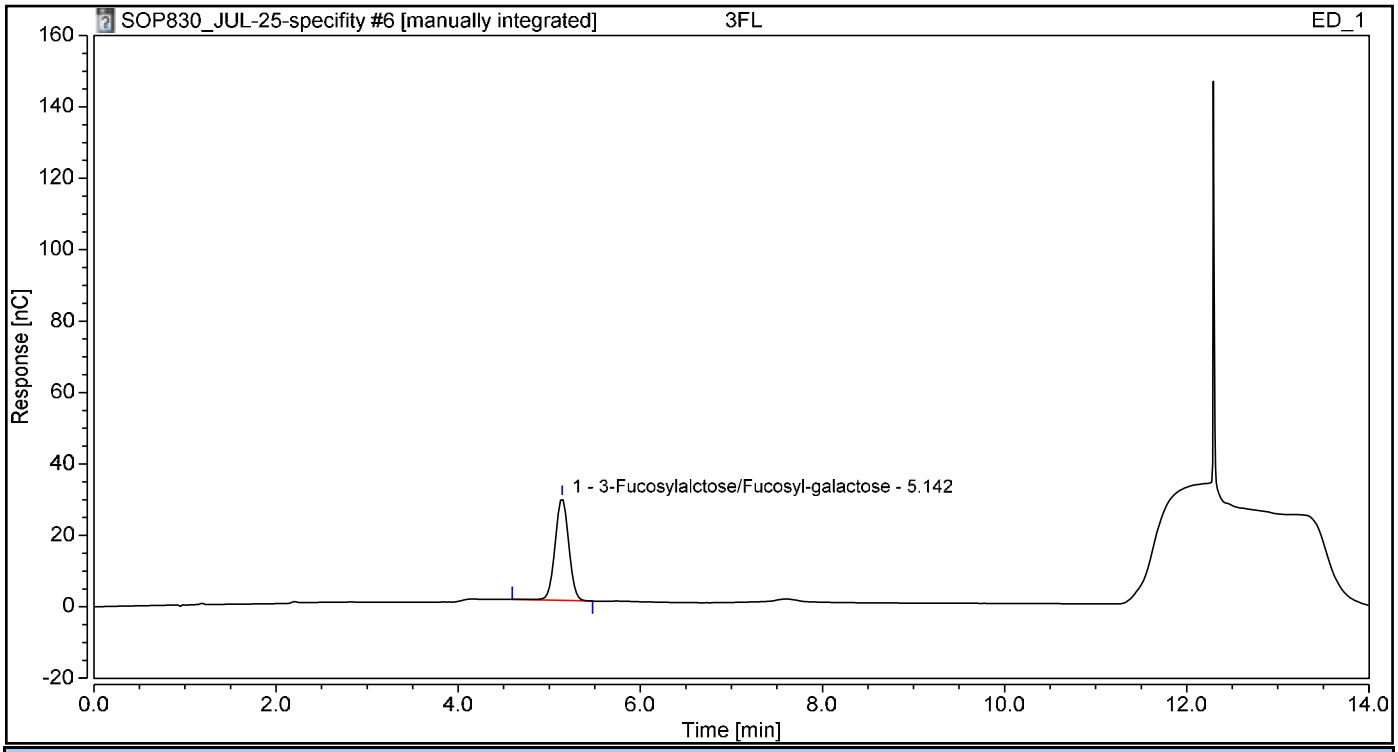
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
n.a.	Xylitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Sorbitol/Galactitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Fucose/Trehalose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Glucose/Galactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	3-Fucosylalctose/Fucosyl-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Allo-Lactose/Lactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	2'-FL	8.859	136.388	377.523	100.00	100.00	n.a.
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**136.388**	**377.523**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**3FL**	*Run Time (min):*	**14.00**
Vial Number:	**BC7**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 17:43**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

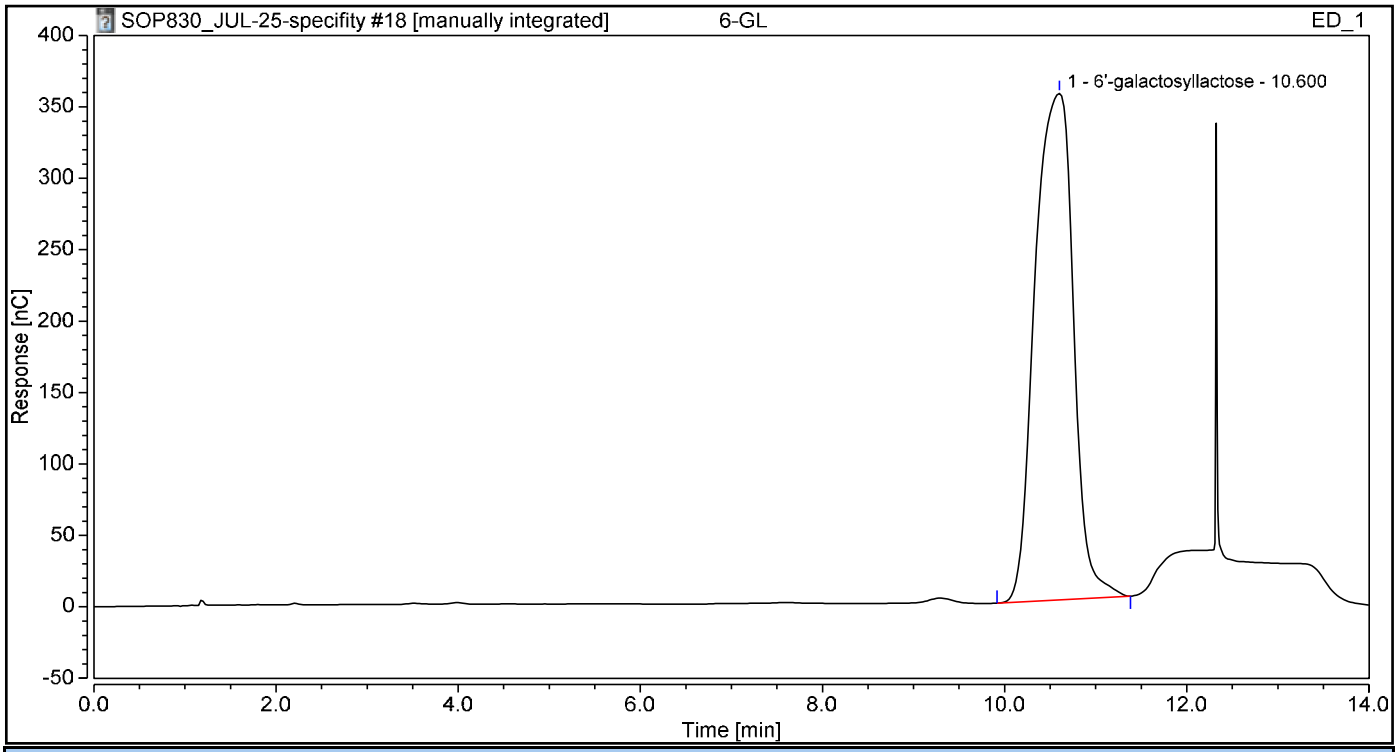
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
n.a.	Xylitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Sorbitol/Galactitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Fucose/Trehalose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Glucose/Galactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	3-Fucosylalctose/Fucosyl-	5.142	4.969	28.604	100.00	100.00	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Allo-Lactose/Lactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	2'-FL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**4.969**	**28.604**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**6-GL**	*Run Time (min):*	**14.00**
Vial Number:	**BE8**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 21:02**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

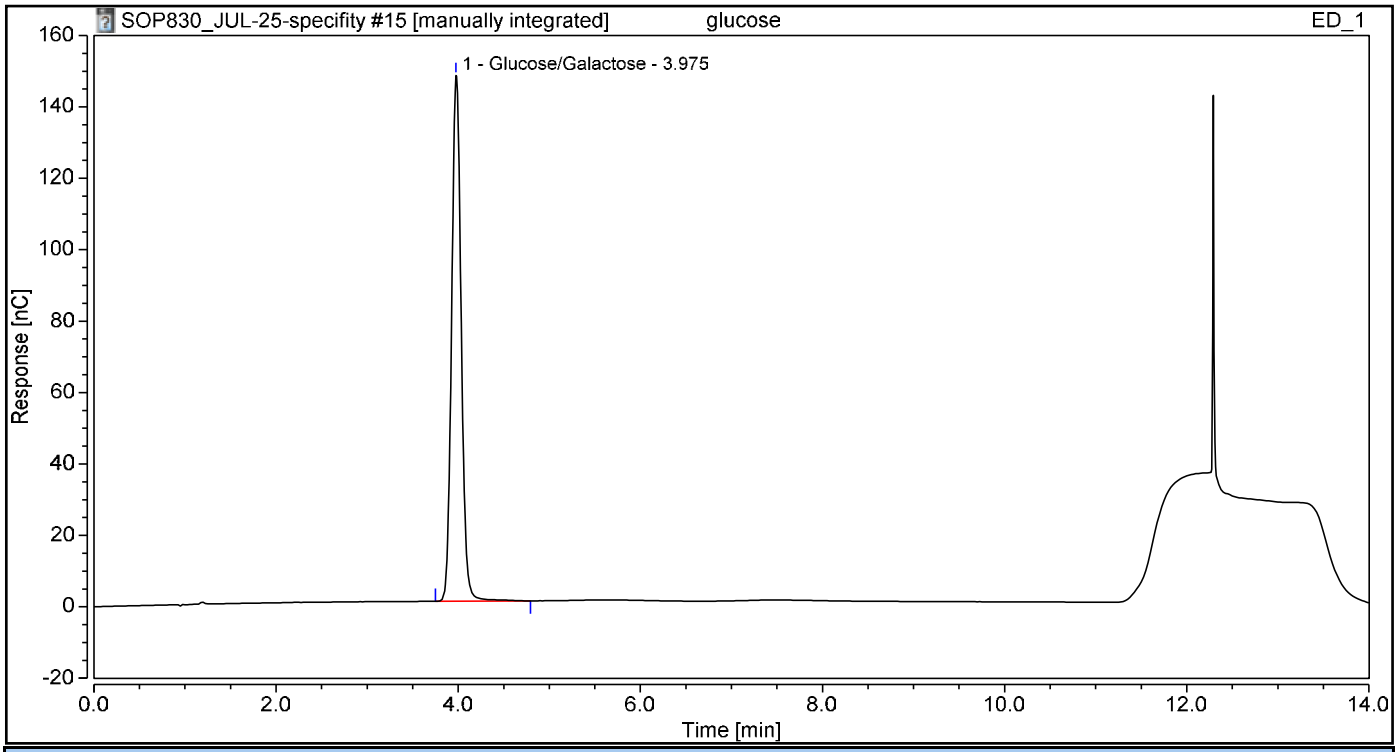
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
n.a.	Xylitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Sorbitol/Galactitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Fucose/Trehalose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Glucose/Galactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	3-Fucosylalctose/Fucosyl-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Allo-Lactose/Lactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	2'-FL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	6'-galactosyllactose	10.600	173.413	354.615	100.00	100.00	n.a.
Total:			**173.413**	**354.615**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**glucose**	*Run Time (min):*	**14.00**
Vial Number:	**BE2**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 20:12**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

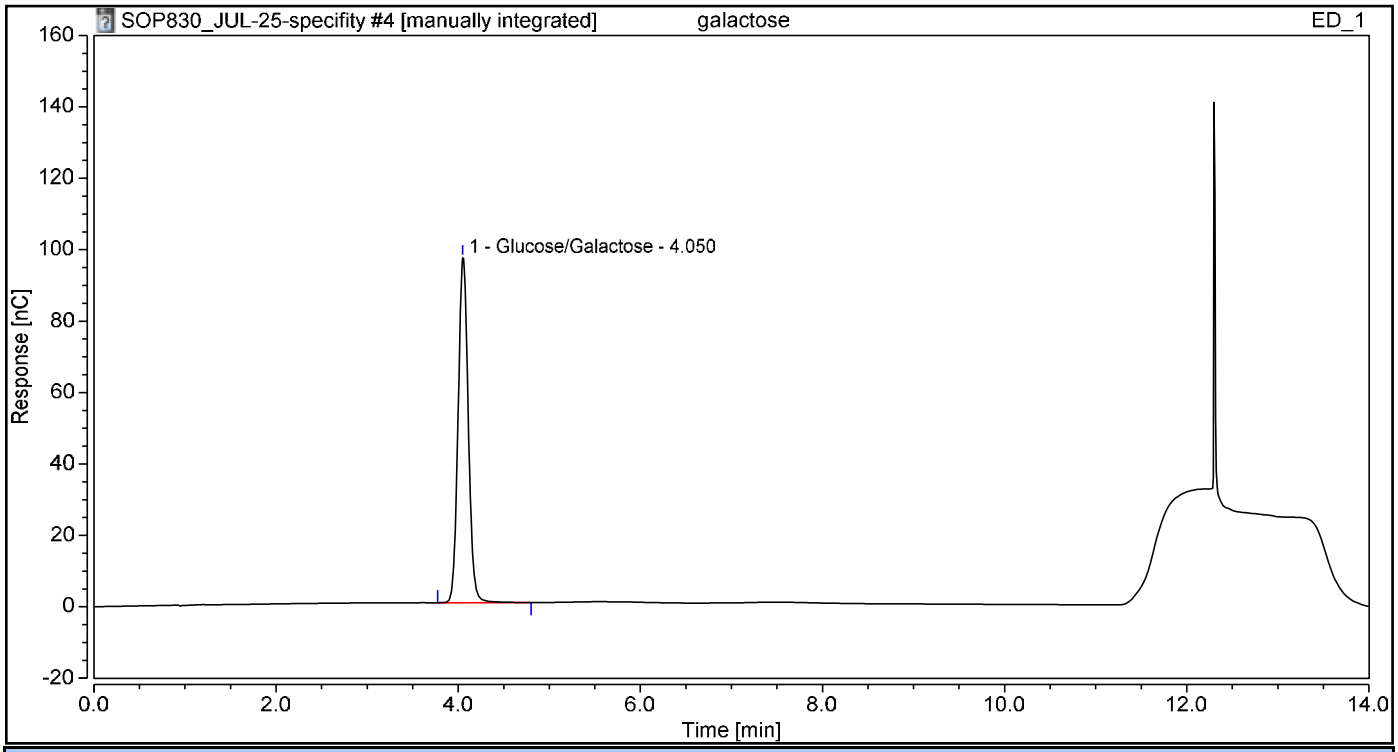
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
n.a.	Xylitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Sorbitol/Galactitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Fucose/Trehalose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	Glucose/Galactose	3.975	18.017	147.250	100.00	100.00	n.a.
n.a.	3-Fucosylalctose/Fucosyl-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Allo-Lactose/Lactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	2'-FL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**18.017**	**147.250**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**galactose**	*Run Time (min):*	**14.00**
Vial Number:	**BC4**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 17:10**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

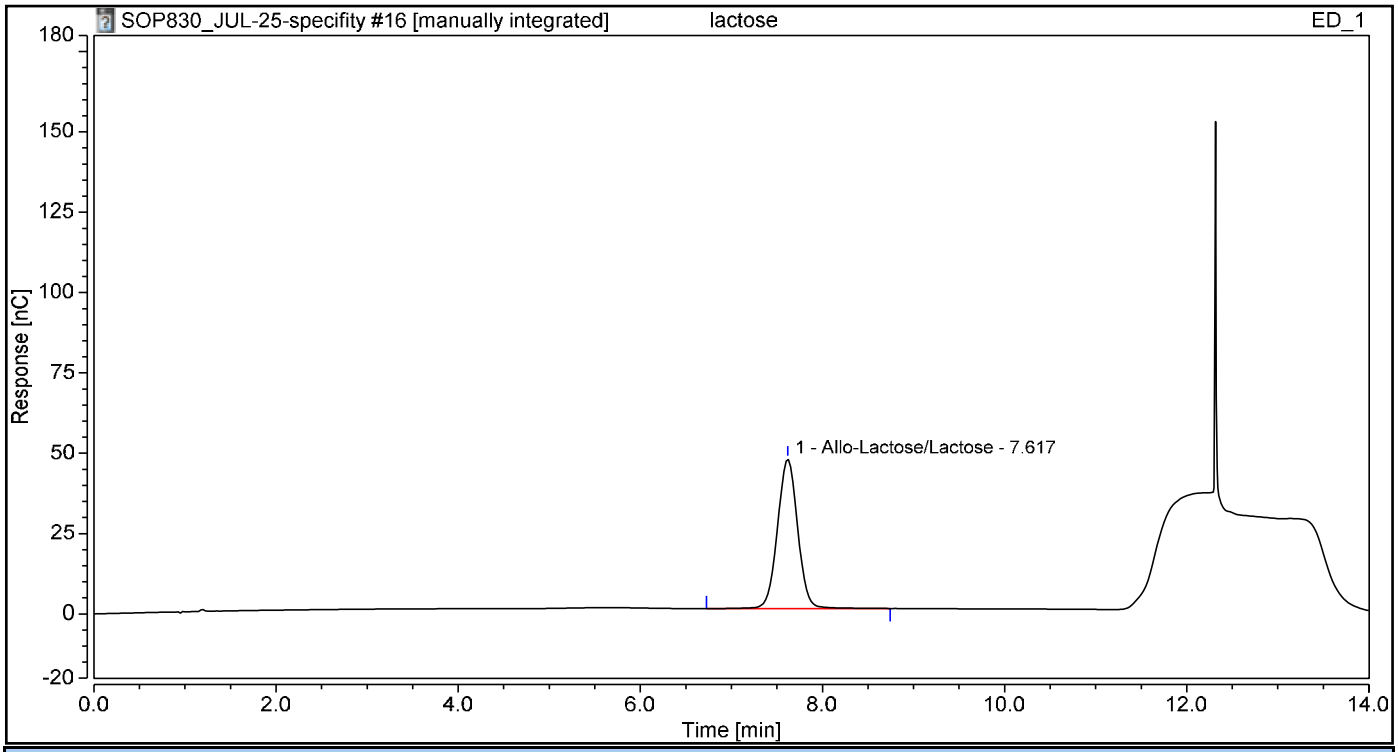
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
n.a.	Xylitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Sorbitol/Galactitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Fucose/Trehalose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	Glucose/Galactose	4.050	12.453	96.559	100.00	100.00	n.a.
n.a.	3-Fucosylalctose/Fucosyl-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Allo-Lactose/Lactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	2'-FL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**12.453**	**96.559**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**lactose**	*Run Time (min):*	**14.00**
Vial Number:	**BE3**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 20:29**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

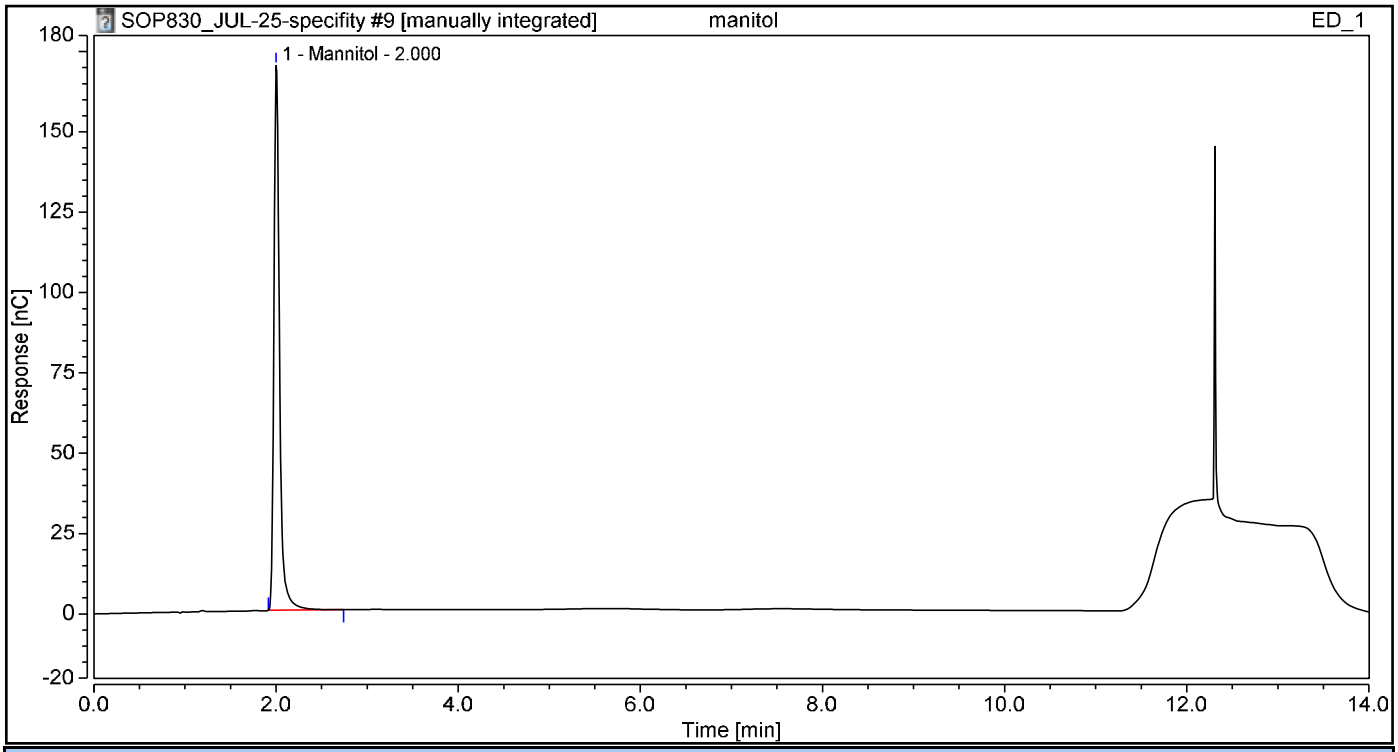
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
n.a.	Xylitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Sorbitol/Galactitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Fucose/Trehalose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Glucose/Galactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	3-Fucosylalctose/Fucosyl-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	Allo-Lactose/Lactose	7.617	11.982	46.567	100.00	100.00	n.a.
n.a.	2'-FL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**11.982**	**46.567**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**manitol**	*Run Time (min):*	**14.00**
Vial Number:	**BD3**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 18:33**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

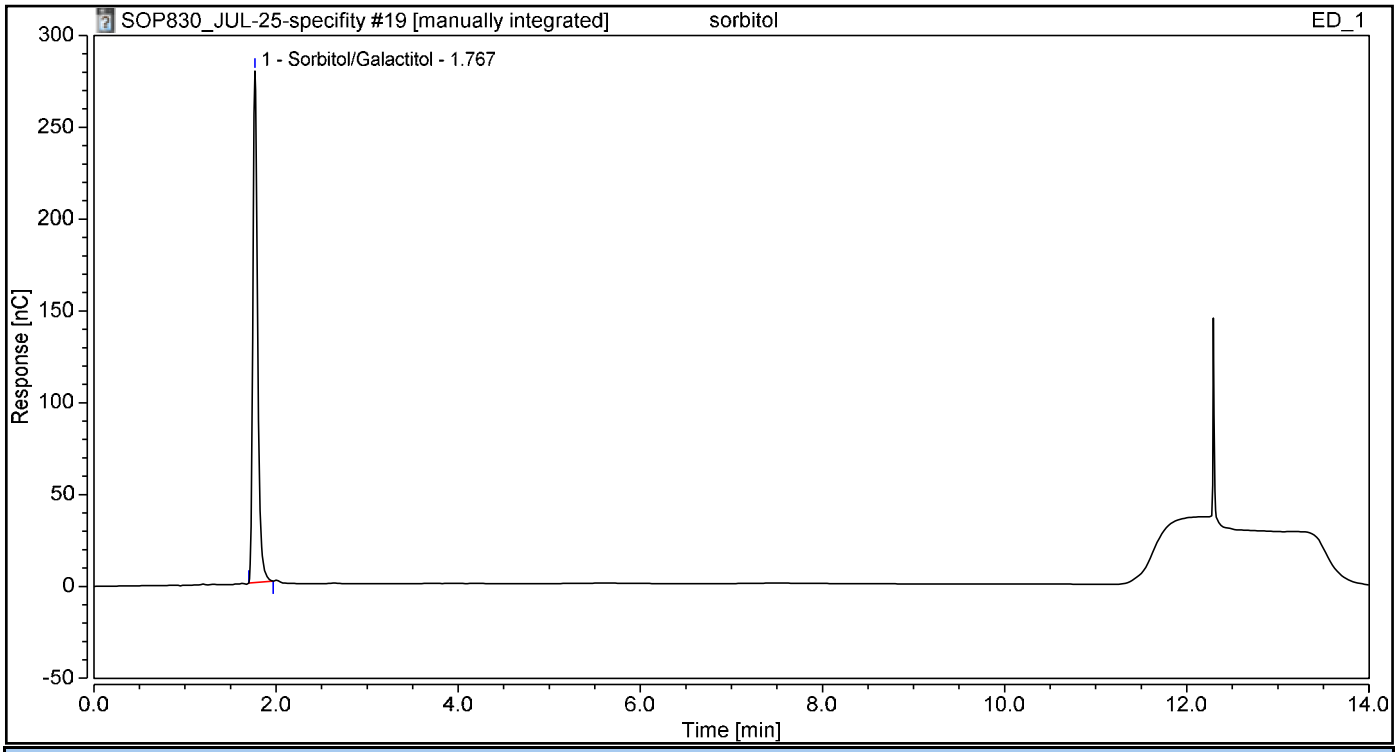
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
n.a.	Xylitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Sorbitol/Galactitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	Mannitol	2.000	12.208	169.527	100.00	100.00	n.a.
n.a.	Fucose/Trehalose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Glucose/Galactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	3-Fucosylalctose/Fucosyl-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Allo-Lactose/Lactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	2'-FL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**12.208**	**169.527**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**sorbitol**	*Run Time (min):*	**14.00**
Vial Number:	**BE5**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 21:18**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

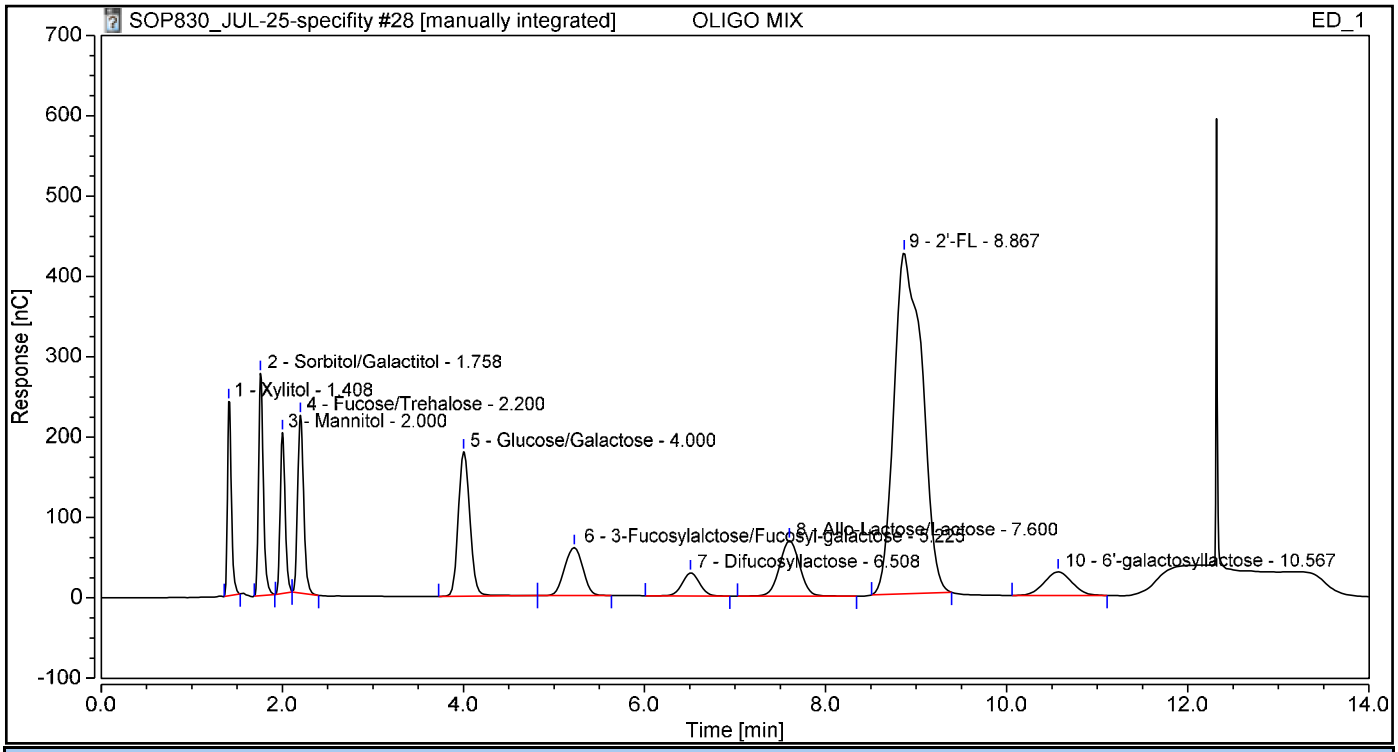
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
n.a.	Xylitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	Sorbitol/Galactitol	1.767	17.323	278.662	100.00	100.00	n.a.
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Fucose/Trehalose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Glucose/Galactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	3-Fucosylalctose/Fucosyl-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Allo-Lactose/Lactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	2'-FL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**17.323**	**278.662**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**OLIGO MIX**	*Run Time (min):*	**14.00**
Vial Number:	**RA3**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**26/Jul/19 18:11**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

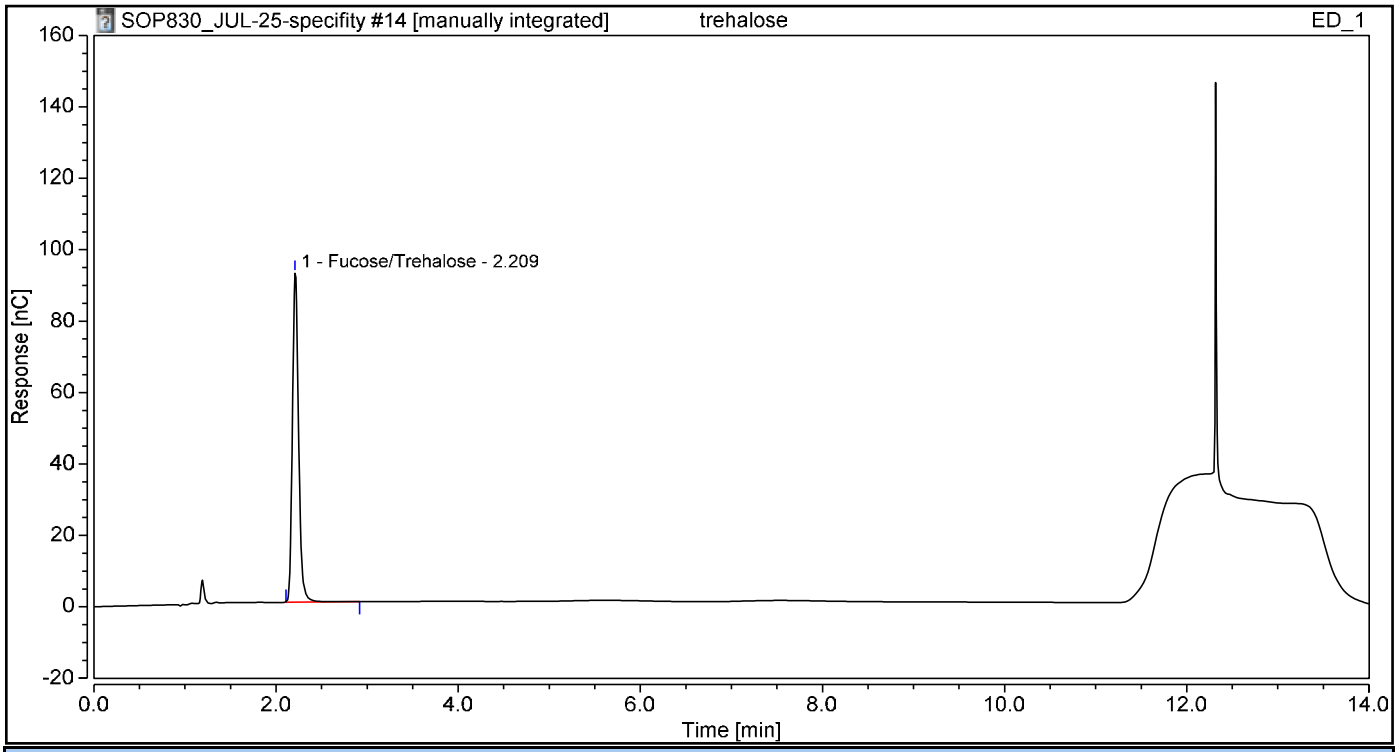
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
1	Xylitol	1.408	12.358	241.667	4.31	13.97	n.a.
2	Sorbitol/Galactitol	1.758	17.042	276.834	5.94	16.00	n.a.
3	Mannitol	2.000	12.706	200.290	4.43	11.57	n.a.
4	Fucose/Trehalose	2.200	16.992	221.058	5.92	12.77	n.a.
5	Glucose/Galactose	4.000	26.628	179.766	9.28	10.39	n.a.
6	3-Fucosylalctose/Fucosyl-	5.225	13.937	59.628	4.86	3.45	n.a.
7	Difucosyllactose	6.508	6.404	28.661	2.23	1.66	n.a.
8	Allo-Lactose/Lactose	7.600	17.693	68.781	6.17	3.97	n.a.
9	2'-FL	8.867	152.628	424.449	53.21	24.53	n.a.
10	6'-galactosyllactose	10.567	10.469	29.277	3.65	1.69	n.a.
Total:			**286.858**	**1730.410**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**trehalose**	*Run Time (min):*	**14.00**
Vial Number:	**BE1**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 19:56**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

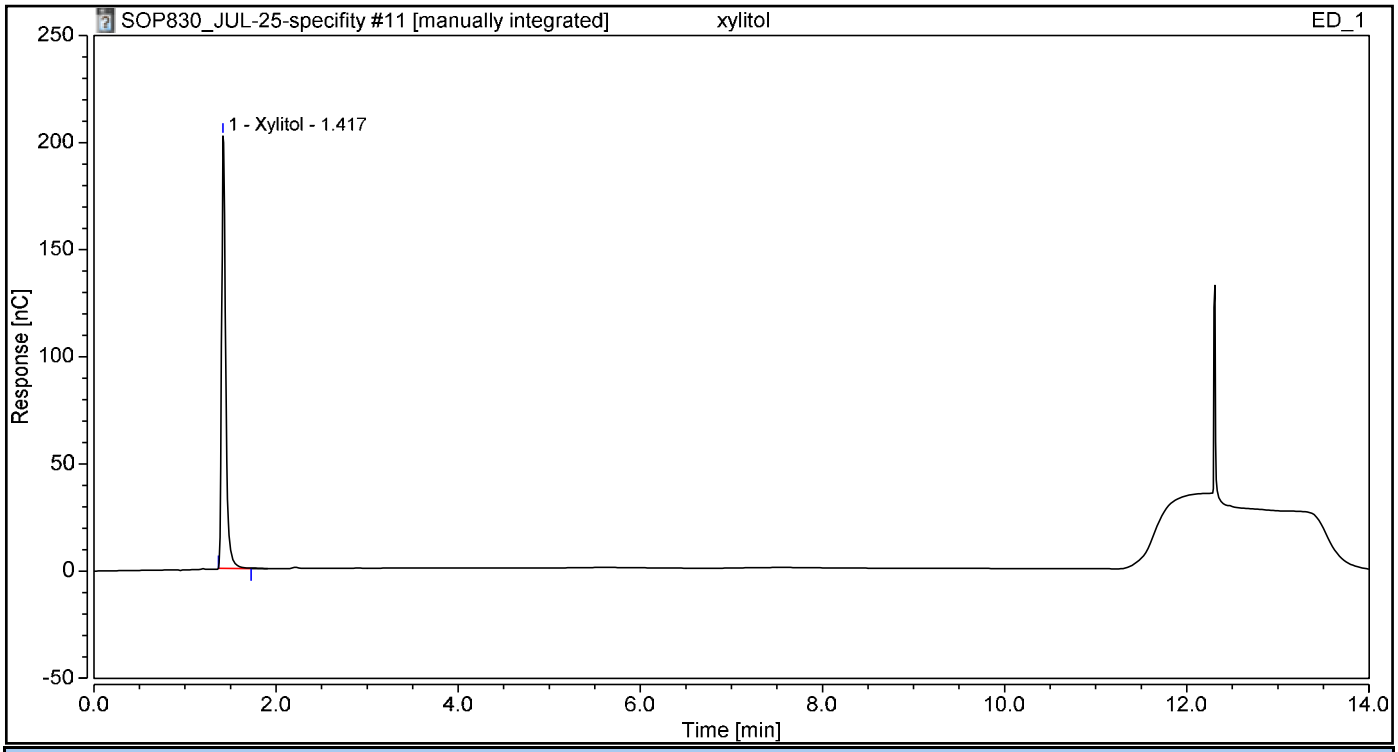
No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
n.a.	Xylitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Sorbitol/Galactitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	Fucose/Trehalose	2.209	7.276	92.110	100.00	100.00	n.a.
n.a.	Glucose/Galactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	3-Fucosylalctose/Fucosyl-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Allo-Lactose/Lactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	2'-FL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**7.276**	**92.110**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**xylitol**	*Run Time (min):*	**14.00**
Vial Number:	**BD6**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Jul/19 19:06**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount
1	Xylitol	1.417	10.487	201.782	100.00	100.00	n.a.
n.a.	Sorbitol/Galactitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Fucose/Trehalose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Glucose/Galactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	3-Fucosylalctose/Fucosyl-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Difucosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Allo-Lactose/Lactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	2'-FL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**10.487**	**201.782**	**100.00**	**100.00**	

APPENDIX E
MICROBIOLOGICAL SPECIFICATIONS AND RESULTS

amyris

Test Report

for

2'Fucosyllactose

Lot Number: H8452 **Manufacturing Date 13-Aug-2019**

Characteristic	Method	Specification	Result
Appearance (powder)	Visual	white to off-white/ivory dry powder	Pass
Appearance in solution (at 5%)	Visual	Clear, colorless to slightly yellow	Pass
pH (20°C, 5% solution)	EP 2.2.3 v9	3.0 – 7.5	5.8
Total Ash	FCC 11 appendix II	< 0.5 % w/w	<0.3% w/w
Lead (Pb)	EP 2.2.58 v9	≤ 0.05 mg/kg	0.00401 mg/kg
Arsenic (As)	EP 2.2.58 v9	≤ 0.2 mg/kg	<0.005 mg/kg
Cadmium (Cd)	EP 2.2.58 v9	≤ 0.05 mg/kg	<0.01 mg/kg
Mercury (Hg)	EP 2.2.58 v9	≤ 0.1 mg/kg	<0.002 mg/kg
Cobalt (Co)	EP 2.2.58 v9	≤ 0.03 mg/kg	<0.01 mg/kg
Total Aerobic Microbial Count / SPC	EP 2.6.12 v9	≤ 1000 cfu/g	240 cfu/g
Total Yeast/ Mold Count	EP 2.6.12 v9	≤ 100 cfu/g	<10 cfu/g
Sulphite Reducing Bacteria	ISO 15213:2003	<100 cfu/g	<10 cfu/g
Bacillus Cereus	ISO7932:2004	<100 cfu/g	<10 cfu/g
Salmonella	EP 2.6.13 v9	Not detected in 25 g	Not detected
Cronobacter sakazaki	ISO/TS 22964	Not detected in 10 g	Not detected
Enterobacteriaceae [Bile Tolerant Gram Negative Bacteria]	EP 2.6.13 v9	Negative in 10 g	Not detected
Coliforms	ISO 11290-1:2017	Not detected in 10 g	Not detected
E.coli	EP 2.6.13 v9	Absent in 10 g	Not detected
Pseudomonas aeruginosa	EP 2.6.13 v9	Absent in 10 g	Not detected
Staphylococcus aureus	EP 2.6.13 v9	Negative in 10 g	Not detected
Listeria monocytogenes	ISO 11290-1:2017	Negative in 10 g	Not detected

Prepared by: Date: 4-Feb-2020

Qian He - Quality Control

Approved by: Date: 4-Feb-2020

Beth Albino - Quality Assurance

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA
P: 510 450 0761 F: 510 225 2645

amyris

Test Report

for

2'Fucosyllactose

Lot Number: H8561 **Manufacturing Date 25-Sep-2019**

Characteristic	Method	Specification	Result
Appearance (powder)	Visual	white to off-white/ivory dry powder	Pass
Appearance in solution (at 5%)	Visual	Clear, colorless to slightly yellow	Pass
pH (20°C, 5% solution)	EP 2.2.3 v9	3.0 – 7.5	5.3
Total Ash	FCC 11 appendix II	< 0.5 % w/w	0.49% w/w
Lead (Pb)	EP 2.2.58 v9	≤ 0.05 mg/kg	0.00683 mg/kg
Arsenic (As)	EP 2.2.58 v9	≤ 0.2 mg/kg	<0.005 mg/kg
Cadmium (Cd)	EP 2.2.58 v9	≤ 0.05 mg/kg	<0.01 mg/kg
Mercury (Hg)	EP 2.2.58 v9	≤ 0.1 mg/kg	<0.006 mg/kg
Cobalt (Co)	EP 2.2.58 v9	≤ 0.03 mg/kg	<0.01 mg/kg
Total Aerobic Microbial Count / SPC	EP 2.6.12 v9	≤ 1000 cfu/g	710 cfu/g
Total Yeast/ Mold Count	EP 2.6.12 v9	≤ 100 cfu/g	<10 cfu/g
Sulphite Reducing Bacteria	ISO 15213:2003	<100 cfu/g	<10 cfu/g
Bacillus Cereus	ISO7932:2004	<100 cfu/g	<10 cfu/g
Salmonella	EP 2.6.13 v9	Not detected in 25 g	Not detected
Cronobacter sakazaki	ISO/TS 22964	Not detected in 10 g	Not detected
Enterobacteriaceae [Bile Tolerant Gram Negative Bacteria]	EP 2.6.13 v9	Negative in 10 g	Not detected
Coliforms	ISO 11290-1:2017	Not detected in 10 g	Not detected
E.coli	EP 2.6.13 v9	Absent in 10 g	Not detected
Pseudomonas aeruginosa	EP 2.6.13 v9	Absent in 10 g	Not detected
Staphylococcus aureus	EP 2.6.13 v9	Negative in 10 g	Not detected
Listeria monocytogenes	ISO 11290-1:2017	Negative in 10 g	Not detected

Prepared by: Date: 4-Feb-2020

Qian He - Quality Control

Approved by: Date: 4-Feb-2020

Beth Albino - Quality Assurance

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA
P: 510 450 0761 F: 510 225 2645

amyris

Test Report

for

2'Fucosyllactose

Lot Number: H8781 **Manufacturing Date 3-Oct-2019**

Characteristic	Method	Specification	Result
Appearance (powder)	Visual	white to off-white/ivory dry powder	Pass
Appearance in solution (at 5%)	Visual	Clear, colorless to slightly yellow	Pass
pH (20°C, 5% solution)	EP 2.2.3 v9	3.0 – 7.5	5.8
Total Ash	FCC 11 appendix II	< 0.5 % w/w	0.33% w/w
Lead (Pb)	EP 2.2.58 v9	≤ 0.05 mg/kg	0.00795 mg/kg
Arsenic (As)	EP 2.2.58 v9	≤ 0.2 mg/kg	<0.005 mg/kg
Cadmium (Cd)	EP 2.2.58 v9	≤ 0.05 mg/kg	<0.01 mg/kg
Mercury (Hg)	EP 2.2.58 v9	≤ 0.1 mg/kg	<0.002 mg/kg
Cobalt (Co)	EP 2.2.58 v9	≤ 0.03 mg/kg	<0.01 mg/kg
Total Aerobic Microbial Count / SPC	EP 2.6.12 v9	≤ 1000 cfu/g	35 cfu/g
Total Yeast/ Mold Count	EP 2.6.12 v9	≤ 100 cfu/g	<10 cfu/g
Sulphite Reducing Bacteria	ISO 15213:2003	<100 cfu/g	<10 cfu/g
Bacillus Cereus	ISO7932:2004	<100 cfu/g	<10 cfu/g
Salmonella	EP 2.6.13 v9	Not detected in 25 g	Not detected
Cronobacter sakazaki	ISO/TS 22964	Not detected in 10 g	Not detected
Enterobacteriaceae [Bile Tolerant Gram Negative Bacteria]	EP 2.6.13 v9	Negative in 10 g	Not detected
Coliforms	ISO 11290-1:2017	Not detected in 10 g	Not detected
E.coli	EP 2.6.13 v9	Absent in 10 g	Not detected
Pseudomonas aeruginosa	EP 2.6.13 v9	Absent in 10 g	Not detected
Staphylococcus aureus	EP 2.6.13 v9	Negative in 10 g	Not detected
Listeria monocytogenes	ISO 11290-1:2017	Negative in 10 g	Not detected

Prepared by: Date: 4-Feb-2020

Qian He - Quality Control

Approved by: Date: 4-Feb-2020

Beth Albino - Quality Assurance

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA
P: 510 450 0761 F: 510 225 2645

amyris

Test Report

for

2'Fucosyllactose

Lot Number: H8750 **Manufacturing Date 18-Oct-2019**

Characteristic	Method	Specification	Result
Appearance (powder)	Visual	white to off-white/ivory dry powder	Pass
Appearance in solution (at 5%)	Visual	Clear, colorless to slightly yellow	Pass
pH (20°C, 5% solution)	EP 2.2.3 v9	3.0 – 7.5	5.5
Total Ash	FCC 11 appendix II	< 0.5 % w/w	<0.3% w/w
Lead (Pb)	EP 2.2.58 v9	≤ 0.05 mg/kg	0.0123 mg/kg
Arsenic (As)	EP 2.2.58 v9	≤ 0.2 mg/kg	<0.005 mg/kg
Cadmium (Cd)	EP 2.2.58 v9	≤ 0.05 mg/kg	<0.009 mg/kg
Mercury (Hg)	EP 2.2.58 v9	≤ 0.1 mg/kg	<0.002 mg/kg
Cobalt (Co)	EP 2.2.58 v9	≤ 0.03 mg/kg	<0.01 mg/kg
Total Aerobic Microbial Count / SPC	EP 2.6.12 v9	≤ 1000 cfu/g	45 cfu/g
Total Yeast/ Mold Count	EP 2.6.12 v9	≤ 100 cfu/g	<10 cfu/g
Sulphite Reducing Bacteria	ISO 15213:2003	<100 cfu/g	<10 cfu/g
Bacillus Cereus	ISO7932:2004	<100 cfu/g	<10 cfu/g
Salmonella	EP 2.6.13 v9	Not detected in 25 g	Not detected
Cronobacter sakazaki	ISO/TS 22964	Not detected in 10 g	Not detected
Enterobacteriaceae [Bile Tolerant Gram Negative Bacteria]	EP 2.6.13 v9	Negative in 10 g	Not detected
Coliforms	ISO 11290-1:2017	Not detected in 10 g	Not detected
E.coli	EP 2.6.13 v9	Absent in 10 g	Not detected
Pseudomonas aeruginosa	EP 2.6.13 v9	Absent in 10 g	Not detected
Staphylococcus aureus	EP 2.6.13 v9	Negative in 10 g	Not detected
Listeria monocytogenes	ISO 11290-1:2017	Negative in 10 g	Not detected

Prepared by: Date: 4-Feb-2020

Qian He - Quality Control

Approved by: Date: 4-Feb-2020

Beth Albino - Quality Assurance

5885 Hollis Street, Suite 100, Emeryville, California 94608, USA
P: 510 450 0761 F: 510 225 2645

APPENDIX F
INTERIM REPORT: ACCELERATED STABILITY STUDY REPORT OF 2'FUCOSYLLACTOSE

Interim Report: Accelerated Stability
Study Report of 2'Fucosyllactose

1. Purpose

a. The purpose of this study is to evaluate stability of 2'Fucosyllactose stored at different storage conditions for up to 26 weeks at 40°C/75% Relative Humidity (RH) and 3 years at 25°C/60% RH, for regulatory submission and to determine shelf-life of the final product.
b. In addition, samples will be stored in similar material as final packaging. Therefore, this will support final packaging material compatibility study as well.
c. This interim report provides data for four (4) lots of samples stored in accelerated conditions up to 13 weeks at 40°C/75% RH, which represents 1.5 years in real-time.
d. This study will continue to monitor and evaluate samples stored at 40°C/75% RH for up to 26 weeks, and at 25°C/60% RH (real-time) for up to 3 years, that will provide data to support a 3-year shelf-life claim.

2. Study Details

a. Samples were aliquoted and stored per STAB-2019-0012P (STAB-2019-0012P: Real-time and Accelerated Stability Study of 2'Fucosyllactose stored at different storage condition for Shelf-Life Study and to Support Regulatory Submission).
b. Sample analysis were shipped from DSM and analyzed at Amyris per STAB-2019-0012P.

c. **Material tested**

Material Description	Lot #	Manufacturer
2'Fucosyllactose	H8452	Amyris
2'Fucosyllactose	H8561	Amyris
2'Fucosyllactose	H9781	Amyris
2'Fucosyllactose	H8750	Amyris

d. **Storage Conditions**

Table 1: Sample quantity and storage information

Containers	4-layer bag consisting of: PET (outside), Alu, OPA, LDPE (inside)
Atmospheres in the Container	Ambient
Temperatures	25°C ± 2°C /60% ± 5% RH and 40°C ± 2°C /75% ± 5% RH
Storage Location	Company stability chambers, the Netherlands

e. **Time Points sampled**

Table 2: Time points for samples stored at 40°C/75% RH

	Sample Pull (Weeks)				
Storage Condition (°C/%RH)	**0**	**1**	**4**	**8**	**13**
40°C/75% RH	X	X	X	X	X

f. **Testing performed:**

Table 3: Tests performed

Carbohydrate Content	Specification	Test Method	Sample Required	Frequency
Carbohydrate Content				
2'-Fucosyllactose	≥ 83 % area	SOP00830	5 g	At each time point
Allo-Lactose/Lactose	≦ 8 % area			
DFL	≦ 7 % area			
2'-Fucosyllactitol	≤ 6 % area			
3'-FL / Fucosylgalactose	< 6 % area			
Fructose				
Glucose/Galactose				
GPE				
Fucose/Trehalose				
Sorbitol/Galactitol				
Xylitol				
Water				
KF (% water)	≦5%	SOP00842	5 g	At each time point

3. Results/Data

Table 5: Stability of Amyris 2'-FL Under Accelerated Storage Conditions

2 FL spray-dried product accelerated stability study (40°C, 75% RH)					
	0 wk	1 wk	4 wk	8 wk	13 wk
H8452					
2'-FL (area %)	91.10	90.70	90.95	90.92	90.55
Allo-Lactose/Lactose (area %)	1.18	1.48	1.64	1.31	1.15
DFL (area %)	1.40	0.97	0.91	1.17	0.98
3'-FL / Fucosylgalactose (area %)	0.33	0.63	0.48	0.34	0.55
Fructose (area %)	0.40	0.37	0.29	0.39	0.55
Glucose/Galactose (area %)	0.26	0.31	0.32	0.24	0.35
GPE (area %)	0.27	0.43	0.47	0.37	0.46
2'-Fucosyllactitol (area %)	3.54	3.77	3.69	3.94	3.9
Fucose/Trehalose (area %)	0.18	0.19	0.18	0.20	0.26
Sorbitol/Galactitol (area %)	0.14	0.17	0.12	0.17	0.31
Xylitol (area %)	0.73	0.76	0.76	0.82	0.82
Water Content (%)	2.66	3.00	2.99	2.84	2.74
H8561					
2'-FL (area %)	92.20	92.10	92.45	92.39	91.96
Allo-Lactose/Lactose (area %)	0.08	0.18	0.24	0.14	0.07
DFL (area %)	1.30	1.04	1.0	1.21	1.02
3'-FL / Fucosylgalactose (area %)	0.38	0.54	0.40	0.30	0.4
Fructose (area %)	0.43	0.43	0.32	0.48	0.61
Glucose/Galactose (area %)	0.33	0.29	0.29	0.23	0.32
GPE (area %)	0.42	0.44	0.47	0.40	0.48
2'-Fucosyllactitol (area %)	3.93	4.13	4.01	4.19	4.22
Fucose/Trehalose (area %)	0.12	0.13	0.12	0.12	0.16
Sorbitol/Galactitol (area %)	0.05	0.03	0.05	0.06	0.11
Xylitol (area %)	0.6	0.59	0.58	0.63	0.62
Water Content (%)	3.07	3.39	3.23	3.10	3.12
H8781					
2'-FL (area %)	91.2	91.0	91.15	90.89	90.94
Allo-Lactose/Lactose (area %)	1.43	1.87	1.85	1.57	1.5
DFL (area %)	1.24	0.82	0.83	1.07	0.84
3'-FL / Fucosylgalactose (area %)	0.23	0.55	0.45	0.31	0.49
Fructose (area %)	0.31	0.37	0.38	0.53	0.49
Glucose/Galactose (area %)	0.22	0.15	0.20	0.16	0.22
GPE (area %)	0.53	0.47	0.31	0.53	0.58
2'-Fucosyllactitol (area %)	3.19	3.30	3.23	3.29	3.37
Fucose/Trehalose (area %)	0.15	0.15	0.14	0.17	0.17
Sorbitol/Galactitol (area %)	0.15	0.17	0.15	0.20	0.21
Xylitol (area %)	0.67	0.67	0.66	0.73	0.78

Water Content (%)	2.53	2.81	2.69	2.64	2.77
H8750					
2'-FL (area %)	91.30	90.70	91.31	90.76	90.47
Allo-Lactose/Lactose (area %)	0.44	0.62	0.61	1.38	0.44
DFL (area %)	1.25	0.91	0.87	1.10	0.92
3'-FL / Fucosylgalactose (area %)	0.18	0.55	0.45	0.33	0.46
Fructose (area %)	0.46	0.47	0.39	0.63	0.68
Glucose / Galactose (area %)	0.52	0.46	0.42	0.24	0.51
GPE (area %)	0.25	0.35	0.34	0.40	0.36
2'-Fucosyllactitol (area %)	3.52	3.75	3.65	3.82	3.84
Fucose / Trehalose (area %)	0.19	0.21	0.20	0.20	0.26
Sorbitol / Galactitol (area %)	0.10	0.11	0.11	0.16	0.24
Xylitol (area %)	1.30	1.36	1.32	0.81	1.44
Water Content (%)	2.66	2.83	2.70	2.68	6.31
Source: Amyris, Inc. Abbreviations: RH = relative humidity; wk = week; wt = weight.					

4. Conclusion

Based on the results shown above and in Appendix 1, 2'-FL is shown to be stable over the 13-week accelerated conditions of 40°C/ 75% RH. The stability study will continue to monitor and evaluate samples held at 40°C/75% RH for up to 26 weeks, and at 25°C/60% RH for up to 3 years, to provide data to support a 3-year shelf-life claim.

Approvals:

[redacted]

28-Feb-2020

Beth Albino (Study Director) Date

[redacted]

13-March-2020

Howard Fuller (Quality Assurance) Date

Appendix 1

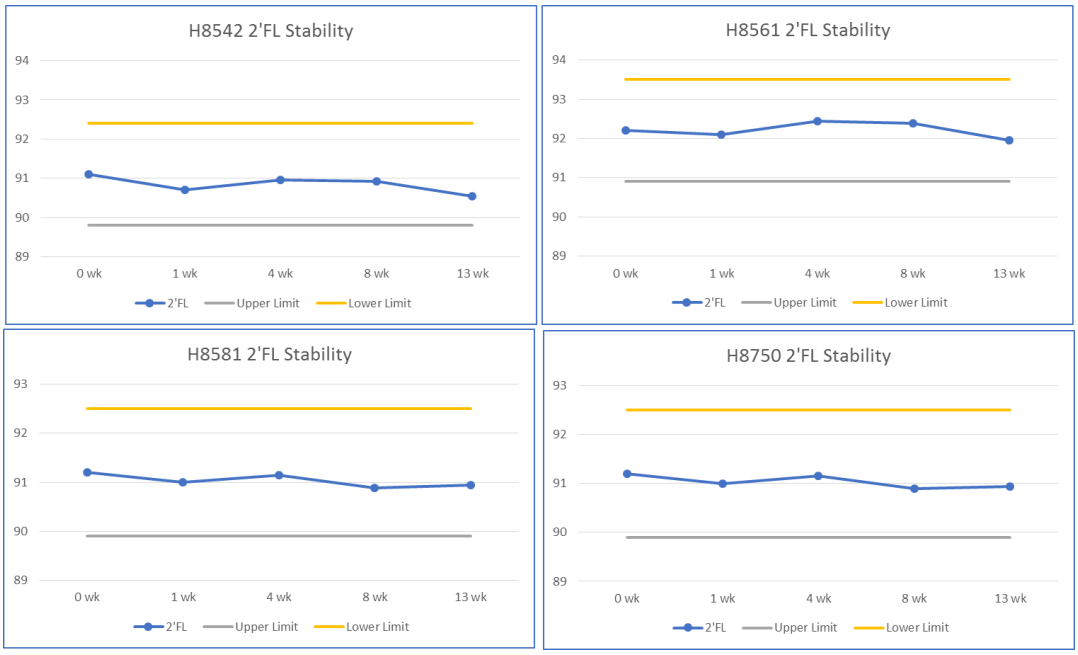
2'-FL assay variation was assessed with results as follows:

Prep No.	2'-FL Area %
RP-Q-1	89.99
RP-Q-2	89.61
RP-Q-3	90.06
RP-Q-4	89.96
RP-Q-5	89.48
RP-Q-6	89.83
RP-S-1	91.03
RP-S-2	90.94
RP-S-3	91.06
RP-S-4	91.27
RP-S-5	91.02
RP-S-6	90.97
Average	90.435
St. Dev (σ)	**0.66**

Using this result, the 95% Confidence Range for assay variation was determined to be:

-/+ 1.96σ = (-1.30, 1.30)

Applying the assay Confidence Range to the T0 result, as given below, shows that there were no changes to the 2'-FL over the 13 weeks accelerated study that is outside the range of the assay variation, demonstrating 2'-FL is stable over 13 weeks accelerated conditions.



APPENDIX G
AMINO ACID CONTENT ANALYSIS

eurofins | Microbiology

Eurofins Microbiology Laboratories (Garden Grove)
11390 Knott Ave
Garden Grove, CA 92841, US
www.eurofinsus.com
Tel:+1 714 892 0208

CERTIFICATE OF ANALYSIS

AR-19-QR-018504-01

Amyris.PACD
Attn: Don Diola
5885 Hollis St. Suite 100
Emeryville, CA 94608

Client Code: QR0000288
Batch Code: EUUSGA2-00032925
PO Number: 97061

Report Date: 09/09/2019
Entry Date: 08/23/2019

Eurofins Sample Code: 111-2019-08230182
Client Sample Code:

Sample Description: FOOD
Condition Upon Receipt: Acceptable

Sample Reference:
DPlowSR
Analysis Date: 8/23/2019

Test	Result
QQ176 - Amino Acids by AH (AOAC, Most Matrices)	
Method Reference: AOAC 982.30 mod.	
Alanine	0.03 %
Arginine	<0.05 %
Aspartic Acid	<0.02 %
Glutamic Acid	0.11 %
Glycine	<0.01 %
Histidine	<0.01 %
Isoleucine	<0.02 %
Leucine	<0.02 %
Phenylalanine	<0.03 %
Proline	0.09 %
Serine	<0.01 %
Threonine	<0.02 %
Total Lysine	0.02 %
Tyrosine	<0.04 %
Valine	<0.02 %

Eurofins Microbiology Laboratories (Garden Grove)
11390 Knott Ave
Garden Grove, CA 92841, US
www.eurofinsus.com
Tel:+1 714 892 0208

CERTIFICATE OF ANALYSIS

AR-19-QR-018504-01

Amyris.PACD
Attn: Don Diola
5885 Hollis St. Suite 100
Emeryville, CA 94608

Client Code: QR0000288
Batch Code: EUUSGA2-00032925
PO Number: 97061

Report Date: 09/09/2019
Entry Date: 08/23/2019

Respectfully Submitted,
Eurofins Scientific. Inc.

Anne Chi
Business Unit Manager

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Measurement of Uncertainity may be obtained upon request.
Retained samples are discarded 30 days after receipt; certain exceptions apply.

eurofins | Microbiology

Eurofins Microbiology Laboratories (Garden Grove)
11390 Knott Ave
Garden Grove, CA 92841, US
www.eurofinsus.com
Tel:+1 714 892 0208

CERTIFICATE OF ANALYSIS

AR-19-QR-018507-01

Amyris.PACD
Attn: Don Diola
5885 Hollis St. Suite 100
Emeryville, CA 94608

Client Code: QR0000288
Batch Code: EUUSGA2-00032925
PO Number: 97061

Report Date: 09/09/2019
Entry Date: 08/23/2019

Eurofins Sample Code: 111-2019-08230185
Client Sample Code:

Sample Description: FOOD
Condition Upon Receipt: Acceptable

Sample Reference:
Radha 8561
Analysis Date: 8/23/2019

Test	Result
QQ176 - Amino Acids by AH (AOAC, Most Matrices)	
Method Reference: AOAC 982.30 mod.	
Alanine	0.02 %
Arginine	<0.05 %
Aspartic Acid	0.02 %
Glutamic Acid	<0.02 %
Glycine	0.01 %
Histidine	<0.01 %
Isoleucine	<0.02 %
Leucine	<0.02 %
Phenylalanine	<0.03 %
Proline	<0.05 %
Serine	0.02 %
Threonine	<0.02 %
Total Lysine	0.07 %
Tyrosine	<0.04 %
Valine	<0.02 %

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Eurofins Microbiology Laboratories (Garden Grove)
11390 Knott Ave
Garden Grove, CA 92841, US
www.eurofinsus.com
Tel:+1 714 892 0208

CERTIFICATE OF ANALYSIS

AR-19-QR-018507-01

Amyris.PACD
Attn: Don Diola
5885 Hollis St. Suite 100
Emeryville, CA 94608

Client Code: QR0000288
Batch Code: EUUSGA2-00032925
PO Number: 97061

Report Date: 09/09/2019
Entry Date: 08/23/2019

Respectfully Submitted,
Eurofins Scientific. Inc.

Anne Chi
Business Unit Manager

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Retained samples are discarded 30 days after receipt; certain exceptions apply.

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11390 Knott Ave
Garden Grove, CA 92841, US
www.eurofinsus.com
Tel:+1 714 892 0208

CERTIFICATE OF ANALYSIS

AR-19-QR-018502-01

Amyris.PACD
Attn: Don Diola
5885 Hollis St. Suite 100
Emeryville, CA 94608

Client Code: QR0000288
Batch Code: EUUSGA2-00032925
PO Number: 97061

Report Date: 09/09/2019
Entry Date: 08/23/2019

Eurofins Sample Code: 111-2019-08230180
Client Sample Code:

Sample Description: FOOD
Condition Upon Receipt: Acceptable

Sample Reference:
Radha 8781
Analysis Date: 8/23/2019

Test	Result
QQ176 - Amino Acids by AH (AOAC, Most Matrices)	
Method Reference: AOAC 982.30 mod.	
Alanine	<0.01 %
Arginine	<0.05 %
Aspartic Acid	<0.02 %
Glutamic Acid	<0.02 %
Glycine	<0.01 %
Histidine	<0.01 %
Isoleucine	<0.02 %
Leucine	<0.02 %
Phenylalanine	<0.03 %
Proline	<0.05 %
Serine	<0.01 %
Threonine	<0.02 %
Total Lysine	<0.01 %
Tyrosine	<0.04 %
Valine	<0.02 %

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11390 Knott Ave
Garden Grove, CA 92841, US
www.eurofinsus.com
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CERTIFICATE OF ANALYSIS

AR-19-QR-018502-01

Amyris.PACD
Attn: Don Diola
5885 Hollis St. Suite 100
Emeryville, CA 94608

Client Code: QR0000288
Batch Code: EUUSGA2-00032925
PO Number: 97061

Report Date: 09/09/2019
Entry Date: 08/23/2019

Respectfully Submitted,
Eurofins Scientific. Inc.

Anne Chi
Business Unit Manager

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www.eurofinsus.com
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Amyris.PACD
Attn: Don Diola
5885 Hollis St. Suite 100
Emeryville, CA 94608

Client Code: QR0000288
Batch Code: EUUSGA2-00032925
PO Number: 97061

Report Date: 09/09/2019
Entry Date: 08/23/2019

Eurofins Sample Code: 111-2019-08230178
Client Sample Code:

Sample Description: FOOD
Condition Upon Receipt: Acceptable

Sample Reference:
Radha 8750-lot 5
Analysis Date: 8/23/2019

Test	Result
QQ176 - Amino Acids by AH (AOAC, Most Matrices)	
Method Reference: AOAC 982.30 mod.	
Alanine	<0.01 %
Arginine	<0.05 %
Aspartic Acid	<0.02 %
Glutamic Acid	<0.02 %
Glycine	<0.01 %
Histidine	<0.01 %
Isoleucine	<0.02 %
Leucine	<0.02 %
Phenylalanine	<0.03 %
Proline	<0.05 %
Serine	<0.01 %
Threonine	<0.02 %
Total Lysine	<0.01 %
Tyrosine	<0.04 %
Valine	<0.02 %

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APPENDIX H
ALLERGENICITY EVALUATION

Risk assessment of genes used in the production of 2'-FL using FAO/WHO guidelines predicts low potential allergenicity.

Introduction

Bioinformatic prediction of allergenicity has become an increasingly popular first step towards the use of GM derived organisms in food/feed products. The FAO/WHO have outlined methodology using common bioinformatics tools, including local alignment and identity/similarity queries against known allergen databases, to assess potential allergenicity. In this study, we show *in silico* that specific engineered gene constructs for 2'-FL production have low risk of potential allergenicity.

Materials, Methods and Results

The FAO/WHO protocol[1] for bioinformatic allergenicity assessment outlines a two-part procedure to be performed against multiple allergen databases. The first part, used to identify potential linear IgE epitopes, searches for any match of 6 consecutive amino acids between the query protein and any allergen database entry. The second part, used to detect potential conformational IgE epitopes, searches for greater than 35% sequence identity, over a sliding 80-mer amino acid window, of the query protein against any documented protein allergen.

The most comprehensive and recently updated allergen database, called AllergenOnline, is maintained by the Food Allergy Research and Resource Program (FARRP) in the Department of Food Science and Technology at the University of Nebraska in Lincoln[3,4,5,6]. The AllergenOnline database contains 2035 peer reviewed allergen sequences from 808 taxonomic groups, and was last updated in January 2017. Part one of the bioinformatics assessment searched for 6-mer matches between the engineered 2'-FL constructs, and the AllergenOnline database. This search returned 161 hits. Part two of the bioinformatics assessment, requiring >35% sequence similarity of any 80-mer amino acid window, returned 179 hits. In addition, total protein sequences queried for >35% similarity against the entire allergen database returned zero hits.

Conclusions

Although the FAO/WHO guidelines published in 2001 continue to be standard practice for bioinformatics allergenicity assessment, the EFSA provides detailed interpretation guidelines based on empirical data published between 2001 and 2010[2]. Specifically, the EFSA notes that the use of a 6-mer amino acid identity search generates too many false positives and is not widely accepted[2,14]. Similarly, the FARRP also suggests that a single identity match of 6 to 8 contiguous amino acids does not imply similar IgE binding in the absence of more extensive identity alignments[6]. Both EFSA and FARRP concur that sequences sharing less than 50% identity over their full-lengths are rarely crosss-reactive[2,3,6], making 35% similarity a stringent threshold. The absence of strong full length identity/similarity evidence suggests the engineered constructs in 2'-FL have low potential for allergenicity.

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APPENDIX I
ENZYMATIC HYDROLYSIS OF 2-FLOL REPORT

DSM Biotechnology Center

Report
Enzymatic hydrolysis of 2'-fucosyllactitol

Study Director: Marco van den Berg

Experimental work and Author: Michael Tabeling

Period experimental work: From 29/08/2019 to 13/11/2019

Project Number: Radha; RD.8803.21

Testing Facility: DSM Biotechnology Center, P.O. Box 1, 2600 MA Delft,The Netherlands

Sponsor: DSM Nutritional Products AG, Wurmisweg 576, 4303-Kaiseraugst, Switzerland

Keywords: [glycosidase hydrolases, fucosidases, 2'-fucsyllactose, 2'-fucosyllactitol, Human Milk Oligosaccharides, Early Life Nutrition, Human Nutrition Health]

Study Monitor: Anette Thiel

Date and signature Study Director:
Marco van den Berg

December 10, 2020

Date and Signature of the Author:
Michael Tabeling

December 17, 2020

Enzymatic hydrolysis of 2'-fucosyllactitol

Executive Summary

A prototype product of 2-fucosyllactose (2'-FL), which is the most predominant HMO present in human milk, has been made. This product contains about 4% ($^{w}/_{w}$) 2´-fucosyl lactitol (2-FLol).
This study showed the hydrolysis of 2-FLol by fucosidases belonging to the glycoside hydrolase family (GH95). A member of this GH95 family is a fucosidase from *Bifidobacterium longum*, which is a common "inhabitant" of the gut of human infants. As a result of this applied enzyme in vitro conditions at pH 6.5 and 37°C, the biological half-life for 2-fucosyllactitol was 1 hour under the conditions reported. The same enzyme was shown to be capable of hydrolyzing 2-FL as well.

Table of Contents

1. Introduction

Human milk oligosaccharides (HMO) consist of a group of more than 200 compounds naturally present in human breast milk. 2´-fucosyl-lactose (2-FL) is generally the most predominant HMO present in human milk.

Laboratory spray dried material, has been made and contains about 4% ($^{w}/_{w}$) 2´-fucosyl lactitol (2-FLol), which is present in negligible amounts in current commercial materials.
Glycoside hydrolases belonging to the family 95 are categorized as fucosidases. Such fucosidases are present in various micro-organisms of the microbiome and therefore are expected to be present in the human gut.

The aim of this study was to evaluate the hydrolysis of 2-FLol in fucose and lactitol and to investigate velocity.

2. Materials & Methods

2.1. Materials

Two different substrates were used:

- Care4U™ (SKU 61011797) from Danisco produced for DuPont Nutrition Biosciences, batch #2FL100517, containing > 98% ($^{w}/_{w}$) 2'-fucosyllactose (Product sheet in Appendix 6.1.1; control)
- Prototype 2'fucosyllactose from DSM, batch HSC-7220 POW [ELN-NBK-024669-004] containing 91.1% ($^{w}/_{w}$) 2'-fucosyllactose and 3.86% ($^{w}/_{w}$) 2'-fucosyllactitol [ELN-NBK-024411-017]. The complete content is outlined in Appendix 6.1.2 (sample).

Commercially available products containing purified glycoside hydrolases from different species/ sources were selected for screening the ability to hydrolyze 2'-fucosyllactitol (table A; certificates of analysis in appendix 6.2).

Table A: Commercially available products containing glycoside hydrolases from different species.

Product	Batch	Supplier	Description	Glycoside Hydrolase Family	EC Number	Species	Enzyme concentration (mg/ml)
CZ0511	18041	NZYtech	BlFuc95A	GH95	3.2.1.51	*Bifidobacterium longum*	1
E-FUCM	101101b	Megazyme	1,2-α-L-Fucosidase (microbial)	GH95	3.2.1.63	*Bifidobacterium* sp.	0.075
CZ0566	17061	NZYtech	LcFuc29A	GH29	3.2.1.51	*Lactobacillus casei*	0.5

2.2. Methods

2.2.1. Enzymatic hydrolysis of 2-FL and/or 2-FLol

Two sets of experiments were performed [ELN (Electronic Lab Notebook) -NBK-026117-002].

2.2.1.1. Screening for enzymes enabling hydrolysis of 2-FLol

At 1-ml volume scale, each commercial available enzyme product was tested at two different concentrations, 2 and 12 µg/ml, for the hydrolysis of 49 mM of 2-FL (control substrate) or 47 mM 2'-FL and 2 mM 2'FLol (sample substrate) in sodium phosphate buffer of pH 6.5 for 24 hours at 37°C and 1000 rpm (thermomixer from Eppendorf). Incubates without enzyme and corresponding final substrate concentrations were taken along for control purposes (no enzyme control).
Aliquots of 125 µl were taken after 10 and 30 minutes and 2, 8 and 24 hours and immediately heated for 5 minutes at 95°C to inactivate the enzyme. These time samples of the enzyme incubates and controls were stored in the freezer (-20°C) until further analysis by HPAEC-PAD. Initially, only the 2 hours samples generated with enzyme dosage of 12 µg/ml were analyzed for all commercially available enzyme products. Based on these results, the remaining time samples related to two different applied dosages of fucosidases from *Bifidobacterium longum* and *Bifidobacterium* species were analyzed as well.

2.2.1.2. Repeatability hydrolysis of 2-FLol in relevant time by GH95 of *Bifidobacterium longum*

Two independent hydrolysis experiments were performed for the sample substrate and 2 µg/ml of GH95 fucosidase from *Bifidobacterium longum* as described in section 2.2.1.1. Incubations without added enzyme were taken along as two independent controls. Heat inactivated time samples of 10 and 30 minutes and 1, 2, 4 and 8 hours were analyzed by HPAEC-PAD.
The average of all the time samples for each of the no enzyme control was set as 100%, resulting in a relative plot for the hydrolysis of 2-FL and 2-FLol in time.

2.2.2. Quantification of the hydrolysis by HPAEC-PAD

Time samples were diluted in milliQ water after inactivation of the enzyme. Different dilutions were applied depending on the detectable range for 2-FL, 2-FLol and lactitol. The analysis was performed according to Amyris document number SOP00830 titled "determination of 2'-FL by IC area%" (Appendix 6.3).

The residual 2-FL and 2-FLol of the two hours samples of the screening were expressed as the relative peak area towards the blanc. In the next series, 2-Flol and lactitol were determined in mg/L using a lactitol standard (Alfa Aesar). Additionally, lactose and 2FL were determined in g/L using a lactose (Sigma) and 2FL (Carbosynth) standard (certificate of the standards in appendix 6.4).

Compounds were quantified using external calibration curves. Calibration points were made in the range 1-20 mg/L. For lactose and 2-fucosyllactose, samples were diluted 20 times more before analysis.

In more detail, samples were analyzed on an Ion Chromatographic system (Thermo) coupled to a pulsed amperometric detector (PAD) (Thermo). Separation was carried out using a Carbopac PA 1 column (250x4 mm) (Thermo) with 100 mM NaOH at 1.5 mL/min.

Missing a standard for 2-FLol, the method is not validated yet. The limit of quantification is depending on the condition of the column and provisional set at 30 or 50 mg/L [ELN NBK-02411-027].

3. Results & Discussion

3.1. Screening for enzymes enabling hydrolysis of 2-FLol

Different glucosidase hydrolases were screened for the hydrolysis of 2-fucosyllactitol next to 2-fucosyllactose after 2 hours hydrolysis at pH 6.5 and 37°C applying a high enzyme dosage.

Pure 2-fucosyllactose was (partly) hydrolyzed by fusosidases from *Bifidobacterium (longum)* (Table 1). Those ones, which hydrolyzed 2-FL for at least 70%, belongs to the GH95 family. Tested fucosidase from *Lactobacillus casei* showed no hydrolysis of 2-FL. This one is member of the GH 29 family. Those fucosidases which can hydrolyze 2-FL are also able to hydrolyze 2-FLol. Incubation of the HMO 2'-fucosyllactose prototype with fucosidase from *Bifidobacterium longum* showed complete hydrolysis of 2'-fucosyllactitol next to 2-fucosyllactose (Table 1). This micro-organism is a common inhabitant of the human body. Additional analysis was done with different enzyme preparations derived from *Bifidobacterium* only.

Table 1: Residual peak area, expressed as percentage compared to the control without enzyme, of 2-FL and 2-FLol obtained after 2 hours hydrolysis of the different substrates applying 12 µg fucosidase from different species per ml incubate at pH 6.5 and 37°C.

Substrate (lot number)	Fucosidase from	Glycoside Hydrolase family	Sample Name 12µg/ 2h @ pH 6.5, 37°C	Area % 2 FL	Area % 2FLol
Care4U (#2FL100517)	*Bifidobacterium longum*	GH95	sample 8C	0	N.A.
Care4U (#2FL100517)	*Bifidobacterium* sp.	GH95	sample 11C	3	N.A.
Care4U (#2FL100517)	*Lactobacillus casei*	GH29	sample 12C	98	N.A.
Care4U (#2FL100517)	None (blanc)	none	sample 14C	100	N.A.
Prototype (#HSC-7220)	*Bifidobacterium longum*	GH95	sample 22 C	0	0
Prototype (#HSC-7220)	*Bifidobacterium* sp.	GH95	sample 25 C	18	14
Prototype (#HSC-7220)	*Lactobacillus casei*	GH29	sample 26 C	99	97
Prototype (#HSC-7220)	None (blanc)	none	sample 28 C	100	100

N.A.: not applicable.
Area %: peak area percentage, relative to peak area of the blanc.

3.2. Hydrolysis of 2-FLol in time by GH95 fucosidases from *Bifidobacterium* species

Two different enzyme preparations containing GH95 fucosidases from *Bifidobacterium* species, both are able to hydrolyze 2-FL(ol) efficiently (Table 1), were tested at two different dosages for the hydrolysis of 2'-FLol, present in the HMO fucosyllactose prototype, in 24 hours at pH 6.5 and 37°C. Both enzyme preparations showed increased levels of lactitol during the hydrolysis of 2'-FLol and concomitant decrease of 2-FLol (see Figures 1 and 2 and Appendix 6.5).

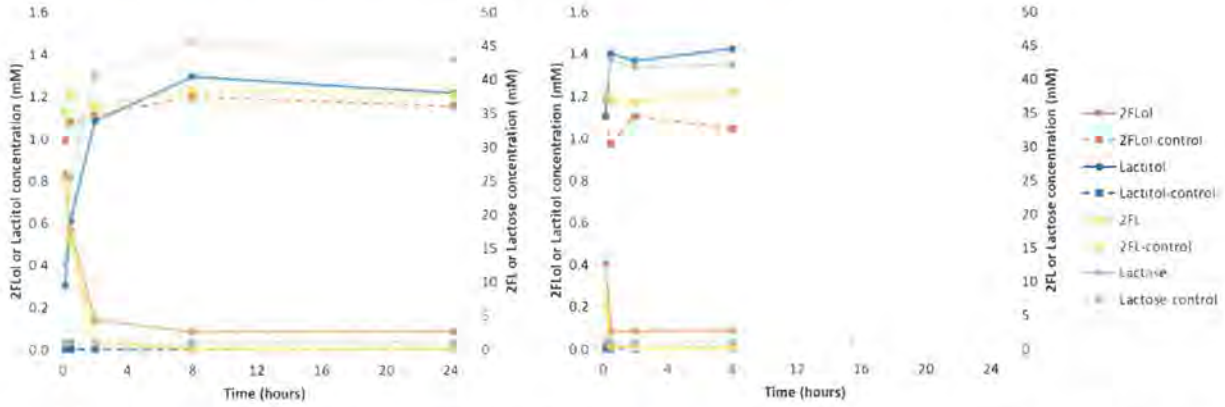

Figure 1: Hydrolysis of 47mM 2FL and 2mM 2FLol by fucosidase (GH95) from *Bifidobacterium longum*, applying dosages of 2 (left) or 12 µg/ml (right), to lactose and lactitol in sodium phosphate buffer pH 6.5 and 37°C (with enzyme, solid lines; no enzyme control, dashed lines).

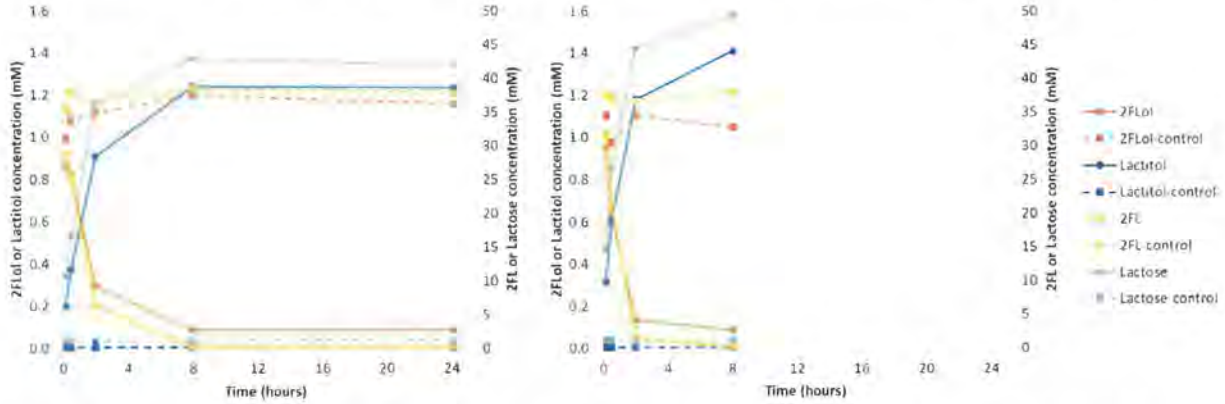

Figure 2: Hydrolysis of 47mM 2FL and 2mM 2FLol by fucosidase (GH95) from *Bifidobacterium* sp., applying dosages of 2 (left) or 12 µg/ml (right), to lactose and lactitol in sodium phosphate buffer pH 6.5 and 37°C (with enzyme, solid lines; no enzyme control, dashed lines)

The incubations without enzyme showed some minor fluctuations around the average value for 2'-FLol in time, likely due to the fact that the used HPLC analysis has not been validated yet.

3.3. Repeatability hydrolysis of 2-FLol in relevant time by GH95 fucosidase from *B. longum*

A duplicate experiment was performed for the hydrolysis of 2FLol and 2FL to lactitol and lactose, respectively, in the relevant digestion time by fucosidase from *Bifidobacterium longum*. Both experiments showed similar hydrolysis profiles suggesting good repeatability (Figure 3 and Appendix 6.6).

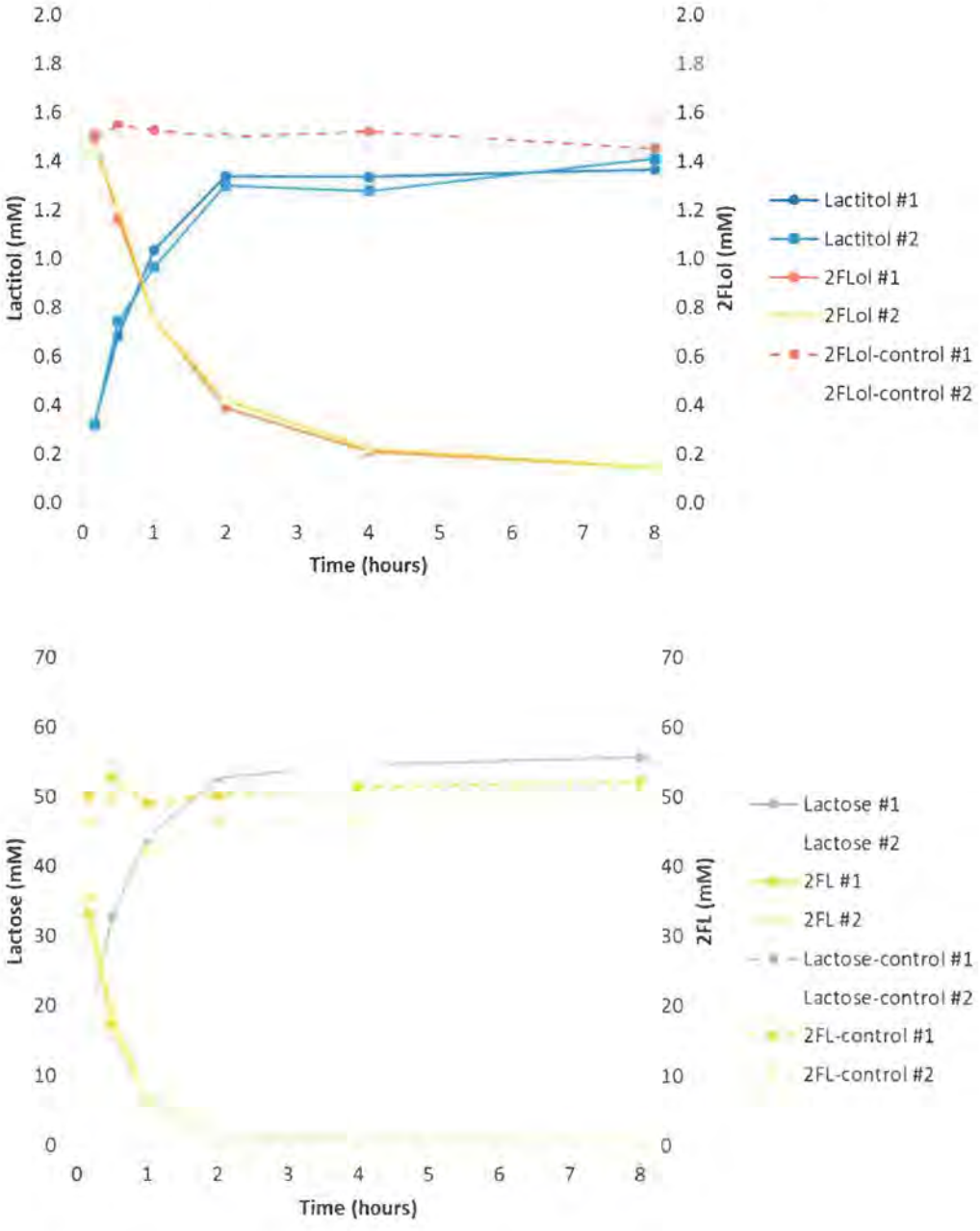

Figure 3: Duplicate hydrolysis of 47mM 2FL (lower part) and 2mM 2FLol (upper part) by fucosidase (GH95) from *Bifidobacterium longum*, applying dosages of 2 µg/ml, to lactose and lactitol in sodium phosphate buffer pH 6.5 and 37°C (with enzyme, solid lines; no enzyme control, dashed lines).

Plotting the data in a relative way showed that half of the 2-fucosyllactitol (50%), corresponding to 1 µmol, has been hydrolyzed by 2 µg fucosidase from Bifidobacterium longum after one-hour incubation at pH 6.5 and 37°C (Figure 4). In other words, the biological half-life is 1 hour at the tested conditions in vitro.

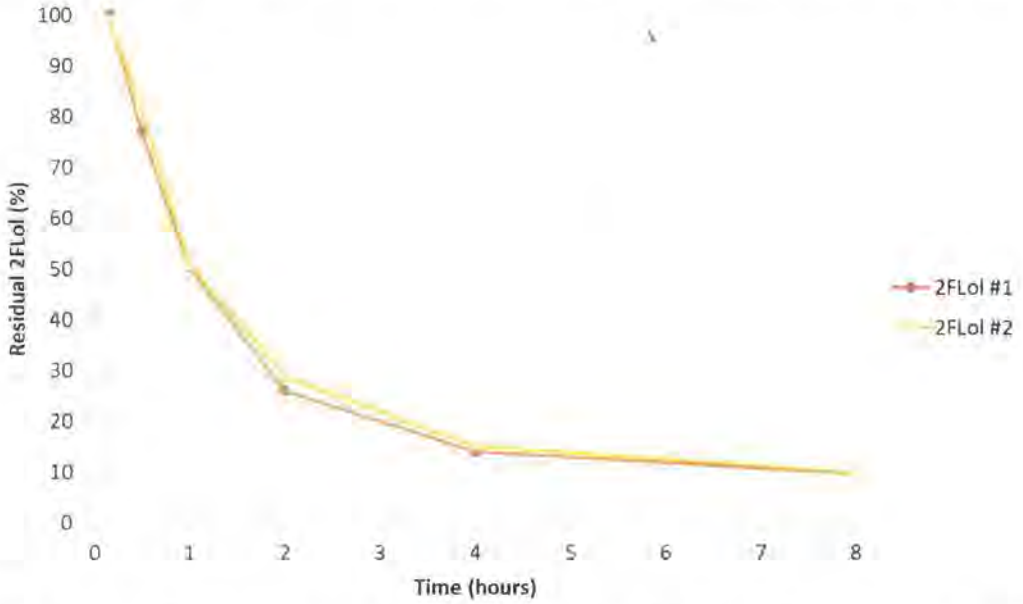

Figure 4: Duplicate relative hydrolysis of 2mM 2FLol by fucosidase from *Bifidobacterium longum*, applying dosages of 2 µg/ml, in sodium phosphate buffer pH 6.5 and 37°C.

Chromatograms of the one-hour hydrolysis samples and corresponding standards are depicted in Appendix 6.7.

4. Conclusions & Outlook

- Fucosidases belonging to the GH95 family can hydrolyze 2-FLol next to 2-FL in the 2-FL-prototype.
- Fucosidase from *Bifidobacterium longum* showed reproducible hydrolysis of 2-FLol to lactitol, next to the hydrolysis of 2-FL to lactose.
- The biological half-life for 2-fucosyllactitol is 1 hour under the tested in vitro conditions of pH 6.5 and 37°C.

5. References

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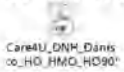
2. Ashida, H., Akiko, M., Kiyohara, M., Wada, J. Yoshida, E., Kumagai, H., Katayama, T., Yamamoto, K. (2009) Two distinct α-L-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates, Glycobiology. 19: 1010-1017.
3. Megazyme (2017) Booklet 1,2-α-L-fucosidase (microbial) (lot 101101b), 03/17 (Appendix 6.8).

6. Appendices

6.1.1. Product sheet Care4U™



6.1.2. Composition DSM prototype batch HSC-7220:

	Threshold set at 0.1%*
Carbohydrate	50 ppm
2-FL	91.1
Difucosyllactose	1.49
lactose	1.4
3-fucosyllactose	0
2FLol	3.86
Xylitol	0.73
Dulcitol/sorbitol	0.11
fucose	0.24
Glycerylphosphoethanolamine	0.36
fructose	0.44
fucosyl galactose	0.12
glucose/galactose	0.17
total other	2.17

*: 0.1% is the minimal peak area of a component in the chromatogram injecting 50 ppm of a sample. In more detail, injection of 50 ppm containing 90% 2-FL (which corresponds to 45 ppm 2-FL) resulted in minimal peak area of 0.005 ppm or 5 ppb (which is 0.1%).

6.2. Certificates of analysis for different commercial products containing glycoside hydrolases.

CZ05111_batch 18041.pdf

E-FUCM_COA_1011 01b.pdf

CZ05661_batch 17061.pdf

6.3. Standard Operation Procedure 00830 titled determination of 2'-FL by IC area%.

SOP00830 Determination of 2

6.4. Certificates of standards for analysis by HPAEC-PAD.

Lactitol monohydrate from /

D-lactose monohydrate from I

2 fucosyllactose sy nthetic from Carbos

6.5. Individual data wrt hydrolysis of 2-FLol in time by GH95 fucosidases from *Bifidobacterium* species.

Table I: Concentrations of different components during hydrolysis of 2FLol and 2-FL with/ without the addition of two different dosages of fucosidases from two different *Bifidobacterium* sources at pH 6.5 and 37°C.

Fucosidase from species	Dosage (µg /ml)	Time (hours)	Lactitol (µg/mL)	2FLol (µg/mL)	Lactose (mg/mL)	2-FL (mg/mL)
Bifidobacterium longum	12	0.17	381	140	12.66	3.22
	12	0.50	482	30	14.69	0.22
	12	2.00	471	< 30	14.28	< 0.1
	12	8.00	490	< 30	14.43	< 0.1
Bifidobacterium sp.	12	0.17	109	328	5.01	15.53
	12	0.50	209	212	9.14	10.27
	12	2.00	406	46	15.22	0.78
	12	8.00	485	< 30	16.88	< 0.1
None (Control)	0	0.17	< 50	381	0.39	18.38
	0	0.50	< 50	336	0.40	18.04
	0	2.00	< 50	381	0.36	17.88
	0	8.00	< 50	360	0.40	18.57
Bifidobacterium sp.	2	0.17	69	298	3.65	14.03
	2	0.50	129	285	5.68	12.37
	2	2.00	313	102	12.42	3.04
	2	8.00	427	< 30	14.67	< 0.1
	2	24.17	425	< 30	14.30	< 0.1
Bifidobacterium longum	2	0.17	105	286	4.38	12.34
	2	0.50	210	197	8.77	7.88
	2	2.00	374	49	13.90	0.55
	2	8.00	446	< 30	15.64	< 0.1
	2	24.17	419	< 30	14.74	< 0.1
None (Control)	0	0.17	< 50	341	0.38	17.21
	0	0.50	< 50	372	0.39	18.55
	0	2.00	< 50	383	0.37	17.62
	0	8.00	< 50	413	0.41	18.82
	0	24.17	< 50	398	0.40	18.40

6.6. Raw data wrt repeatability hydrolysis of 2-FLol in relevant time by GH95 fucosidase from *B. longum*.

Table II: Concentrations of different components during duplicate hydrolysis of 2FLol and 2-FL with/ without the addition of fucosidase from *Bifidobacterium longum* at pH 6.5 and 37°C.

Fucosidase from source	Dosage (µg /ml)	Time (hours)	Lactitol µg/mL	2FLol µg/mL	Lactose mg/mL	2-FL mg/mL
Bifidobacterium longum	2	0.17	111	519	5.9	16
	2	0.5	236	401	11	8.5
	2	1	356	261	15	3.3
	2	2	460	134	18	0.5
	2	4	460	73	19	< 0.4
	2	8	470	< 50	19	< 0.4
None (Control)	0	0.17	< 50	515	0.4	24
	0	0.5	< 50	532	0.4	26
	0	1	< 50	526	0.3	24
	0	2	< 50	516	0.4	25
	0	4	< 50	524	0.5	25
	0	8	< 50	500	0.4	26
Bifidobacterium longum	2	0.17	110	503	6.0	17
	2	0.5	255	415	13	9.5
	2	1	332	259	15	3.2
	2	2	447	145	18	0.4
	2	4	440	77	19	< 0.4
	2	8	486	< 50	21	< 0.4
None (Control)	0	0.17	< 50	489	0.4	23
	0	0.5	< 50	522	0.4	24
	0	1	< 50	486	0.3	21
	0	2	< 50	511	0.4	23
	0	4	< 50	503	0.4	23
	0	8	< 50	541	0.4	25

6.7. Example Chromatograms

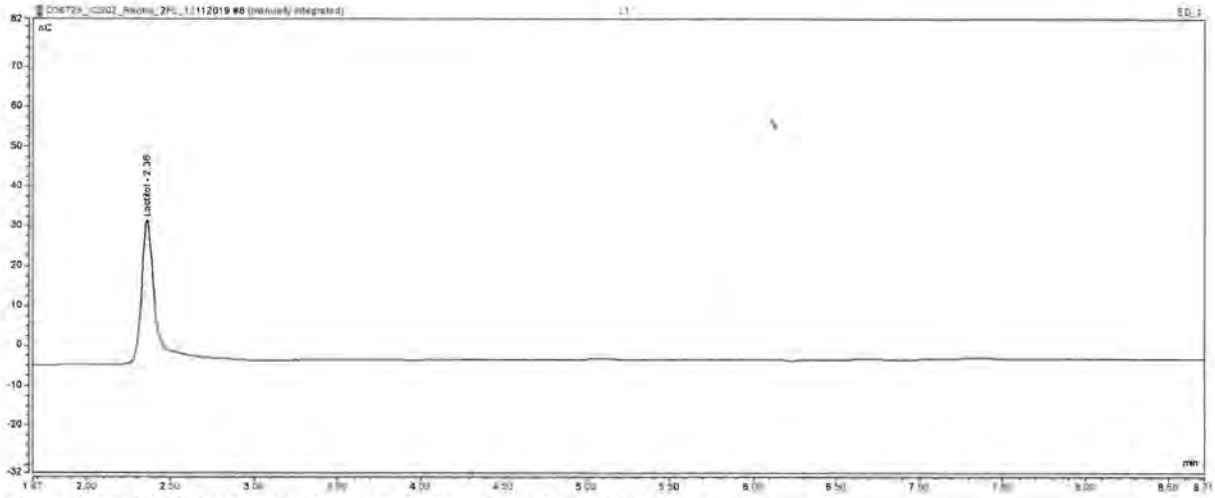


Figure A: HPAEC-PAD chromatogram for the used lactitol standard

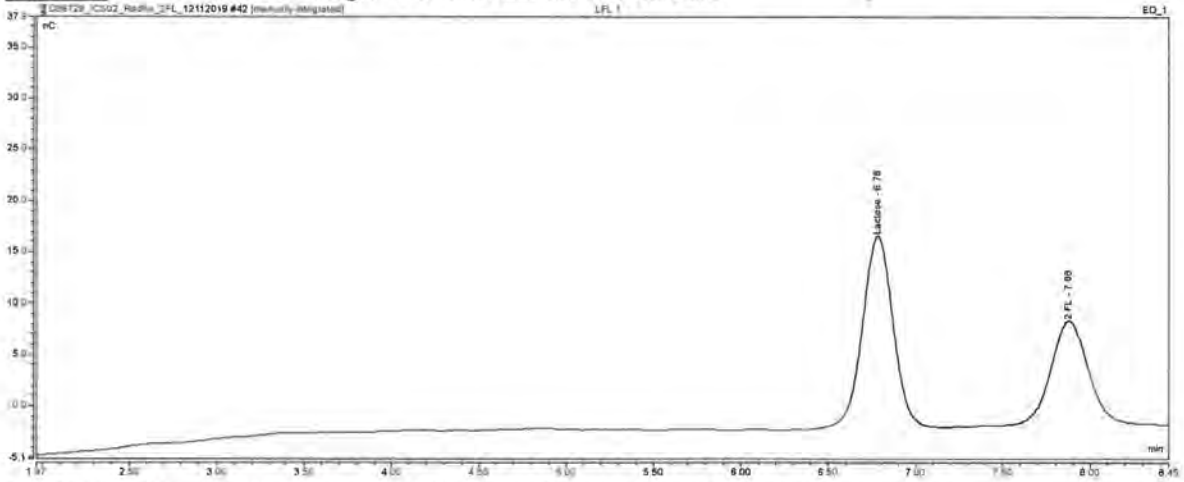


Figure B: HPAEC-PAD chromatogram for the used lactose and 2-fucosyllactose standard

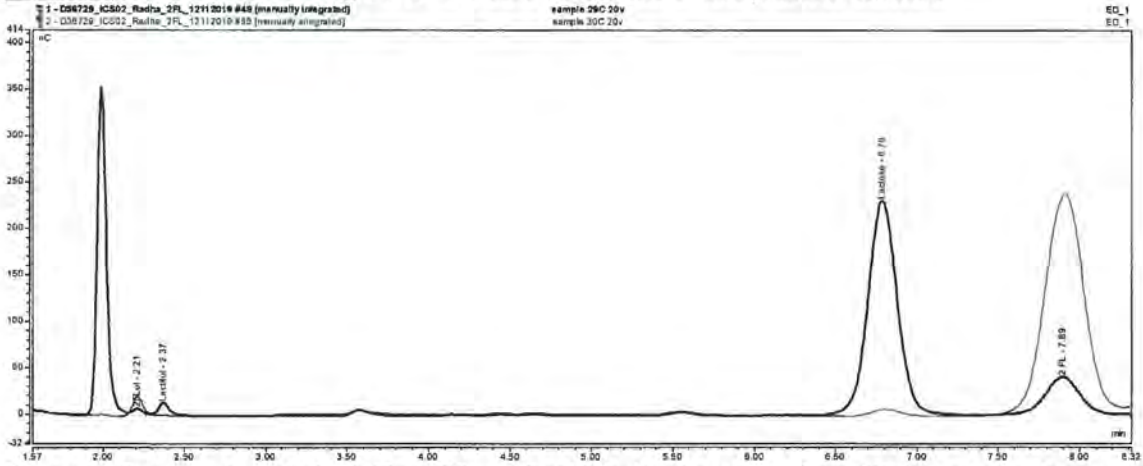


Figure C: One-hour hydrolysis of 47mM 2FL and 2mM 2FLol by fucosidase from *Bifidobacterium longum*, dosed at 2 µg/ml, to lactose and lactitol in sodium phosphate buffer pH 6.5 and 37°C (black line). One-hour incubation of 47mM 2FL and 2mM 2FLol without added enzyme (control; blue line).

6.8. Booklet 1,2-α-L-fucosidase from Megazyme

Alpha Fucosidase from Megazyme.pdf

APPENDIX J
CONSENSUS REPORT OF THE GRAS PANEL

Consensus Statement of the GRAS Panel on the Generally Recognized as Safe Status of the Proposed Uses of Amyris's 2'-Fucosyllactose

INTRODUCTION

The undersigned, an independent panel of experts, qualified by their scientific training and national and international experience to evaluate the safety of food and food ingredients (the GRAS Panel), was specially convened by Amyris, Inc., to conduct a critical and comprehensive evaluation of the available pertinent data and information, and to determine whether under the conditions of intended use as a food ingredient Amyris's 2'-Fucosyllactose (2'-FL), produced using a genetically engineered strain of *Saccharomyces cerevisiae* is safe and "generally recognized as safe" (GRAS) based on scientific procedures. For purposes of this evaluation, "safe" or "safety" as it relates to GRAS within the terms of the Federal Food, Drug, and Cosmetic Act means that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2012a).

Amyris, Inc. performed a comprehensive search of the literature relating to the safety of 2'-Fucosyllactose (2'-FL). Amyris summarized the results of the literature search and prepared a dossier, "**Safety Assessment and Generally Recognized as Safe (GRAS) Notification of 2'-Fucosyllactose (2'-FL) for Use as an Ingredient in Foods**" for independent consideration and review by the GRAS Panel.

The GRAS Panel consisted of the following individuals: Joseph F. Borzelleca, PhD, Emeritus Professor Virginia Commonwealth University School of Medicine; Roger A. Clemens, DrPH, PolyScience Consulting and Adjunct Professor, University of Southern California School of Pharmacy; and Stanley M. Tarka, Jr., PhD, ATS, (The Pennsylvania State University College of Medicine, Tarka Group, Inc). The GRAS Panel critically evaluated the safety documentation (the dossier), and other available data and information the members of the GRAS Panel believed to be pertinent to the safety of Amyris's 2-FL and its intended use as an ingredient in specified foods.

Following its independent and collective critical evaluation of the available information, the GRAS panel, convened on April 24 and May 5, 2020. Following its deliberations, the GRAS panel unanimously agreed to the conclusions described herein. A summary of the basis for these conclusions follows.

AMYRIS'S 2'-FL: MANUFACTURING PROCESS AND PRODUCT SPECIFICATIONS

The substance in this GRAS determination is 2'-Fucosyllactose (2'-FL) produced using a genetically engineered strain of *Saccharomyces cerevisiae* (strain CEN.PK113-7D). *S. cerevisiae*, also known as brewer's yeast or baker's yeast, has an extensive history of safe use in the food industry (21 CFR §172.896, 21 CFR §172.325 21 CFR §172.898, 21 CFR §184.1983). *S. cerevisiae* also has been granted Qualified Presumption of Safety (QPS) status in the European Union by the European Food Safety Authority (EFSA)[1] and, therefore, is considered safe for the derivation of genetically modified strain lineages intended for use in the production of food additives and enzymes. In Amyris's production strain, the DNA construct was inserted by homologous recombination, and the introduced genetic elements are stable. The production strain is not toxigenic or pathogenic, and does not contain or produce any known pathogenicity-related proteins, toxins, allergens, pyrogens, or antibiotic resistance markers. The manufacturing process consists of a fermentation process where food-grade sugar, lactose, and fermentation nutrients are fed to a culture of the production strain and fermented to produce 2'-FL and other carbohydrates. The fermentation process is conducted under strictly controlled temperature and pH conditions with appropriate heat treatment and purification steps. After fermentation, the 2'-FL

[1] Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Girones, R., ... & Robertson, L. (2017). Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. *EFSA Journal*, *15*(3). https://doi.org/10.2903/j.efsa.2017.4664

fermentation broth is separated from the aqueous phase by centrifugation. Any residual yeast is removed though separation and purification steps, and residual solids, proteins, DNA, salts, and organic acids are removed through various filtration steps. Chromatographic and polishing steps then remove any additional salts, metals, proteins, organic acids, and colorants. The 2'-FL then goes through additional filtration, evaporation, heat treatment, and sterilization steps before the concentrated product is spray-dried to reduce moisture to ≤ 5.0 % by weight (w/w). All processing aids in the post-fermentation process are approved for use in food processing as noted in the dossier.

Amyris's 2'-FL has a molecular weight of 488.44 AMU and conforms to 2'-FL structure (CAS# 41263-94-9). A comparison of the proton NMR spectra for Carbosynth 2'-FL (reference standard) and the Amyris 2'-FL demonstrate structural equivalence (Appendix A of dossier). Amyris's 2'-FL was not evaluated in any toxicology studies; however, numerous other sources of 2'-FL (Tables 11a and 11b in the dossier) were test materials for toxicity studies. These studies are evaluated and summarized in the dossier. To ensure that the toxicology studies are appropriate for evaluating Amyris's 2'-FL, the profiles of the 2'-FL substances (i.e., the distribution of non-2'-FL carbohydrates) were compared and found to be sufficiently similar to base safety conclusions on the results of these studies.

To ensure that a consistent food-grade ingredient is produced, Amyris has established specifications for their 2'-FL. The chemical, physical and microbiological specifications are presented in **Table 1**. Batches were analyzed for conformity to the established specifications and all batches meet specifications.

Table 1: Specifications of Amyris's 2'-Fucosyllactose

Parameter	Specification	Method
Carbohydrate content (% area)		
2'-fucosyllactose	≥ 86% area	Ion chromatography (Amyris SOP 830)
Difucosyllactose (DFL)	< 8% area	
Lactose/allo-lactose	< 7% area	
2'-fucosyllactitol	≤ 6% area	
3-Fucosyllactose (3FL)	< 7% area	
Fucosyl-galactose		
Xylitol		
Dulcitol/sorbitol		
Glucose/Galactose		
Fucose		
Glycerophosphoethanolamine (GPE)		
Fructose		
Chemical		
Water Content (KF titration)	≤5.0% w/w	Karl Fischer titration (Amyris SOP 842)
pH (20 °C, 5% solution)	3.0 – 7.5	EP 2.2.3 v9
Protein Content	≤0.01% w/w	Modified Bradford Assay (Amyris SOP 843)
Total Ash	≤0.5% w/w	FCC 11 appendix II
Arsenic	≤ 0.2 mg/kg	EP 2.2.58 v9
Cadmium	≤ 0.05 mg/kg	EP 2.2.58 v9
Lead	≤ 0.05 mg/kg	EP 2.2.58 v9
Mercury	≤ 0.1 mg/kg	EP 2.2.58 v9
GMO detection (rDNA from production strain)	Negative	PCR (Amyris SOP 844)

Table 1: Specifications of Amyris's 2'-Fucosyllactose		
Parameter	**Specification**	**Method**
Microbial Specifications		
Total Aerobic Microbial Count/Standard Plate Count	≤ 1000 cfu/g	EP 2.6.12 v9
Total Yeast/Mold Count	≤ 100 cfu/g	EP 2.6.12 v9
Sulfite Reducing Bacteria	< 100 cfu/g	ISO 15213: 2003
Enterobacteriaceae	Negative in 10 g	EP 2.6.13 v9
Salmonella	Not detected in 25 g	EP 2.6.13 v9
Cronobacter sakazakii	Not detected in 10 g	ISO/TS 22964
Coliforms	Not detected in 10 g	ISO 4831: 2006
E. coli	Absent in 10 g	EP 2.6.13 v9
Listeria monocytogenes	Absent in 10 g	ISO 11290-1: 2017
Pseudomonas aeruginosa	Absent in 10 g	EP 2.6.13 v9
Staphylococcus aureus	Negative in 10 g	EP 2.6.13 v9
Bacillus cereus	< 100 cfu/g	ISO 7932: 2004
Abbreviations: °C = degrees Celsius; cfu = colony-forming unit; EP = European Pharmacopoeia; EU = endotoxin units; FCC = Food Chemicals Codex; g = grams; ISO = International Organization for Standardization; KF = Karl Fischer; m = milli; SOP = Standard Operating Procedure; w/w = by weight; v = version.		

In addition to batch testing, batches were analyzed in an accelerated mode stability study (13 weeks, 40°C, 75% Relative Humidity (RH)). Data from this study confirmed that Amyris 2'-FL powder is stable for 1.5 years (Appendix F in the dossier).

USES AND EXPOSURES

History of Use and Exposure

Approximately 85% of the world's population is exposed to 2'-FL from human milk. Human milk oligosaccharides (HMOs) are the third largest component of breast milk solids after lactose and lipids and 2-'FL is the most abundant HMO in human breast milk.[2] Most infants have been exposed to 2'-FL because it is a naturally occurring component of human breast milk, synthesized in the mammary glands of secretor mothers.[3] Even infants fed breast milk from non-secretor mothers excrete 2'-FL in the urine and in the stool, indicating the infant can produce alpha--1,2-epitope containing glycans.[4] The mean

[2] Coppa GV, Bruni S, Morelli L, Soldi S, Gabrielli O (2004) The first prebiotics in humans: human milk oligosaccharides. J Clin Gastroenterol 38(6 Suppl) S80-83. https://doi.org/10.1097/01.mcg.0000128926.14285.25.

[3] Castanys-Munoz E, Martin MJ, Prieto PA (2013) 2'-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. Nutr Rev 71(12) 773-789. https://doi.org/10.1111/nure.12079

[4] Kunz C, Rudloff S (2017) Compositional analysis and metabolism of human milk oligosaccharides in infants. Intestinal microbiome: functional aspects in health and disease 88 137-148. Karger Publishers. https://doi.org/10.1159/000455398.

concentration of 2'-FL in human breast milk ranges from 1.1 g/L[5,6] to 4.26 g/L,[7] with levels up to 7.3 g/L reported.[8]

Proposed Uses and Estimated Daily Intakes

The intended uses and maximum use levels of Amyris's 2'-FL were presented in one or more 2'-FL GRNs. Amyris intends to use its 2'-FL as a food ingredient in term infant formulas (non-exempt), toddler formulas (12-36 months) at a maximum level of 2.4 g 2'-FL per liter. Amyris also intends to use its 2'-FL in baby foods for infants and young children (children older than one year of age) and beverages for young children. Other uses in infant and toddler food and beverage products include processed cereals, infant meal replacement products, ready-to-eat hot cereals, yogurts and drinks, desserts such as fruit desserts and cobblers, snack crackers and cookies, milk modifiers, and milk-based drinks. Amyris is proposing to use its 2'-FL in conventional foods and beverages intended for children and adults such as in milk substitutes, flavoring in milk-based beverages such as coffees and smoothies, frozen dairy desserts such as ice cream and frozen yogurt, fruit pie fillings, fruit preserve products, meal replacement beverages and meal replacement bars, breakfast bars, cereal products (hot and ready-to-eat), energy drinks, sports drinks, and fruit drinks/juices. Amyris also proposes to use its 2'-FL as an ingredient in medical foods as a component of oral nutritional supplements for enteral feeding for ages 11 years and older. Specific food uses and use levels are presented in **Table 2.**

Table 2. Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the U.S.

Food Category	Proposed Food Use	RACC[a]	Proposed Use Level (g/RACC or g/serving)	Proposed Maximum Use level (g/kg or g/L)[b]
Dairy	Unflavored Pasteurized and Sterilized milk	240 mL	0.28	1.2
	Buttermilk	240 mL	0.28	1.2
	Yogurt	225 g	1.2	5.3
	All acidophilus or fortified milks, non-fat and low-fat milk fluids, including fluid milk and reconstituted milk powder	240 mL	0.28	1.2
	Flavored milks, including chocolate milk, coffee drinks, cocoa, smoothies (dairy and fruit based), other fruit and dairy combinations, yogurt drinks and fermented milk drinks including kefir	240 mL	0.28	1.2
	Frozen dairy desserts including ice cream and frozen yogurts, frozen novelties	~70 g	1.2	17
	Milk product for pregnant women ("mum formulas") -9 to 0 months	200 mL	1.2	6.0
Dairy analogs	Milk substitutes such as soymilk and imitation milks	240 mL	0.28	1.4

[5] McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, Kamau-Mbuthia EW, Kamundia EW, Mbugua S, Moore SE, Prentice AM (2017) What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. The American journal of clinical nutrition 105(5):1086-100. https://doi.org/10.3945/ajcn.116.139980.

[6] Bao, Y C. Chen, D. S. Newburg, *Anal. Biochem.* 2013, *433* (1), 28-35. *Quantification of neutral human milk* oligosaccharides *by graphitic carbon HPLC with tandem mass spectrometry.* https://doi.org/10.1016/j.ab.2012.10.003.

[7] Galeotti, F., Coppa, G. V., Zampini, L., Maccari, F., Galeazzi, T., Padella, L., ... & Volpi, N. (2014). Capillary electrophoresis separation of human milk neutral and acidic oligosaccharides derivatized with 2-aminoacridone. *Electrophoresis*, *35*(6), 811-818. https://doi.org/10.1002/elps.201300490.

[8] Gabrielli, O., Zampini, L., Galeazzi, T., Padella, L., Santoro, L., Peila, C., ... & Coppa, G. V. (2011). Preterm milk oligosaccharides during the first month of lactation. *Pediatrics*, *128*(6), e1520-e1531. https://doi.org/10.1542/peds.2011-1206.

Table 2. Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the U.S.				
Food Category	**Proposed Food Use**	**RACC[a]**	**Proposed Use Level (g/RACC or g/serving)**	**Proposed Maximum Use level (g/kg or g/L)[b]**
	Non-dairy yogurt	225 g	1.2	5.3
Other	Syrups used to flavor milk beverages	40 g	0.28	7.0
	Dairy based pudding custards and mousses	~70 g	1.2	17
	Fruit pie filling	85 g	1.2	14.1
	Fruit preparation such as fruit filing in bars, cookies, yogurt and cakes	~40 g	1.2	30
	Jellies and jams, fruit preserved and fruit butters	~20 g	1.2	60
Infant formulas, Follow-on formula, and baby foods	Infant formula (non-exempt formula)	100 mL	0.24	2.4
	Toddler formulas, growing-up milks (12-36 months)	100 mL	0.24	2.4
	Processed cereal-based food and baby food for infants and young children	7 to 170 g	0.084 to 2.04	12
	Other Foods for Infants and Young Children	7 to 170 g	0.084 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2
	Infant meal replacement products	100 mL	0.24	2.4 (0.4 g/100kcal)
	Ready-to-eat, ready-to-serve, hot cereals	15 g (dry) 110 g (ready-to-serve)	1.2	10.9 (as consumed)
	Yogurt and juice beverages ("baby drinks")	120 mL	1.2	10
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type dessert")	110 g or mL	1.2	10.9
	Baby crackers, pretzels, cookies and snacks items	7 g	0.4	57
	Milk-based drinks and similar products intended for young children	120 mL	0.14	1.2
	Milk modifiers (i.e. powder for addition in milk such as cacao-based powders, etc.)	120 mL (ready to serve)	0.14	1.2
Medical Foods	Oral nutritional supplements and enteral tube feeding (11 years and older)	200 g or mL	4.0	20
Meal substitutes	Milk-based meal replacement beverages or diet beverages / meal replacement drinks for weight reduction (milk-based and non-milk-based)	240 mL	1.2	5.0
	Meal replacement bars for weight reduction	30g	1.2	40
Grain products	Grain bars, including snack bars, meal replacement bars, and breakfast bars	40g	0.48	12
Breakfast cereals	Ready-to-eat breakfast cereals for adults and children - puffed	15g	1.2	80
	Ready-to-eat breakfast cereals for adults and children – high-fiber	40g	1.2	30

Table 2. Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the U.S.				
Food Category	**Proposed Food Use**	**RACC[a]**	**Proposed Use Level (g/RACC or g/serving)**	**Proposed Maximum Use level (g/kg or g/L)[b]**
	Ready-to-eat breakfast cereals for adults and children – biscuit types	60g	1.2	20
	Hot cereals for adults and children	40g (dry) (~240g prepared)	1.2	4.8 (as consumed)
Beverage	Flavored drinks	360 mL	0.28	0.8
	Energy drinks	360 mL	0.28	0.8
	Fitness and thirst quenchers, sport and isotonic drinks / sport, isotonic drinks	360 mL	0.28	0.8
	Fruit drink, including vitamin and mineral-fortified products	240 mL	0.28	1.2
	Fruit juices / fruit juices and nectar	240 mL	0.28	1.2

[a] RACC = Reference Amounts Customarily Consumed per Eating Occasion in the U.S. Code of Federal Regulations, 2018 (21 CFR 101.12).

[b] Proposed maximum use level is presented on g/kg basis for solids, and g/L basis for liquids and forms. The basis for the calculation of Estimated Daily Intake is presented in Tables 3a and 3b.

The estimated daily intake (EDI) of Amyris's 2'-FL was estimated using food consumption data reported in the United States Department of Health and Human Service's 2013-2016 National Health and Nutrition Examination Surveys (NHANES), is presented in **Tables 3a and 3b**.

Table 3a: Summary of the Estimated Daily Intake of 2'-FL from All Proposed Food and Beverage Uses in the U.S. by Population Group (2013-2016 NHANES Data)					
Population Group	**Age Group**	**All-Users Consumption (g/day)**			
		% Users	**N**	**Mean**	**95th Percentile**
Infants	0-6 mo.	100	241	2.73	5.87
Infants	7-12 mo.	99.66	228	3.82	8.43
Toddlers	1 to 3 yr.	98.77	1117	2.30	5.49
Children	4 to 10 yr.	99.34	2315	2.61	7.08
Male Teenager	11-18 yr.	99.49	1213	3.12	8.99
Female Teenager	11-18 yr.	99.13	1216	2.36	7.28
Female Adults of childbearing age	19-40 yr.	99.30	1807	1.78	5.79
Female Adults	19-64 yr.	99.49	3767	1.72	5.45
Male Adults	19-64 yr.	99.30	3313	2.38	7.84
Elderly Adults	65 yr. and up	98.95	1215	2.25	6.30
Abbreviations: 2'-FL = 2'-fucosyllactose; g = grams; mo. = months; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; yr. = years					

Table 3b: Summary of the Estimated Daily Intakes of 2'-FL per Kilogram Body Weight from All Proposed Food and Beverage Uses in the U.S. by Population Group (2013-2016 NHANES Data)							
		Mean	95th Percentile	% Users	N	Mean	95th Percentile
Infants	0-6 mo.	6.8	8.9	100	241	0.40	0.82
Infants	7-12 mo.	9.3	11.3	99.6	227	0.42	0.88
Toddlers	1 to 3 yr.	13.8	18.5	98.77	1103	0.18	0.45
Children	4 to 10 yr.	28.9	48.5	99.34	2303	0.10	0.29
Male Teenager	11-18 yr.	60.4	94.5	99.49	1210	0.054	0.18
Female Teenager	11-18 yr.	65.6	106.0	99.13	1205	0.043	0.15
Female Adults of childbearing age	19-40 yr.	76.5	120.2	99.30	1791	0.025	0.082
Female Adults	19-64 yr.	78.2	120.3	99.49	3739	0.024	0.076
Male Adults	19-64 yr.	89.6	130.3	99.30	3295	0.028	0.093
Elderly Adults	65 yr. and up	79.5	113.9	98.95	2283	0.029	0.086
Abbreviations: 2'-FL = 2'-fucosyllactose; bw = body weight; g = grams; k = kilo; mo. = months; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; yr. = years							

SAFETY ASSESSMENT

The addition of Amyris's 2'-FL in infant formula will enable the infant formula to more closely approximate the composition of human milk. 2'-FL as an ingredient in infant formula and general population uses have been reviewed by the FDA in at least six GRNs that all received letters of no objection from FDA.[9]

Absorption, distribution, metabolism and excretion of 2'-FL

It has been consistently demonstrated in studies specifically evaluating ADME of infant formula oligosaccharides that HMOs are not readily absorbed by infants and arrive intact in the colon where they are metabolized by resident microbiota and/or excreted in the feces.[10,11] Studies report that 40 to 50% of the 2'-FL in breast milk consumed by infants was reported unchanged in fecal samples.[12,13,14] Less than 5% of 2′-FL and other HMOs consumed by infants were absorbed from the GI tract and most were

[9] GRNs 546, 571, 650, 735, 749, 852

[10] Engfer MB, Stahl B, Finke B, Sawatzki G, Daniel H (2000) Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr 71(6) 1589-1596. https://doi.org/10.1093/ajcn/71.6.1589.

[11] Gnoth MJ, Kunz C, Kinne-Saffran E, Rudloff S (2000) Human milk oligosaccharides are minimally digested in vitro. J Nutr 130(12) 3014-3020. https://doi.org/10.1093/jn/130.12.3014.

[12] Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, Newburg DS (2001) Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology 11(5) 365-372. https://doi.org/10.1093/glycob/11.5.365.

[13] Coppa, G. V., Pierani, P., Zampini, L., Bruni, S., Carloni, I., & Gabrielli, O. (2001). Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. In *Bioactive components of human milk* (pp. 307-314). Springer, Boston, MA. https://doi.org/10.1007/978-1-4615-1371-1_38.

[14] Albrecht S, Schols HA, van Zoeren D, van Lingen RA, Groot Jebbink LJ, van den Heuvel EG, Voragen AG, Gruppen H (2011) Oligosaccharides in feces of breast- and formula-fed babies. Carbohydr Res 346(14) 2173-2181. https://doi.org/10.1016/j.carres.2011.06.034.

detected intact in the large intestine where they are subjected to partial fermentation by indigenous microbiota.[15] The unabsorbed 2'-FL is metabolized by gut microbiota to short-chain fatty acids.

Toxicological and Clinical Studies

The toxicological and clinical studies evaluating the safety and tolerance of 2'-FL support the determination that 2'-FL is safe for its intended food uses and proposed use levels. The toxicological studies (Tables 11a and 11b in the dossier) and clinical studies (Tables 11c and 11d in the dossier) are summarized and discussed below.

The toxicology studies were performed on 2'-FL produced either by microbial fermentation or chemical synthesis by various technologies. Although Amyris's 2'-FL was not the test substance evaluated, the other 2'-FLs that were evaluated are appropriate for evaluating Amyris's 2'-FL because the profiles of 2'-FL and the associated substances, i.e., the non-2'-FL carbohydrates (Table 5b in the dossier), were sufficiently similar to base safety interpretations on the results of these studies. This substantial chemical equivalency supports bridging to the published studies.

In vitro assays for genotoxicity and mutagenicity tests on other sources of 2'-FL suggest that 2'-FL produced either by chemical synthesis or microbial fermentation is not mutagenic and is not genotoxic. Using concentrations up to 5000 µg/plate of 2'-FL, with and without metabolic activation, there were no signs of cytotoxicity and no increase in revertant colony numbers reported in any of the test strains when compared to control counts.[16, 17, 18, 19, 20, 21]

Repeated dose toxicity studies demonstrated that 2'-FL does not induce toxic effects after ingestion by rats for 90 days. The studies evaluated the safety of 2'-FL produced via microbial fermentation or chemical synthesis administered by gavage or as a dietary admixture to rats. Subchronic oral toxicity studies in which 2'-FL was administered by gavage at doses of 0, 1000, 2000, and 5000 mg

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[15] Brand-Miller , J. C., McVeagh, P., McNeil, Y. & Messer, M. 1998. Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. *J. Pediatr.,* 133, 95-98. https://doi.org/10.1016/s0022-3476(98)70185-4.

[16] Verspeek-rip, M. (2015). Evaluation of the Mutagenic Activity of 2'FL in the Salmonella Typhimurium Reverse Mutation Assay and the Eshcerichia Coli Reverse Mutation Assay. (Laboratory Project Identification: Project 507432; Substance 206374/B). Prepared by DD 's Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S. (discussed in GRN 650)

[17] Verbaan, A.J. (2015a). An In Vitro Micronucleus Assay with 2'-0-Fucosyllactose In Cultured Peripheral Human Lymphocytes: Confidential. (Laboratory Project Identification: Project 507398; Substance 206096/A). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom *Al* S (discussed in GRN 650)

[18] Verbaan, A.J. (2015b). An In Vitro Micronucleus Assay with 2'FL in Cultured Peripheral Human Lymphocytes. (Laboratory Project Identification: Project 507433; Substance 206374/B). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S (discussed in GRN 650).

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[20] Phipps KR, Baldwin N, Lynch B, Flaxmer J, Šoltésová A, Gilby B, Mikš MH, Röhrig CH (2018). Safety evaluation of a mixture of the human-identical milk oligosaccharides 2′-fucosyllactose and difucosyllactose. Food and chemical toxicology. 120:552-65. https://doi.org/10.1016/j.fct.2018.07.054.

[21] van Berlo D, Wallinga AE, van Acker FA, Delsing DJ (2018) Safety assessment of biotechnologically produced 2′-Fucosyllactose, a novel food additive. Food and Chemical Toxicology. 118:84-93. https://doi.org/10.1016/j.fct.2018.04.049.

2'-FL/kg·bw/day in juvenile rats (strain Crl:WI(Han) or Crl:CD(SD)) reported a NOAEL of 5000 mg/kg·bw/day, the highest dose tested.[22,23,24]

In a 90-day oral (feeding) toxicity study in juvenile rats (strain Crl:WI(Han)), the mean intakes of 2'-FL were 0, 2.17, 4.27, and 7.25 g/kg·bw/day 2'-FL, and 0, 2.45, 5.22, and 7.76 g/kg·bw/day in male and female juvenile rats respectively. A NOAEL of 7.76 g/kg·bw/day 2'-FL was reported by the authors.[25] The safety of 2'-FL was evaluated in a 21-day repeated dosing study in neonatal pigs and no observed adverse effects were reported at 2'-FL concentrations of 0, 200, 500, and 2000 mg 2'-FL/L/day for 21 days. These doses are equivalent to 29.37, 72.22 and 291.74 mg/kg/day in males and 29.30, 74.31, and 298.99 mg/kg/day in females.[26]

Four clinical studies evaluated the growth and tolerance of infants fed formulas containing 2'-FL and other oligosaccharides. The studies evaluated concentrations at 0.2 to 1.2 g 2'-FL/L formula and the combined effects of 2'-FL with GOS,[27] short-chain fructo-oligosaccharides (scFOS),[28] LNnT,[29] or whey.[30] No adverse effects or alterations in growth were reported.

A clinical study evaluated the tolerability of 2'-FL administered at doses of 5, 10, and 20 g per day 2'-FL, LNnT, or 2'-FL with LNnT (2:1 mass ratio) for 14 days in adults ages 19 to 57 years.[31] Participants receiving the highest dose of 20 g/day 2'-FL and LNnT (2:1 mass ratio) reported significantly higher occurrence of bloating and gas compared to baseline, and those receiving 20 g/day 2'-FL reported softer stools as compared to baseline. The authors determined these effects were clinically irrelevant, however, these observations may suggest there is a tolerance limit of 20 g 2'-FL/day.

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[23] Coulet M, Phothirath P, Allais L, Schilter B (2014) Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-Fucosyllactose (2'-FL). Regulatory Toxicology and Pharmacology 68(1) 59-69. https://doi.org/10.1016/j.yrtph.2013.11.005.

[24] Phipps KR, Baldwin N, Lynch B, Flaxmer J, Šoltésová A, Gilby B, Mikš MH, Röhrig CH (2018). Safety evaluation of a mixture of the human-identical milk oligosaccharides 2′-fucosyllactose and difucosyllactose. Food and chemical toxicology. 120:552-65. https://doi.org/10.1016/j.fct.2018.07.054.

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[27] Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA (2015) Infants fed a lower calorie formula with 2′ FL show growth and 2′ FL uptake like breast-fed infants. Journal of pediatric gastroenterology and nutrition. 61(6):649. https://doi.org/10.1097/mpg.0000000000000889.

[28] Reverri EJ, Devitt AA, Kajzer JA, Baggs GE, Borschel MW (2018) Review of the clinical experiences of feeding infants formula containing the human milk oligosaccharide 2′-fucosyllactose. Nutrients. 10(10):1346. https://doi.org/10.3390/nu10101346.

[29] Puccio G, Alliet P, Cajozzo C, Janssens E, Corsello G, Sprenger N, Wernimont S, Egli D, Gosoniu L, Steenhout P (2017) Effects of infant formula with human milk oligosaccharides on growth and morbidity: a randomized multicenter trial. Journal of pediatric gastroenterology and nutrition. 64(4):624. https://doi.org/10.1097/mpg.0000000000001520.

[30] Storm HM, Shepard J, Czerkies LM, Kineman B, Cohen SS, Reichert H, Carvalho R (2019) 2′-Fucosyllactose Is Well Tolerated in a 100% Whey, Partially Hydrolyzed Infant Formula with Bifidobacterium lactis: A Randomized Controlled Trial. Global pediatric health. https://doi.org/10.1177/2333794x19833995.

[31] Elison E, Vigsnaes LK, Krogsgaard LR, Rasmussen J, Sørensen N, McConnell B, Hennet T, Sommer MO, Bytzer P (2016) Oral supplementation of healthy adults with 2′-Ofucosyllactose and lacto-N-neotetraose is well tolerated and shifts the intestinal microbiota. British Journal of Nutrition. 116(8):1356-68. https://doi.org/10.1017/s0007114516003354.

Comparison of Safe Intake Levels to Proposed Intake Levels

Based on Amyris's proposed uses of 2'-FL in infant formula at up to 2.4 g/L, the mean EDI for an infant 0 to 6 months of age (6.8 kg average weight; approximately 1 L/day) is 400 mg/kg·bw/day and an infant 7 to 12 months of age (9.3 kg average weight; approximately 1.5 L/day) is 420 mg/kg·bw/day. For comparison, background levels of intake of 2'-FL as consumed in human milk based on human secretor mothers' milk ranges from 1.1 to 4.26 g/L.[32,33] At an intake of 1.1 to 4.26 g/L for a 6.8 kg weight infant (0 to 6 months of age), this results in consumption of 162 to 627 mg/kg·bw/day. At an intake of 1.1 to 4.26 g/L for an 9.3 kg weight infant (7 to 12 months of age), this results in consumption of 118 to 458 mg/kg·bw/day. Thus, the highest consumption of 2'-FL for the proposed infant formula use does not exceed the range of consumption of 2'-FL in human breastmilk for breastfed infants. These proposed uses and intake levels are consistent with other safety assessments by authoritative bodies: as presented in other 2'-FL GRNs which received no-questions letters from FDA; as presented in EFSA opinions for 2'-FL as a novel food in 2019.[34]

All toddler, children, and adult intakes based on the proposed uses are well within the range of background intakes for infants described above. As presented in Table 3b, the 95th percentile EDI for toddlers is 450 mg/kg·bw/day, for children is 290 mg/kg·bw/day, for male teenagers is 180 mg/kg·bw/day, for female teenagers is 150 mg/kg·bw/day, for female adults of child-bearing age is 82 mg/kg·bw/day, for female adults is 76 mg/kg·bw/day, for male adults is 93 mg/kg·bw/day, and elderly adults is 86 mg/kg·bw/day (age ranges, body weights, and other information are provided in Table 3b). These 95th percentile EDIs for children, toddlers, and adults do not exceed the range of intakes on a per kg bw basis in breastfed infants. Because the intake of 2'-FL from Amyris's intended uses at the proposed use levels is unlikely to exceed the intake level of naturally occurring 2'-FL in breastfed infants per kilogram body weight, the GRAS Panel concluded that the specified uses and use levels of Amyris's 2'-FL are suitable, safe, and GRAS.

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[32] Bao, Y C. Chen, D. S. Newburg, *Anal. Biochem.* 2013, *433* (1), 28-35. *Quantification of neutral human milk* oligosaccharides *by graphitic carbon HPLC with tandem mass spectrometry.* https://doi.org/10.1016/j.ab.2012.10.003.

[33] Galeotti, F., Coppa, G. V., Zampini, L., Maccari, F., Galeazzi, T., Padella, L., ... & Volpi, N. (2014). Capillary electrophoresis separation of human milk neutral and acidic oligosaccharides derivatized with 2-aminoacridone. *Electrophoresis*, *35*(6), 811-818. https://doi.org/10.1002/elps.201300490.

[34] EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA), Turck, D., Castenmiller, J., De Henauw, S., Hirsch-Ernst, K. I., Kearney, J., ... & Pelaez, C. (2019). Safety of 2'-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283. *EFSA Journal*, *17*(6), e05717. https://doi.org/10.2903/j.efsa.2019.5717

CONCLUSIONS

We, the undersigned independent qualified members of the GRAS Panel, have individually and collectively, critically evaluated the data and information summarized above, and other data and information that we deemed appropriate to the safety of the intended use of Amyris's 2'-FL in specified foods. We unanimously conclude that the proposed uses and use levels of Amyris's 2'-FL produced via fermentation using a genetically engineered strain of *Saccharomyces cerevisiae* (as presented in the GRAS dossier and summarized herein and based on parental strain CEN.PK113-7D) in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate established specifications as presented in the supporting dossier *["Safety Assessment and Generally Recognized as Safe (GRAS) Notification of 2'-Fucosyllactose (2'-FL) for Use as an Ingredient in Foods"]* is safe.

We further unanimously conclude that the proposed uses and use levels as an ingredient in specified foods of Amyris's 2'-FL, produced via fermentation using a genetically engineered strain of *Saccharomyces cerevisiae* in a manner that is consistent with current cGMP and meeting the food grade specifications as presented in the supporting dossier are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other experts, qualified by scientific training and experience to evaluate the safety of food and food ingredients, and evaluating the same data and information, would concur with these conclusions.

Joseph F. Borzelleca, PhD
Professor Emeritus of Pharmacology and Toxicology
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signature: [redacted]

Date:

Roger A Clemens, DrPH
Co-founder and Executive Vice President
PolyScience Consulting
Adjunct Professor, Pharmacology & Pharmaceutical Sciences
Professor, Regulatory and Quality Sciences
The D.K. Kim International Center for Regulatory Science
University of Southern California School of Pharmacy
Los Angeles, California

Signature [redacted]

Date:

Stanley M. Tarka, Jr. PhD, ATS
President of The Tarka Group, Inc.
Adjunct Associate Professor, The Pennsylvania State University, College of Medicine
Hershey, Pennsylvania

Signature: [redacted]

Date:

amyris

15 October 2021

Ellen Anderson
U.S. Food & Drug Administration
Division of Food Ingredients
Office of Food Additive Safety Center for Food Safety and Applied Nutrition

Re: Amyris, Inc Response to the FDA Letter of Deficiencies – GRAS Notice No. GRN 000987

Dear Ellen Anderson:

This correspondence provides comprehensive responses to your Letter of Deficiencies, dated 23 September 2021, which pertains to GRN 000987 as filed by Amyris, Inc. (Amyris).

Certain responses where cited, are supported by supplemental information and provided as attachments. Also, note that certain information where noted is provided as Confidential Business Information (CBI) as the information can be used by experts skilled in the art to reverse engineer the proprietary strain design. It is requested that the FDA first inform Amyris prior to the release of any of the CBI requested information.

Amyris Response to FDA Letter of Deficiencies – GRN 00987

FDA Question-1. Please clarify the food category "infant meal replacement products." What type of foods does this category include? It is our understanding that foods such as PediaSure are not intended for use by infants (<1 year of age).

> **Response-1.** Amyris agrees with the FDA that products such as PediaSure are not intended for infants < 1 year of age. Rather, the "infant meal replacement products" food category is already captured by the "toddler formulas, growing-up milks (12-36 months)" category as well as the "other drinks for young children" category both listed in Table 1. Thus, Amyris is revising GRN 987 to remove the "infant meal replacement products" category.

FDA Question-2. Is the intended use level of 1.4 g/L in milk substitutes intentionally higher than current use levels (1.2 g/L)?

> **Response-2.** Amyris agrees that the current use level should be 1.2 g/L. Amyris has revised the exposure estimate reported in Tables 9a and 9b to reflect this change (please see response to question 11).

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FDA Question-3. On page 9, you state that "promoters and terminators used to express the genes are native to S. cerevisiae, and include but are not limited to, promotors of GAL1 and GAL10 proteins, and terminators of PGK1 and CYC1." Please describe the names of the additional native promoters and terminators used to generate the production strain.

Response-3. Please see response in the CBI attachment.

FDA Question-4a. We request additional information about the genes used to create the production strain: Define the gene that is derived from the assembled metagenome (page 9) and further describe why you believe that gene is not likely to be either toxigenic or allergenic, preferably at the genetic level.

Response-4a. Please see response in the CBI attachment.

FDA Question-4b. On page 10 you note "The genes used to create the production strain are found in Table 4." (You have described the taxonomy in Table 3). Please correct this reference and also add the source organism for each inserted gene. The enzymes and their technical effects are listed in Table 3 (page 11). Please clarify the role of dihydrofolate reductase in the production of 2'-FL.

Response-4b. Please see response in the CBI attachment.

FDA Question-5. On page 14, you note that 2'-fucosyllactitol is ≤6% area and other impurities combined, including 3-FL, fucosylgalactose, xylitol, dulcitol/sorbitol, and glycerophosphoethanolamine (GPE), are <7% area. In Table 5b on page 16, you indicate that 3-FL and fucosylgalactose are present in the notified substance described in GRN 000571 but the other components are not described in GRNs 000546, 000650, 000571, 000735, 000749, or 000852. We request that the following be addressed:

FDA Question-5a.We note that dulcitol (galactitol) and sorbitol are in fact present in the notified substances described in GRN 000749. Please correct the information in Table 5b accordingly.

Response-5a. Table 5b has been revised below to specify that the 'Other Carbohydrates' specifications in GRN 000749 include dulcitol (galactitol) and sorbitol as well as 3FL, 2'-Fucosyl-D-lactulose, Fucosylgalactose, Glucose/galactose, Fucose, Mannitol and Trihexose.

Allo-Lactose has been removed in the specification list below as it is not produced in the manufacturing process of the Amyris 2'-FL.

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Table 5b: Comparison of Amyris' 2'-FL and Other GRAS 2'-FL Specifications									
Parameter	**Units**	**Amyris**	**Glycom (546)**	**Glycom (650)**	**Jennewein (571)**	**Glycosyn (735)**	**DuPont (749)**	**BASF (852)**	**EFSA (2019)[a]**
Carbohydrate content									
2'-fucosyllactose	%	≥ 86	≥ 95	≥ 94	≥ 90	≥ 90	≥ 82	≥ 90	≥ 75
Difucosyllactose (DFL)	%	< 8	--	≤ 1	≤ 5	--	≤ 7	≤ 2	≥ 5
Other carbohydrates	%	--	--	--	--	--	< 6*	--	≤ 6
2'-Fucosyl-D-lactulose	%	--	--	≤ 1	--	--	--	≤ 2	≤ 2
Lactose	%	< 7	--	≤ 3	≤ 5	≤ 2	≤ 8	≤ 3	≤ 10
2'-fucosyllactitol	%	≤ 6	--	--	--	--	--	--	--
3-Fucosyllactose (3FL)	%	< 7	--	--	≤ 5	--	--	--	--
Fucosyl-galactose	%		--	--	≤ 3	--	--	--	--
Xylitol	%		--	--	--	--	--	--	--
Dulcitol/sorbitol	%		--	--	--	--	--	--	--
Glucose/Galactose	%		--	--	≤ 3	≤ 2	--	--	--
Fucose	%		--	≤ 1	≤ 3	≤ 2	--	≤ 2	≤ 1
Glycerophosphoethanolamine (GPE)	%		--	--	--	--	--	--	--
Fructose	%		--	--	--	--	--	--	--
*Other Carbohydrate specifications include 3FL, 2'-Fucosyl-D-lactulose, Fucosylgalactose, Glucose/galactose, Fucose, Sorbitol/galactitol, Mannitol and Trihexose									
Chemical									
Water Content (KF titration)	%	≤5.0	≤ 9.0	≤ 5.0	≤ 9	≤ 5.0	≤ 9.0	≤ 9.0	≤ 6.0
pH (20 °C, 5% solution)	--	3.0 – 7.5	3.0 – 7.5	3.2 - 5.0	--	3.0 – 7.5	--	3.2 – 7.5	4.0 - 6.0
Protein Content	% or µg/g	≤0.01 %	0.01 %	0.01%	≤ 100 µg/g	≤0.01%	≤ 100 µg/g	≤0.01%	≤0.01 %
Total Ash	%	≤0.5	≤ 0.2	≤ 1.5	≤ 0.5	≤ 0.2	≤ 0.5	≤ 1.5	≤ 0.8
Acetic Acid	%	--	≤ 0.3	≤ 1	--	--	--	≤ 1	--
Arsenic	mg/kg	≤ 0.15	--	--	≤ 0.2	≤ 0.1	≤ 0.2	≤ 0.1	--
Cadmium	mg/kg	≤ 0.05	--	--	≤ 0.1	≤ 0.1	≤ 0.05	≤ 0.05	--
Lead	mg/kg	≤ 0.05	≤ 0.8	≤ 0.1	≤ 0.02	≤ 0.05	≤ 0.05	≤ 0.05	--
Mercury	mg/kg	≤ 0.1	--	--	≤ 0.5	≤ 0.05	≤ 0.1	≤ 0.05	--

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Cobalt	mg/kg	≤ 0.03	--	--	--	--	--	--	--
GMO detection (rDNA from production strain)	--	Negative	--	--	Negative	Negative	Negative	--	--
Microbial Specifications									
Total Aerobic Microbial Count/Standard Plate Count	mg/kg or cfu/g	≤ 10^3 cfu/g	--	≤ 0.1 mg/kg	--	--	≤ 1000 cfu/g	≤ 500 cfu/g	--
Aerobic mesophilic total count	cfu/g	--	≤ 500	≤ 500	≤ 10000	≤ 3000	--	--	≤ 1000
Total Yeast/Mold Count	cfu/g	≤ 10^2	≤ 10	≤ 10	≤ 100	≤ 10	≤ 100	≤ 100	≤ 100
Sulfite Reducing Bacteria	cfu/g	< 100	--	--	--	< 30	--	--	
Enterobacteriaceae	grams or cfu/g	Negative in 10 g	Absent in 10 g	Absent in 10 g	Absent in 11 g	Absent in 10 g	ND in 10 g	Absent in 10 g	≤ 10 cfu/g
Salmonella	grams	ND in 25	Absent in 25	Absent in 25	Absent in 100	Absent in 25	ND in 100	Absent in 25	Absent in 25
Cronobacter sakazakii	grams	ND in 10	Absent in 10	Absent in 10	Absent in 100	Absent in 25	ND in 10	Absent in 10	Absent in 10
Coliforms	grams	ND in 10	--	--	Absent in 11	--	ND in 10	--	--
E. coli	grams	Absent in 10	--	--	--	Absent in 10	--	--	--
Listeria monocytogenes	grams	Absent in 10	Absent in 25	Absent in 25	--	--	Absent in 25	Absent in 25	--
Pseudomonas aeruginosa	grams	Absent in 10	--	--	--	--	--	--	--
Staphylococcus aureus	grams	Negative in 10	--	--	--	Absent in 1	--	--	--
Bacillus cereus	cfu/g	< 100	≤ 50	≤ 50	--	≤ 100	≤ 10	--	--
Source: Amyris, Inc.									

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a = EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA). Safety of 2'-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283. This substance is a 2'-FL/DFL mixture.
Abbreviations: -- = not specified or not applicable; % = percent; °C = degrees Celsius; cfu = colony-forming unit; g = grams; k = kilo; KF = Karl Fischer; m = milli; ND = not detected; w/w = by weight.

FDA Question 5b. What accounts for the presence of the sugar alcohols — is it attributable to the production organism, a difference in the purification steps compared with other 2'-FL ingredients, or both?

Response-5b. The production organism, *S. cerevisiae*, expresses native reductases that act on sugars to produce sugar alcohols.

FDA Question-5c. What accounts for the variation in levels of sorbitol & galactitol (0.05-0.79%) and xylitol (0.42-1.30%) observed in the batch analyses of 2'-FL?

Response-5c. Sugar alcohols (sorbitol, galactitol, xylitol, etc.) are naturally produced by *S. cerevisiae.* Levels of sugar alcohols can vary due to very minor variations in cultivation conditions (pH, oxygen concentration, sugar concentration) over the course of fermentation.

FDA Question-5d. What accounts for the presence of GPE in 2'-FL?

Response-5d. GPE is a native molecule in *Saccharomyces cerevisiae* phospholipid metabolism.[1] Trace amounts of this native phospholipid carry through purification and are found in the final 2'-FL.

Please see our Question #13 below also related to these impurities.

FDA Question-6. The method of purification described in GRN 000987 is in general terms. Please discuss how the methods of purification influence levels of impurities in 2' -FL, and include the following in your response:

FDA Question-6a. Does your method include treatment with activated carbon?

Response-6a. Yes, activated carbon is used in the V. Chromatography/Polishing unit operations. Activated carbon reduces the level of residual proteins and product color.

[1] K Stålberg, AC Neal, H Ronne, and U Ståhl. Journal of Lipid Research. 2008. 49: 1794 – 1806.

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FDA Question-6b. Does your method include purification steps with both cationic and anionic exchange resins?

Response-6b. Yes, cation and anionic exchange resins are used in the V. Chromatography/Polishing unit operations. The ion exchange resins are used to remove residual salts, trace metals, organic acids, protein, and colorants.

FDA Question-6c. You state on page 13, "The filtered product is then processed through chromatographic and polishing steps to remove additional salts, metals, proteins, organic acids, and colorants (V) (Appendix D)." However, Appendix D consists of chromatographs of carbohydrate and sugar alcohol standards. Please clarify this statement and provide additional supporting discussion, including the type of filtration materials (e.g., nano-, micro- or ultra-filtration).

Response-6c. After centrifugation, the product stream is processed through the following filtration unit operations:

Microfiltration – to remove residual solids

Ultrafiltration – to remove protein and residual DNA

Nanofiltration – to remove salts, organic acids, carbohydrate impurities, and a portion of residual protein.

The chromatographic and polishing steps referenced on page 13 consist of processing the intermediate product stream with cation exchange resin, anion exchange resin, and activated carbon. These steps are used to remove the residual impurities listed in the above answers. Removal of impurities by the filtration and chromatography steps is verified by the analysis reported in Table 6.

The chromatographs shown in Appendix D are the output of running carbohydrate and sugar alcohol standards through analytical columns. These analytical columns are used for resolving and quantifying 2'-FL product and carbohydrate impurities. The results of analysis of the regulatory lots are reported in Table 6.

FDA Question-7. Please provide a statement that all materials used in the manufacturing process are approved for their respective uses via a regulation in Part 21 of the U.S. Code of Federal Regulations, are the subject of an effective food contact notification, or are GRAS for that use in the U.S.

Response-7. Amyris confirms that all materials used in the manufacturing process of 2'-FL are approved for their respective uses via a regulation in Part 21 of the U.S. Code of Federal Regulations, are the subject of an effective food contact notification, or are GRAS for that use in the U.S.

Below is the list of raw materials and their regulatory status.

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Raw Material	Regulatory Status
Magnesium sulfate heptahydrate	No limitation other than cGMP as flavor enhancer, nutrient supplement, and processing aid, 21 CFR § 184.1443
Ammonium sulfate	GRAS when used in accordance with cGMP, 21 CFR § 184.1143
Monopotassium phosphate (KH_2PO_4)	GRAS when used in accordance with cGMP, 21 CFR §160.110
Succinate Buffer	GRAS when used in accordance with cGMP, 21 CFR §184.1091
Ethylenediaminetetraacetic acid (EDTA)	Permitted in a number of foods as a food additive at specified levels, 21 CFR §172.135
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §182.8997
Copper sulfate ($CuSO_4$) anhydrous	Used as a nutrient supplement and processing aid with no limitation other than cGMP, 21 CFR §184.1261
Manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §184.1446
Cobalt (II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	As an animal feed trace mineral (21 CFR §582.80)
Sodium molybdate dihydrate ($NaMoO_4 \cdot 2H_2O$)	As an agricultural chemical additive, chemical additive, processing aid; considered a plant nutrient under 40 CFR §180.920 and exempt from a tolerance in food
Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Used as a nutrient supplement and processing aid with no limitation other than cGMP, 21 CFR §184.1315
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Used as an anticaking agent, antimicrobial agent, curing or pickling agent, firming agent, flavor enhancer, humectant, nutrient supplement, pH control agent, processing aid, stabilizer and thickener, surface-active agent, synergist, texturizer in accordance with cGMP, 21 CFR §184.1193
Biotin	GRAS when used in accordance with cGMP, 21 CFR §182.8159
para-amino-benzoic acid	EAFUS listed

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Calcium pantothenate	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §184.1212
Nicotinic acid	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §184.1530
Myo-inositol	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §184.1370
Thiamine.HCl	Used as a flavoring agent and nutrient supplement with no limitation other than cGMP, 21 CFR §184.1875
Pyridoxine.HCl	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §184.1676
Ammonium phosphate monobasic ($NH_4H_2PO_4$)	GRAS when used in accordance with cGMP, 21 CFR §184.1141a
Food grade sugar (sucrose)	GRAS
Lactose	GRAS

FDA Question-8. You state that batch analyses confirm that cobalt is removed during processing and not detected (ND<0.01 mg/kg) in the final product. Please address if cobalt chloride is added during fermentation.

Response-8. Cobalt chloride is a trace element that may be added to the fermentation media.

FDA Question-9. For characterizing the carbohydrate content, you note the method as ion chromatography with pulse amperometry detection (Amyris SOP 830). Please provide a statement that the method has been validated for this purpose.

Response-9. SOP00830 is an ion chromatography with pulse amperometry detection method for the quantitation of carbohydrate content on an area% basis in 2'-FL final product. This method was validated at Amyris, Inc (Emeryville) following ICH Guideline Q2(R1) - Validation of Analytical Procedures: Text and Methodology. The validation covered linearity, accuracy, precision (repeatability), specificity, limit of quantitation, limit of detection, sample/standard solution stability and system suitability of the method. All acceptance criteria set in the validation protocol were met and SOP00830 is considered a validated method.

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FDA Question-10. In the batch analyses shown in Appendix B (pages 15, 18, 20, 22, 24) you list "lactose/allo-lactose" in your results of analyses. Do you expect allo-lactose to be formed in the fermentation process? Please address.

Response-10. The analytical method does not resolve lactose and allo-lactose. However, Amyris does not expect allo-lactose (allolactose) to be formed in the Amyris *S. cerevisiae* fermentation process. The correct annotation should be lactose, not lactose/allolactose. The original designation was a carry-over from GRN 735 that is not applicable here given that *S. cerevisiae* does not possess the necessary enzyme activity to form allo-lactose from lactose. Table 6 has been updated to properly designate the compound as lactose only.

FDA Question-11. You note on page 25 that "The proposed uses of Amyris's 2'-FL do not increase the cumulative EDI already reviewed in previous GRNS of chemically equivalent 2'-FL." While you do not cite specific GRNs to support this statement, we note that previous GRNs have provided lower estimates for the mean cumulative exposure to 2'-FL and have not provided an estimate of the 95th percentile. Please provide the 90th percentile dietary exposure estimates instead of the 95th percentile values and address the points below.

Response-11. As requested, Amyris provides revised Tables 9a and 9b, which provide 90th percentile consumption estimates on a g/day and g/kg bw/day basis for all target populations.

Table 9a: Summary of the Estimated Daily Intake of 2'-FL from All Proposed Food and Beverage Uses in the U.S. by Population Group (2013-2016 NHANES Data)

Population Group	Age Group	All-Users Consumption (g/day)			
		% Users	N	Mean	90th Percentile
Infants	0-6 mo.	100	241	2.73	4.90
Infants	7-12 mo.	99.66	228	3.82	6.72
Toddlers	1 to 3 yr.	98.77	1117	2.29	4.39
Children	4 to 10 yr.	99.34	2315	2.61	5.34
Male Teenager	11-18 yr.	99.49	1213	3.12	7.50
Female Teenager	11-18 yr.	99.13	1216	2.36	5.93
Female Adults of childbearing age	19-40 yr.	99.30	1807	1.78	4.22
Female Adults	19-64 yr.	99.49	3767	1.72	3.90
Male Adults	19-64 yr.	99.30	3313	2.38	5.42
Elderly Adults	65 yr. and up	98.95	2315	2.25	4.79

Abbreviations: 2'-FL = 2'-fucosyllactose; g = grams; mo. = months; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; yr. = years

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Table 9b: Summary of the Estimated Daily Intakes of 2'-FL per Kilogram Body Weight from All Proposed Food and Beverage Uses in the U.S. by Population Group (2013-2016 NHANES Data)							
Population Group	Age Group	Body Weight (kg)			All-Users Consumption (g/kg·bw/day)		
		Mean	90th Percentile	% Users	N*	Mean	90th Percentile
Infants	0-6 mo.	6.8	8.6	100	241	0.40	0.68
Infants	7-12 mo.	9.3	10.9	99.6	227	0.42	0.75
Toddlers	1 to 3 yr.	13.8	16.9	98.77	1103	0.18	0.35
Children	4 to 10 yr.	28.9	42.9	99.34	2303	0.10	0.22
Male Teenager	11-18 yr.	60.4	84.7	99.49	1210	0.054	0.13
Female Teenager	11-18 yr.	65.6	91.9	99.13	1205	0.043	0.10
Female Adults of childbearing age	19-40 yr.	76.5	107.3	99.30	1791	0.025	0.061
Female Adults	19-64 yr.	78.2	107.6	99.49	3739	0.024	0.055
Male Adults	19-64 yr.	89.6	115.4	99.30	3295	0.028	0.064
Elderly Adults	65 yr. and up	79.5	105.2	98.95	2283	0.029	0.064
Abbreviations: 2'-FL = 2'-fucosyllactose; bw = body weight; g = grams; mo. = months; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; yr. = years Note: *Body weights were not recorded for all survey respondents, therefore, N in Table 9b is less than N in Table 9a for some groups of consumers.							

FDA Question-11a. We note that your estimates (2.7 and 3.82 g/p/d at the mean for ages 0-6 and 7-12 months, respectively) are higher by approximately 25-50% the mean than those reported in previous GRNs.1 We request that you discuss any differences between your estimates and those reported in previous GRNs, particularly since the uses are largely substitutional.

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Response-11a. First, Amyris notes that two possible sources of variability are the NHANES database itself and how the data was obtained for the previous GRAS notices. Specifically, GRN 932 and 852 cite use of data between 2013-2014 while Amyris' GRAS notice utilized a larger data set from 2013-2016. Further, these previous GRAS notices appear to have "binned" the age groups in a slightly different way than was done for the current notice. Specifically, GRNs 932 and 852 list target populations as "Infant (0-5 months)", "Infant (6-11 months)", and "Toddlers (12-35 months)" while the current notice separates these same populations slightly differently as "Infant (0-6 months)", "Infant (7-12 months)", and "Toddlers (1-3 years)". The combination of the different starting data set, and the apparent differences in how the data were queried between the current notice and previous notices could account for some of the exposure differences.

In addition to these two potential factors, the differences in exposure are also likely due to the additional uses contemplated in the current notice in comparisons to the previous notices. Specifically, "processed cereal-based food and baby food for infants and young children", "other baby foods for infants and young children", "other drinks for young children", and "milk modifiers (i.e., powder for addition in milk such as cacao-based powders, etc.)" are all uses which do not appear on previous notices but are included in the current notice and may directly impact exposure to infant and toddler populations. The Agency indicates correctly that additional uses are largely substitutional, however this is the case only in a caloric intake sense (e.g., an increase in caloric intake of dry cereal would be accompanied by a concurrent decrease in caloric intake from infant formula). The use levels however for these food items are not necessarily the same, so that these changes would result in the same caloric intake for the target populations, the intake of 2'-FL could increase. In conclusion the intended uses in the current notice are largely identical to previous notices, and the increase in exposure calculated would still be considered safe when viewed in light of the extensive safety data that is presented in the GRAS notice.

FDA Question-11b. Please provide additional discussion to support your statement that the cumulative EDI you present in Table 9a has already been reviewed in previous GRNs.

Response-11b. While the exposures discussed in 9a is slightly higher than those covered in previous GRAS notices, they are not excessively so. As discussed above, the increases in exposure are likely due in to a newer NHANES data set utilized herein. The differences in exposure are minor and supported by the available safety data.

FDA Question-11c. Please also provide a 90th percentile dietary exposure estimates to 2'-FL for infants 0-6 months, infants 7-12 months, children 1-3 years of age.

Response-11c. Please see revised Tables 9a and 9b above.

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FDA Question-12. We note that in Table 6 you have an entry for endotoxins (Endotoxins (total, EU/g); however, you have not set a specification for endotoxins. Please confirm that you do not provide a specification for endotoxins because they are not produced by the production organism used in the manufacturing process. We suggest that you supply a revised Table 6 that does not contain the endotoxin specification analysis.

Response-12. Amyris states that *Saccharomyces cerevisiae* does not produce endotoxins and Table 6 is revised as suggested and presented below.

Table 6. Analysis of Amyris' 2'-FL Production Batches

Parameter	Specification	Batch Number					Method
		H8163*	H8452	H8561	H8781	H8750	
Batch Date		2/6/19	8/13/19	9/25/19	10/3/19	10/18/19	
Carbohydrate content (% area)							
2'-fucosyllactose	≥ 86% area	86.06	91.1	92.2	91.2	91.3	By ion chromatography (Amyris SOP 830)
Lactose	< 8% area	2.18	1.18	0.08	1.43	0.44	
Difucosyllactose (DFL)	< 7% area	0.52	1.40	1.30	1.24	1.25	
2'-fucosyllactitol	≤ 6% area	4.92	3.54	3.93	3.19	3.52	
3-Fucosyllactose (3FL) & Fucosyl-galactose	< 7% area	0.92	0.33	0.38	0.23	0.18	
Sorbitol & Galactitol		0.79	0.14	0.05	0.15	0.10	
Xylitol		0.42	0.73	0.59	0.67	1.30	
Fucose		3.36	0.18	0.12	0.15	0.19	
Glucose & Galactose		0.20	0.26	0.33	0.22	0.52	
Glycerophosphoethanolamine (GPE)		0.24	0.27	0.42	0.53	0.25	
Fructose		0.24	0.40	0.43	0.31	0.46	
Sugar alcohols, other		0.15	--	--	--	--	
Sub-total, minor oligosaccharides and sugar alcohols		6.32	2.31	2.32	2.26	3.0	
Total		100	99.53	99.83	99.32	99.51	
Appearance							
Color	White to off-white/ivory	N/A	Pass	Pass	Pass	Pass	Visual
Form	Dry powder	N/A	Pass	Pass	Pass	Pass	Visual
Appearance in solution (at 5%)	Clean, colorless to slightly yellow	N/A	Pass	Pass	Pass	Pass	Visual

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Chemical							
Water Content (KF titration)	≤5%	N/A	2.66	3.07	2.53	2.66	Karl Fischer titration (Amyris SOP 842)
pH (20 °C, 5% solution)	3.0 – 7.5	N/A	5.8	5.3	5.8	5.5	EP 2.2.3 v9
Protein Content (% w/w)	≤0.01% w/w	N/A	<0.004	<0.004	<0.004	<0.004	Modified Bradford Assay (Amyris SOP 843)
Total Ash (% w/w)	≤0.5% w/w	N/A	<0.3%	0.49%	0.33%	<0.3%	FCC 11 appendix II
Arsenic (mg/kg)	≤ 0.15 mg/kg	N/A	ND <0.005	ND <0.005	ND <0.005	ND <0.005	EP 2.2.58 v9
Cadmium (mg/kg)	≤ 0.05 mg/kg	N/A	ND <0.01	ND <0.01	ND <0.01	ND <0.009	EP 2.2.58 v9
Cobalt (mg/kg)	≤ 0.03 mg/kg	N/A	ND <0.01	ND <0.01	ND <0.01	ND <0.01	EP 2.2.58 v9
Lead (mg/kg)	≤ 0.05 mg/kg	N/A	0.004	0.00683	0.00795	0.0123	EP 2.2.58 v9
Mercury (mg/kg)	≤ 0.1 mg/kg	N/A	ND <0.002	ND <0.006	ND <0.002	ND <0.002	EP 2.2.58 v9
GMO detection (rDNA from production strain)	Negative	N/A	Negative	Negative	Negative	Negative	PCR (Amyris SOP 844)
Microbial Specifications							
Total Aerobic Microbial Count/Standard Plate Count (cfu/g)	≤ 10^3 cfu/g	N/A	240	710	35	45	EP 2.6.12 v9
Total Yeast/Mold Count (cfu/g)	≤ 10^2 cfu/g	N/A	ND <10	ND <10	ND <10	ND <10	EP 2.6.12 v9
Sulfite Reducing Bacteria (cfu/g)	< 100 cfu/g	N/A	ND <10	ND <10	ND <10	ND <10	ISO 15213: 2003
Bacillus cereus (cfu/g)	<100 cfu/g	N/A	ND <10	ND <10	ND <10	ND <10	ISO 7932: 2004
Enterobacteriaceae	Negative in 10 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9
Salmonella	ND in 25 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9
Cronobacter sakazakii	ND in 10 g	N/A	ND	ND	ND	ND	ISO/TS 22964

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Coliforms	ND in 10 g	N/A	ND	ND	ND	ND	ISO 4831: 2006
E. coli	Absent in 10 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9
Listeria monocytogenes	Absent in 25 g	N/A	ND	ND	ND	ND	ISO 11290-1: 2017
Pseudomonas aeruginosa	Absent in 10 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9
Staphylococcus aureus	Negative in 10 g	N/A	ND	ND	ND	ND	EP 2.6.13 v9

Source: Amyris, Inc.
*Batch results are from an initial pilot plant manufacturing lot.
Abbreviations: °C = degrees Celsius; cfu = colony-forming unit; EU = endotoxin units; FCC = Federal Communications Commission Food Chemicals Codex; g = grams; KF = Karl Fischer; k = kilo; m = milli; ND = not detected; w/w = by weight.
N/A = Not analyzed.

FDA Question-13. On page 34, you state, "The toxicology studies were performed on 2'-FL from various sources. Although Amyris 2'-FL was not the test substance evaluated, the other 2'-FLs that have been evaluated are appropriate for evaluating Amyris's 2'-FL because the profiles of 2'-FL and the associated substances, i.e., non-2'-FL carbohydrates (Table 5b), were sufficiently similar to base safety interpretations on the results of these studies. This substantial chemical equivalency supports bridging to the published studies." However, as we noted in our Question #5 above, Table 5b (page 16) indicates that several non-2'-FL impurities present in the notified ingredient have not been present in previous GRAS notices for 2'-FL ingredients (i.e., 2'-fucosyllactitol, xylitol, fructose and GPE).

FDA Question-13a. Given these differences in composition, it is unclear how previously published toxicology studies (i.e., in vivo, repeated dose) can support the safe use of Amyris's notified ingredient. For xylitol, fructose, and GPE, please provide a comprehensive safety narrative that addresses: (1) why these impurities are not expected to be a safety concern for infants consuming Amyris's 2'-FL; and (2) which published safety study has a 2'-FL test article that most closely approximates the composition of the notified ingredient.

Response-13a. All 3 molecules are native yeast metabolites. Yeast extract as a whole, and each of these compounds individually, are present in many foods and has a long history of safe use.

- **Fructose**

Fructose is a simple sugar which is ubiquitous in foods. Fructose exposure in foods comes both from direct exposure and exposure to common "table sugar" which is a disaccharide of fructose and glucose. Fructose is present in human milk at mean concentrations of c. 5-7 mg/L reflecting

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dietary intake of fructose by the lactating mother.[2] Goran et al. estimated that these human milk levels translate into an intake of 1 mg fructose per kg of body weight for a one-month-old, breastfed infant and that higher levels of fructose in mother's milk has been associated with improved body composition (higher body weight, higher lean mass, etc.). Walker and Goran report infant formula typically provide a significant percentage (25-50%) of total calories in the form of various sugars, particularly sucrose. While fructose is typically present at relatively low levels (0-1.2% of total sugar), sucrose is commonly present at much higher levels (up to 100% of total sugar).[3] Further, follow on foods typically consumed by older infants (6-12 months old) were shown to contain even higher levels of fructose which many commercially available baby foods containing 25-80% of total calories from sugars, of which fructose was commonly 10-40% of total sugar.

The most exposed population to Amyris' 2'-FL on a g/kg bw basis is infants 7-12 months old. In the notice, Amyris conducted an exposure estimate which calculated a mean and 90th percentile exposures of 0.42 and 0.75 g/kg bw/day respectively (see response to question #11 above). Using the highest reported level of fructose present in the submitted batch analyses (Appendix B of the GRAS notice) of 0.46%, this would represent a potential exposure of 1.9 and 3.5 mg/kg bw/day of fructose from the intended uses of Amyris' notified 2'-FL. This exposure is in line with exposures to fructose already present in the diet of infants. Further, as the caloric needs of this target population are typically well known and controlled, exposure to fructose from the notified uses would be expected to be substitutional to other exposures rather than additional. Therefore, no increase in fructose exposure would be expected.

- **Xylitol**

Xylitol is a sugar alcohol commonly used as a "low-calorie" sweeter and is cleared under 21 CFR 172.395 for special dietary uses at a level that does not exceed the intended effect. In adults, doses of up to 100g/day is well tolerated.[4] Similarly, xylitol is well tolerated in children, with studies indicating few adverse events at does at or below 45 g/day.[5] In all target populations,

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[2] Goran, M.I., et al. (2017) Fructose in Breast Milk Is Positively Associated with Infant Body Composition at 6 Months of Age. *Nutrients* 9(2): p. 146; Berger, P.K., et al. (2018) High-Fructose Corn-Syrup-Sweetened Beverage Intake Increases 5-Hour Breast Milk Fructose Concentrations in Lactating Women. *Nutrients*. 10(6):669.

[3] Walker RW, and Goran MI. (2015) Laboratory determined sugar content and composition of commercial infant formulas, baby foods and common grocery items targeted to children. *Nutrients*. 7:5850-67; doi:10.3390/nu7075254.

[4] Wang YM, van Eys J. (1981) Nutritional significance of fructose and sugar alcohols. *Annu Rev Nutr*. 1:437–75; Asano T, Levitt MD, Goetz FC. (1973) Xylitol absorption in healthy men. *Diabetes*. 22:279–81; Forster H, Quadbeck R, Gottstein U. (1982) Metabolic tolerance to high doses of oral xylitol in human volunteers not previously adapted to xylitol. *Int J Vitam Nutr Res Suppl*. 22:67–88.

[5] Akerblom HK et al. (1982) The tolerance of increasing amounts of dietary xylitol in children. *Int J Vitam Nutr Res Suppl*. 22:53–66.

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the most commonly reported adverse events occurring upon consumption of larger amounts of xylitol are mild gastrointestinal effects which occur when fluid is drawn into the distal small bowel and colon causing osmotic diarrhea.[6] A recent study examined the tolerability of xylitol in children 6-36 months of age.[7] Children were randomized into groups receiving placebo, 7.5 g/day xylitol (once per day), or 15 g/day xylitol (given as 5 g TID) for three months. Incidences of reported gastrointestinal effects (gas, diarrhea, etc.) were not statistically different between placebo and either xylitol group over the course of the study, indicating up to 15 g/day xylitol is well tolerated in this target population.

The most exposed population to Amyris' 2'-FL on a g/day basis is infants 7-12 months old. In the notice, Amyris conducted an exposure estimate which calculated a mean and 90th percentile exposures of 3.8 and 6.7 g/day respectively (see response to question #11 above). Using the highest reported level of xylitol present in the submitted batch analyses (Appendix B of the GRAS notice) of 1.3%, this would represent a potential exposure of 0.049 and 0.087 g/day of xylitol from the intended uses of Amyris' notified 2'-FL. Given the available safety data, this exposure would be of no safety concern.

- **GPE**

Glycerophosoethanolamine (GPE) is the "polar head group" portion of phosphatidylethanolamine (PE), which composes up to 25% of phospholipids present in animal cell membranes. PE is converted to GPE and its constituent fatty acids during normal metabolism of consumed phospholipids, typically through hydrolysis by pancreatic enzymes.[8] GPE is further converted to ethanolamine sn-glycerol 3-phosphate which are utilized by gut bacteria for oxidation and resynthesis of glycerolipids respectively.[9]

PE is known to be a component of food ingredients such as soy lecithin and is known to be present in human milk concentrations between c. 50 to 150 mg/L and is present as component

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[6] Tsampalieros, A., et al. (2008) Dietary fructose intolerance in children and adolescents. *Arch Dis Child*. 93(12): 1078.

[7] Vernacchio L., Vezina R.M., and Mitchell M.D. (2006) Tolerability of oral xylitol solution in young children: implications of otitis media prophylaxis. *Int J. Pediatr Otorhinolaryngol*. 71 (1):89-94.

[8] Andersson L, Sternby B, and Nilsson A. (1994) Hydrolysis of phosphatidylethanolamine by human pancreatic phospholipase A2. Effect of bile salts. *Scand J Gastroenterol*. 29(2):182-7.

[9] Nilsson, Å., R.-D. Duan, and L. Ohlsso. (2021) Digestion and Absorption of Milk Phospholipids in Newborns and Adults. *Frontiers in Nutrition*. 8(532); Yu, J., et al. (2018) Update on glycerol-3-phosphate acyltransferases: the roles in the development of insulin resistance. *Nutrition & Diabetes*. 8(1): p. 34.

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of milk fat globule membranes.[10] Bruschetta et al identified and quantified fully deacetylated GPE in human milk at concentrations up to 43.03 mg/L.[11]

The most exposed population to Amyris' 2'-FL on a g/kg bw basis is infants 7-12 months old. In the notice, Amyris conducted an exposure estimate which calculated a mean and 90th percentile exposures of 0.42 and 0.75 g/kg bw/day respectively (see response to question #11 above). Using the highest reported level of GPE present in the submitted batch analyses (Appendix B of the GRAS notice) of 0.53%, this would represent a potential exposure of 2.2 and 4.0 mg/kg bw/day of fructose from the intended uses of Amyris' notified 2'-FL. In summary, GPE is a natural breakdown product of cellular membranes in many foods including human milk and building block for resynthesis of membranes in the consuming human and would not be a safety concern at the levels contained in the notified substance.

Amyris has been unable to identify a safety study which indicates that the test substance contained the same impurities (i.e., fructose, xylitol, and/or GPE). Many studies do not contain the granularity of impurity content provided by Amyris. For instance, many contain statements describing "other carbohydrates" which may or may not include compounds such as fructose and xylitol. As described above, PE is a common component of cell membranes and in bacteria represent and even larger proportion of phospholipid content than in eukaryotic cells. Therefore, Amyris believes that studies describing 2'-FL produced via bacterial fermentation would most closely represent the substance described in the current notice. GRNs 571, 650, 735, 749, and 852 describe 2'-FL production via bacterial fermentation with *E. coli* K-12 while GRN 932 describes 2'-FL production via bacterial fermentation with *Corynebacterium glutamicum*. Several published safety studies which are summarized in the current GRAS notice describe studies in which the test substance was produced via microbial fermentation. These include Phipps *et al.* 2018, Van Berlo *et al.* 2018, and Penard *et al.* 2015.

FDA Question-13b. We also note that in the memorandum for the pre-submission meeting between Amyris and FDA on November 20, 2019, FDA suggested that Amyris avoid the phrase "substantial chemical equivalence" when discussing the identity of the notified ingredient. Please provide an updated statement that avoids this specific phrase.

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[10] Demmelmair, H. and B. Koletzko (2018) Lipids in human milk. *Best Practice & Research Clinical Endocrinology & Metabolism*. 32(1):57-68; Fallbrook, A., et al. (1999) Phosphatidylcholine and phosphatidylethanolamine metabolites may regulate brain phospholipid catabolism via inhibition of lysophospholipase activity. *Brain Research*. 834(1):207-210; Selvalatchmanan, J., et al., (2011) Variability of Lipids in Human Milk. *Metabolites*. 11(2):104.

[11] Bruschetta, G., et al., (2021) A promising 31P NMR-multivariate analysis approach for the identification of milk phosphorylated metabolites and for rapid authentication of milk samples. *Biochemistry and Biophysics Reports*. 27:101087.

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Response-13b. Amyris states that the phrase "substantial chemical equivalence" should be replaced with "This similarity in the 2'-FL profiles supports bridging to the published studies." at page 34 section 6.3.

FDA Question-14. On page 52, you provide a discussion regarding the safety of 2'-fucosyllactitol (2'-fiol). However, we have identified several issues with this safety narrative.

FDA Question-14a. Please completely and comprehensively address each point below., Lactitol oligosaccharides: The notice lacks a detailed description of the test article, study design, methods, and results of the Yanahira et al., 1995 and Yanahira et al., 1997 studies. Please provide a sufficiently detailed discussion of these studies to support the safety of 2'-fiol.

Response-14a. Lactitol-oligosaccharide (LO) was prepared from lactitol by *Aspergillus oryzae* ,β-galactosidase. The LO was purified from the reaction mixture by charcoal column chromatography. After purification, a purity of 95% was determined by HPLC analysis. The studies of Yanahira et al. are investigative studies and not classical toxicity studies and should be considered as additional information with limited relevance in the overall weight of evidence approach. Yanahira et al. 1995 describe the effects of LO on the intestinal microflora in rats compared with those of lactitol. Body weights and food intake were not significantly different. Yanahira et al. 1997 determined the effects of LO, lactose, lactitol and galactooligosaccharide on calcium and magnesium absorption by feeding 8-week-old Sprague-Dawley male rats diets containing 5% of the above carbohydrates for two weeks. The investigated body weights and food intake were not significantly different.

The investigative studies by Yanahira et al. support the overall weight of evidence approach for 2'-fiol based on the lack of observed adverse events in these publications.

FDA Question-14b. In silico predictions: You state that multiple in silico tests were performed to confirm the safety of 2'-fiol. However, the notice lacks published, peer-reviewed information supporting the general recognition of these in silico models and predictions as appropriate and relevant for food safety assessments. Additionally, we note that the ICH M7 assessment applies to mutagenic impurities in pharmaceuticals and is not necessarily relevant for a food safety assessment. Please provide a discussion of how this approach is based on generally recognized scientific principles for food additive safety assessments.

Response-14b. The use of (Q)SAR models as useful information source to assess mutagenic properties of impurities is widely accepted by many regulatory bodies. In 2016, EFSA published a guidance document on the establishment of the residue definition for dietary risk assessment[12] and advised that the genotoxicity assessment should be assisted by application of (Q)SAR and

[12] EFSA Journal 2016;14(12):4549

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read-across of metabolites. In 2019, EFSA published an external scientific report on the applicability of existing (Q)SAR models[13]. Here, an expert group tested the performance of combinations of QSAR models for Ames test prediction and could show that the combination of DEREK and SARAH had a sensitivity of 75.86% and a specificity of 88.97% against a recently published EFSA genotoxicity database, indicating that these two systems in combination produce a valid and reliable prediction regarding bacterial mutagenicity. In the ICH M7 (R1) guideline, it is stated that a computational toxicology assessment should be performed using (Q)SAR methodologies that predict the outcome of a bacterial mutagenicity assay with two (Q)SAR prediction methodologies that complement each other. One methodology should be expert rule-based, and the second methodology should be statistical-based.

Based on these different guidance documents, Amyris has utilized two commercially available *in silico* models to assess the safety of 2'-fIol. Derek Nexus is a knowledge-based system applying structure–activity relationship and expert knowledge rules to derive a reasoned conclusion about the potential toxicity of the query chemical. Sarah Nexus is a statistical-based software aimed at giving fast, automatic predictions for Ames mutagenicity. This combination has been shown to have a reliable sensitivity and specificity (see above) and complies with the guidance on how to assess the mutagenic property of a compound provided by the ICH M7. Although the ICH M7 is providing guidance specifically for pharmaceuticals, the general guidance on how to assess the mutagenic property of a compound can be applied for impurities in food as well. The ICH M7 guidance concludes that the absence of structural alerts from two complementary (Q)SAR methodologies (expert rule-based and statistical) is sufficient to conclude that the impurity is of no mutagenic concern, and no further testing is recommended. In line with this guidance, the impurity 2'-fIol is regarded as not mutagenic since both Derek Nexus and Sarah Nexus gave reliable negative predictions regarding mutagenicity.

In a scientific report prepared by the Joint Research Centre of the European Commission in 2010[14], it is concluded, that when using computational models for regulatory purposes, predictions of genotoxicity and carcinogenicity should not be based on the use of any single model alone, but on a weight of evidence approach including information from all available sources including read across. It was therefore considered to use the main compound 2'Fucosyllactose as a potential read-across candidate and to use the experimental data available with 2'Fucosyllactose as data source to describe the toxicological properties of 2'-fIol. To that end, a comparative investigation of the structural properties of both compounds has been performed using the OECD Toolbox 4.2. As shown in the figure below, the structural features between 2'Fucosyllactose and 2-fIol as described by profilers related to genotoxicity are identical:

[13] EFSA Supporting publication 2019:EN-1598

[14] Applicability of QSAR analysis to the evaluation of the toxicological relevance of metabolites and degradates of pesticide active substances for dietary risk assessment, 2010.

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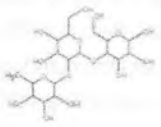
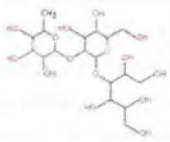
Filter endpoint tree...	1 [target]	2
Structure		
Profile		
General Mechanistic		
DNA binding by OASIS	No alert found	No alert found
DNA binding by OECD	No alert found	No alert found
Toxic hazard classification by Cramer	Low (Class I)	Low (Class I)
Toxic hazard classification by Cramer (extended)	Low (Class I)	Low (Class I)
Endpoint Specific		
Aquatic toxicity classification by ECOSAR	Neutral Organics	Neutral Organics
DNA alerts for AMES by OASIS	No alert found	No alert found
DNA alerts for CA and MNT by OASIS	No alert found	No alert found
in vitro mutagenicity (Ames test) alerts by ISS	No alert found	No alert found
in vivo mutagenicity (Micronucleus) alerts by ISS	H-acceptor-path3-H-acceptor	H-acceptor-path3-H-acceptor
Protein binding alerts for Chromosomal aberration by OASIS	No alert found	No alert found
Protein binding alerts for skin sensitization according to GHS	No alert found	No alert found
Protein binding alerts for skin sensitization by OASIS	No alert found	No alert found
Protein Binding Potency h-CLAT	No alert found	No alert found

The identical structural profile together with the negative predictions observed by two different QSAR models (an expert rule-based model and a statistical model), strongly indicate that 2-flol is not genotoxic.

In addition, when calculating the structural similarity between 2'Fucosyllactose and 2'-flol with the commonly used Dice formula and atom centered fractions, a similarity score of 87.879 % is specified, further demonstrating that both the parent compound 2'Fucosyllactose and the impurity 2'-flol show strong structural similarities:

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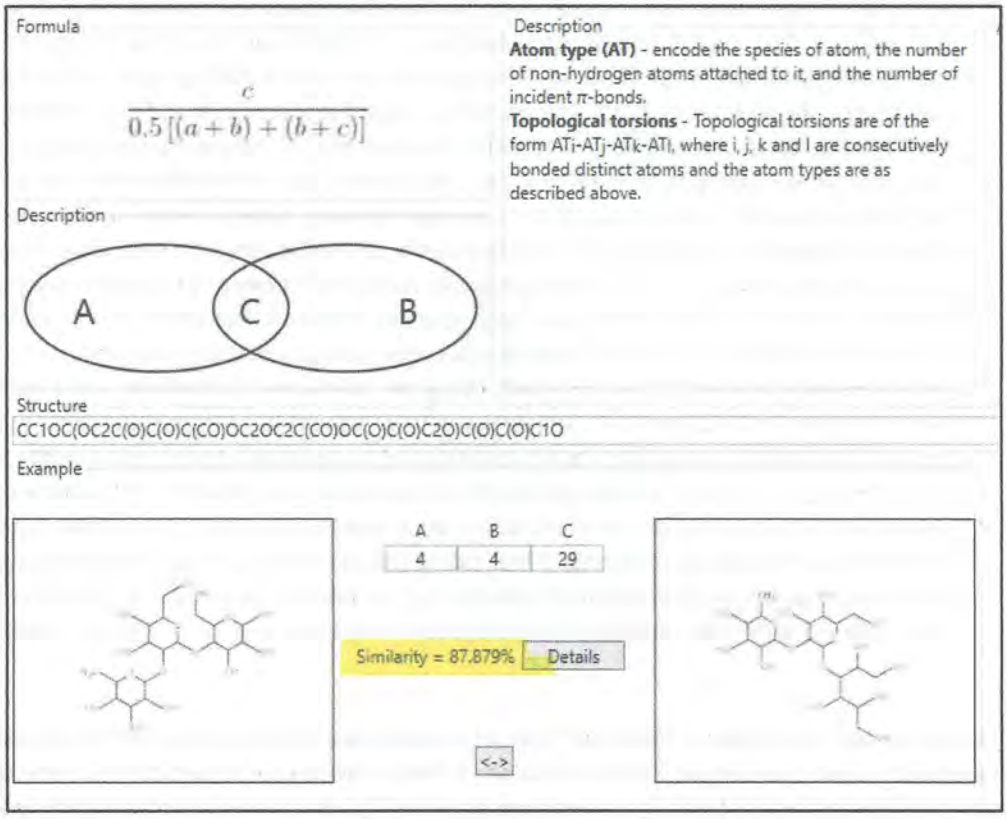

Therefore, it can be concluded that the experimental data obtained with 2'Fucosyllactose can be used to characterize 2'-flol in a read-across approach. Together with the negative predictions observed with Derek Nexus and Sarah Nexus it can be further concluded that 2'-flol is not genotoxic. In addition, other toxicological endpoints such as repeated dose toxicity are most likely comparable between these two compounds as well, indicating that 2'-flol is of low toxicological concern.

FDA Question-14c.Threshold of Toxicological Concern: You state that Toxtree software characterized 2'-flol as a Cramer Class I compound with a Threshold of Toxicological Concern (TTC) of 1,800 µg/d (30 µg/kg bw/d). However, you do not provide a comparison of this TTC value with the estimated dietary exposure of 2'-flol to infants which are a sensitive subpopulation.2 If the dietary exposure to 2'-flol is greater than the TTC value, please provide a narrative that explains why this is not a safety concern for sensitive populations, such as young infants.

Response-14c. The specification of 2'-fucosyllactitol (2'-flol) in the product 2'-FL is ≤ 6%. Based on Amyris's proposed uses of 2'-FL in infant formula at a concentration of up to 2.4 g/L, the

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mean EDI for an infant 0 to 6 months of age (6.8 kg average weight; approximately 1 L/day) is 400 mg/kg·bw/day for 2'FL and 24 mg/kg·bw/day for 2'-flol. For an infant 7 to 12 months of age (9.3 kg average weight; approximately 1.5 L/day) the mean EDI is 420 mg/kg·bw/day for 2'-FL and 25.2 mg/kg·bw/day for 2'-flol. Therefore, the dietary exposure with 2'-flol is clearly above the TTC value for a Cramer Class I compound. However, the TTC concept is considered of minor relevance for the safety assessment of 2'-flol. The Cramer Class determination by Toxtree was performed as part of an overall weight of evidence approach. Substances of Cramer Class I are simple chemical structures for which efficient modes of metabolism exist with a low order of oral toxicity. In addition, TTC values are generally very conservative and represent a worst-case scenario as their data base consists of a wide range of chemicals. Therefore, the TCC concept is considered less relevant for 2'flol, especially since the data rich parent compound can serve as a source chemical in a read-across approach. Using the Dice formula, a similarity score of 87.88 % was calculated for 2'-flol and 2'-FL (see response to question 14b), showing that these molecules are characterized by a strong structural similarity and a comparable toxicological profile and a similar biological behavior can be assumed for. Based on data available for 2'FL and the similar chemical structure, an equally low absorption rate is assumed for 2'flol. To summarize, based on this weight of evidence approach for 2'-flol, taking the lack of toxicological relevant features, a low absorption rate and the chemical similarity to 2'-FL into account, 2'-flol is considered to be of no safety concern for consumers, including sensitive populations, such as young infants.

FDA Question-14d. Fucosidases: You state, "Several fucosidases are described in the literature and these would be able to hydrolyze 2'-flol, and some of these activities are expected to be present in the human gut (Schopohl et al., 1992; Ogata-Arakawa et al., 1977)." However, we note that Schopohl et al., 1992 describes properties of an α-L-fucosidase from Dictyostelium discoideum and Ogata-Arakawa et al., 1977 describes α-L-fucosidases from almond emulsin. Thus, it is unclear what "activities" are being referenced and why they would be expected to be present in the human gut. Please provide a more detailed discussion of α-L-fucosidase enzymes, including organisms in which fucosidase enzymes are found (relevant to the infant gut), substrate specificities, and glycoside hydrolase families, etc., to support the safety of 2'-flol.

Response-14d. Several fucosidases are described in the literature and these would be able to hydrolyze 2'-flol (Schopohl et al., 1992; Ogata-Arakawa et al., 1977), and some of these activities are expected to be present in the human gut.

"Ashida, H., Akiko, M., Kiyohara, M., Wada, J. Yoshida, E., Kumagai, H., Katayama, T., Yamamoto, K. (2009) Two distinct α-L-fucosidases from Bifidobacterium bifidum are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates, Glycobiology. 19: 1010-1017", and *"Katayama T, Sakuma A, Kimura T, Makimura Y, Hiratake J, Sakata K, Yamanoi T, Kumagai H, Yamamoto K. 2004. Molecular cloning and characterization of Bifidobacterium bifidum 1,2-α-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95). J Bacteriol. 186:4885–4893"*, describes the initial characterization of the relevant GH95 enzyme.

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Together these documents confirm that the gut microbe Bifidobacterium has such GH95 α-L-fucosidase enzyme activities with the correct substrate specificity.

Amyris demonstrated with the enzymatic hydrolysis the enzymatic conversion of 2'-fIol by Bifidobacterium GH95 α-L-fucosidase (the same as in Ashida etal) and suggesting that Bifidobacterium can perform this hydrolysis in the gut.

In the neonate intestine, HMOs provide a substrate for bacteria including Bifidobacterium and Bacteroides spp. that are capable of metabolizing the HMOs using glycoside hydrolases and other specific enzymes (Marcobal and Sonnenburg, 2012)." This reference describes several hydrolases in gut microbiota, including the relevant GH95 α-L-fucosidase in table 1:

TABLE 1. Annotated gene coding for glycoside hydrolases putatively involved in HMO degradation as described at the CAZy database (http://www.cazy.org)

	GH2	GH16	GH18	GH20	GH29	GH33	GH95
Bacteroides fragilis ATCC25285	15	6	2	12	9	3	4
Bacteroides vulgatus ATCC8483	25	1	2	8	8	2	4
Bifidobacterium infantis ATCC15697	2	–	1	3	3	2	1
Bifidobacterium bifidum NCIMB 4117	3	–	–	4	1	1	1

The selected glycoside hydrolase families include the following activities: GH2, β-galactosidase; GH16, endo-β-1,4-galactosidase; GH18, endo-β-*N*-acetylglycosaminidase; GH20, β-hexosaminidase; GH29, α-1,3/4-fucosidase; GH33, sialidase; GH95, α-1,2-fucosidase.

FDA Question-14e. Polyols: In 1983, a JECFA report stated, "The Committee had before it extensive data on polyols including xylitol, lactitol, and hydrogenated glucose syrup. On previous occasions the Committee had considered sorbitol and mannitol. The Committee was aware of human animal studies clearly demonstrating that excessive consumption of these substances led to diarhoea."3 A JECFA toxicological monograph similarly discusses the laxative effects of lactitol, xylitol, and sorbitol.4 From the results of the unpublished in vitro fermentation study, you conclude that 2'-fIol is hydrolyzed to fucose and lactitol. Additionally, Table 5b (page 16), indicates the presence of xylitol, dulcitol, and sorbitol in Amyris's 2'-FL ingredient. However, no safety narrative is provided that discusses potential adverse effects in young infants given the exposure to polyol impurities present in the 2'-FL ingredient as well as the lactitol produced from bacterial fermentation of the 2'-fIol impurity. Additionally, given that all data and information pivotal to a GRAS conclusion must be generally available and generally accepted, please discuss how this in vitro study supports a GRAS conclusion given that it is an unpublished report.

Response-14e.

- **Xylitol**

Refer to answer 13.a.

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- **Sorbitol/Galactitol (Dulcitol)**

Sorbitol occurs naturally in many edible fruits and berries and is widely used in the food, confectionery, oral care, and pharmaceutical industries because of its unique physical and chemical properties[15].

Osmotic laxative effects after oral intake have been observed at doses >20 g/day in adults[19]. Natural exposure to sorbitol occurs during early life: sorbitol is produced by the maternal placenta and passed into fetal circulation in normal human pregnancies[16]. A recent study has shown that some of the polyols are in relatively high concentration in human embryonic fluids, which suggests an important but yet unknown biologic role during early development[17]. Sorbitol has also been reported to be present in human milk albeit in unknown concentrations[18].

Lactitol and sorbitol/Galactitol are not new compounds and can also be found in other 2FL products:

- GRN 815 (Glycom 2'-FL/DFL): page 15: "The small remaining portion of the product consists mainly of other carbohydrate-type compounds structurally related to 2'-FL and DFL [e.g., 2'-fucosyl-D-lactulose, 3- fucosyllactose (3-FL), 2'-fucosyl-galactose, **lactitol**, 2'-fucosyllactitol, difucosyllactitol, glucose, isomaltose, **sorbitol** and fucosyl-fucosyllactose (FFL)]." NB: sum of other carbohydrates average = 2.2 +/- 0.1
- GRN 749 (Dupont 2FL): page 12/13: **Sorbitol/Galactitol (dulcitol)** mentioned in the "other carbohydrates" specification of < 6%; page 14 levels mentioned are 1.02 % to 1.55%
- Not mentioned in other GRAS notices

In the submitted GRAS Dossier, depending on the batch, the presence of Sorbitol/Galactitol (Dulcitol) in Amyris 2FL is reported at levels between 0.05 to 0.79% area.

Since no experimental toxicological data are available for Dulcitol (Galactitol), QSAR analyses was performed using DEREK Nexus v.6.0.1 and SARAH Nexus v.3.0.0 (Lhasa Ltd) and structural alerts were investigated with the OECD QSAR Toolbox v.4.2.

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[15] Le, A.S. and K.B. Mulderrig, Sorbitol and mannitol. FOOD SCIENCE AND TECHNOLOGY-NEW YORK-MARCEL DEKKER-, 2001: p. 317-334.

[16] Brusati, V., et al., Fetal and maternal non-glucose carbohydrates and polyols concentrations in normal human pregnancies at term. Pediatr Res, 2005. 58(4): p. 700-4.

[17] Jauniaux, E., et al., Polyol Concentrations in the Fluid Compartments of the Human Conceptus during the First Trimester of Pregnancy: Maintenance of Redox Potential in a Low Oxygen Environment. The Journal of Clinical Endocrinology & Metabolism, 2005. 90(2): p. 1171-1175.

[18] Cavalli, C., et al., Free Sugar and Sugar Alcohol Concentrations in Human Breast Milk. Journal of Pediatric Gastroenterology and Nutrition, 2006. 42(2): p. 215-221.

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Based on the results of these analyses, dulcitol is not expected to be genotoxic. No relevant structural alerts regarding genotoxicity were fired by the OECD Toolbox, and both DEREK and SARAH gave negative predictions for bacterial mutagenicity. In addition, no alerts regarding chromosomal damage or carcinogenicity were fired by DEREK, indicating an overall lack of genotoxic potential for this compound.

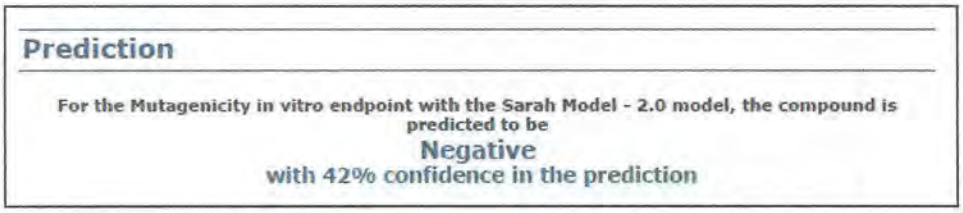

Figure 1: QSAR Prediction by SARAH

No indications for dermal sensitization or irritation potentials were given by DEREK and the OECD QSAR Toolbox.

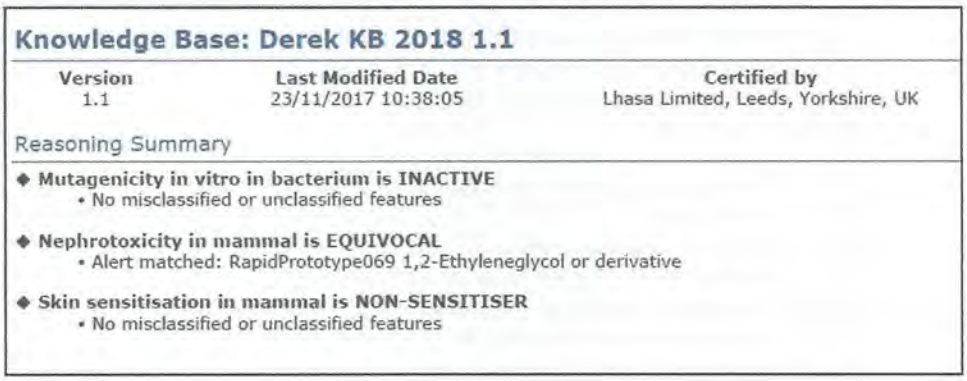

Figure 2: QSAR Prediction by DEREK

One alert regarding nephrotoxicity was fired by DEREK with a reliability of equivocal, suggesting a potential for kidney damage. This alert describes the nephrotoxicity of 1,2-ethyleneglycol and its derivatives and was derived using a proprietary data set of 731 chemicals, classified on the basis of the presence or absence of histopathologic lesions in the kidney in oral rat repeat dose studies mostly of 28-days duration. Eleven chemicals in this data set activated this rapid prototype alert and five of these were nephrotoxic. Since no further details are given by DEREK, the reliability of this alert remains unclear.

Renal toxicity was also suggested by the HESS repeated dose toxicity profiler implemented in the OECD QSAR toolbox due to structural similarity of dulcitol with Glycerin. Glycerin is a normally non-toxic, simple, viscous, polyol compound that is a colorless, odorless and sweet tasting. Glycerin causes metabolic imbalance of electrolytes [Munekazu Gemba, Folia Pharmacol. Jpn.,127, 433-440(2006)]. This alert is triggered for compounds with a structural

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similarity of >50% (DICE, atom centered fragments); dulcitol shows a similarity of 55%. Since Glycerin is widely used in the food industry as a sweetener and humectant and in pharmaceutical formulations, and since the similarity was just above the threshold of significance, this alert is considered to be of less relevance.

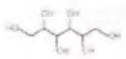
Filter endpoint tree...	1 [target]
Structure	
General Mechanistic	
DNA binding by OASIS	No alert found
DNA binding by OECD	No alert found
Estrogen Receptor Binding	Non binder, non cyclic structure
Protein binding by OASIS	No alert found
Protein binding by OECD	No alert found
Protein binding potency Cys (DPRA 13...	DPRA less than 9% (DPRA 13%)
Protein binding potency GSH	Not possible to classify according to these rules (G...
Protein binding potency Lys (DPRA 13%)	DPRA less than 9% (DPRA 13%)
Toxic hazard classification by Cramer	Low (Class I)
Toxic hazard classification by Cramer (...	Low (Class I)
Endpoint Specific	
Aquatic toxicity classification by ECOS...	Neutral Organics
Carcinogenicity (genotox and nongen...	No alert found
DART scheme	Not known precedent reproductive and developm...
DNA alerts for AMES by OASIS	No alert found
DNA alerts for CA and MNT by OASIS	No alert found
Eye irritation/corrosion Exclusion rules...	Group C Melting Point > 55 C
Eye irritation/corrosion Inclusion rules...	Inclusion rules not met
in vitro mutagenicity (Ames test) alert...	No alert found
in vivo mutagenicity (Micronucleus) alerts by ISS	H-acceptor-path3-H-acceptor
Protein binding alerts for Chromosom...	No alert found
Protein binding alerts for skin sensitiz...	No alert found
Protein binding alerts for skin sensitiz...	No alert found
Protein Binding Potency h-CLAT	No alert found
Skin irritation/corrosion Exclusion rule...	Group C Melting Point > 55 C
Skin irritation/corrosion Inclusion rule...	Inclusion rules not met
Empiric	
Lipinski Rule Oasis	Not bioavailable
Structure similarity	[90%,100%]
Toxicological	
Repeated dose (HESS)	Galactosamine (Hepatotoxicity) Alert Glycerin (Renal Toxicity) Alert

Figure 3: Structural profiling with the OECD QSAR Toolbox 4.2

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The OECD Toolbox fired an additional structural alert regarding hepatotoxicity triggered by the structural similarity of dulcitol to galactosamine. After acute treatment in the rat, galactosamine causes focal necrosis of hepatocytes[19]. However, while Galactosamine is bioavailable based on Lipinksi's Rule of Five and as shown by the observed systemic effects, dulcitol was classified as not bioavailable by Lipinski's Rule of Five, indicating that dulcitol is of much less bioavailability than galactosamine. The potential of dulcitol to induce hepatotoxicity of a similar magnitude is therefore regarded as unlikely, also taking the structural similarity of only 66.7% (DICE, atom centered fragments) into account.

No alerts regarding endocrine activity were triggered by DEREK, and no structural elements involved in reproduction toxicity were identified by the OECD Toolbox.

- **Lactitol**

Lactitol is not absorbed in the small intestine but is broken down by microflora in the large intestine[20]. Similar to sorbitol, doses producing a laxative effect are >10–20 g daily[24,21]. At doses of 250 to 400 mg/kg/d lactitol is used as a therapeutic laxative in in infancy and childhood functional constipation[22].

The body of safety evidence summarized here is all either publicly available, peer reviewed literature or generated from commonly used *in silico* QSAR analysis tools. As such, the safety information is appropriate for supporting a GRAS conclusion for Amyris' 2'-FL.

FDA Question-15. On pages 5 and 25, you indicate that the notified 2'-FL will be used in enteral tube feeds for persons ages 11 years and older; however, no safety discussion regarding this use is provided. Please provide a narrative that discusses the safe use of 2'-FL in enteral tube feeds.

Response-15. There are no published studies which assess the safety and/or tolerability of the addition of 2'-FL to enteral formulas. However, given the similarity of 2'-FL to other low or non-digestible carbohydrates (such as scFOS, lcFOS, GOS, etc.) with regard to ADME we can rely upon the literature for these substances to establish safety of 2'-FL use in enteral feeding. To

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[19] Wanda M. Haschek, Handbook of Toxicologic Pathology -2nd ed, volume 2, p187-225, Academic Press, USA(2002).

[20] Grimble, G.K., D.H. Patil, and D.B. Silk, Assimilation of lactitol, an 'unabsorbed' disaccharide in the normal human colon. Gut, 1988. 29(12): p. 1666-1671.

[21] Patil, D.H., G.K. Grimble, and D.B.A. Silk, Lactitol, a new hydrogenated lactose derivative: intestinal absorption and laxative threshold in normal human subjects. British Journal of Nutrition, 2007. 57(2): p. 195-199.

[22] Yachha, S.K., et al., Management of Childhood Functional Constipation: Consensus Practice Guidelines of Indian Society of Pediatric Gastroenterology, Hepatology and Nutrition and Pediatric Gastroenterology Chapter of Indian Academy of Pediatrics. Indian Pediatr, 2018. 55(10): p. 885-892.

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date, all evidence bears out the belief that risks of adverse effects from judicious addition of low-digestible carbohydrates to enteral formula GRAS. In fact in a recent report (O'Keefe 2018) the need to assess dietary fiber requirements in patients receiving enteral feeding is discussed.[23] The author states that enteral feeding "generally overlook the metabolic needs of the colon, and when combined with antibiotics may predispose patients to dysbiosis, bacterial overgrowth with pathogens such as *C. difficile*, and acute colitis". There is no mention in the article of any risk of adverse effects due to excessive intake of such fibers, and it is clear that the author does not believe that such risks are significant. Below we summarize a number of randomized clinical trials in which a variety of non-digestible carbohydrates were added to enteral feedings given to preterm infants, children, healthy adults, bed-ridden elderly adults, and patients hospitalized for a variety of serious medical conditions. The test articles include partially hydrolysed guar gum (PHGG), fructooligosaccharides (FOS), galactooligosaccharides (GOS), and GOS/FOS blends, with ingestion levels exceeding 20 g/day in adults and up to 3.5g/day in children. No adverse effects were reported in any study.

Rushdi *et al.* 2004 followed 20 adult IBS patients who were given either standard enteral nutrition or a formulation containing 2% (22g/L) PHGG, which resulted in an exposure of 22 to 37g PHGG/day for 2 days.[24] Supplementation with PHGG reduced reported gastrointestinal issues, though no statistical significance was determined.

Karaken *et al.* 2007 followed 30 adult patients with severe acute pancreatitis which required cessation of oral feeding.[25] Participants received either 0 or 24g/day scFOS for 2 days. Formulations were well tolerated with no reported adverse events. Khoshoo *et al.* 2010 describes 14 children 1-15 years old who received at least 75% of daily calories from a feeding tube.[26] Participants were given enteral formula containing 3.5 g FOS/L, with FOS intake approximately 3.5g/day for 2 weeks. There were no reported adverse events associated with consumption of FOS containing formula.

Akatsu *et al.* 2016 follows elderly patients receiving enteral feeding for 10 weeks containing GOS.[27] The amount of GOS was not discussed, but no adverse events were reported. Modi *et*

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[23] O'Keefe, SJD. (2018) The need to reassess dietary fiber requirements in healthy and critically ill patients. *Gastroenterol Clin North Am.* 47(1):219-29.

[24] Rushdi TA, Prichard C, Khater YH. (2004) Control of diarrhea by fiber-enriched diet in ICU patients on enteral nutrition: a prospective randomized controlled trial. *Clin Nutr* 23:1344-1352.

[25] Karakan et al. (2007) Comparison of early enteral nutrition in severe acute pancreatitis with prebiotic fiber supplementation versus standard enteral solution: a prospective randomized double-blind study. *World J Gastroenterol.* 13:2733- 2737.

[26] Khoshoo V, Sun SS, Storm H. (2010) Tolerance of an enteral formula with insoluble and prebiotic fiber in children with compromised gastrointestinal function. *J Am Diet Assoc* 110:1728-1733.

[27] Akatsu et al. (2016) Enhanced vaccination effect against influenza by prebiotics in elderly patients receiving enteral nutrition. *Geriatr Gerontol Int.* 16:205-213.

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al. 2010 followed 77 preterm infants (gestational age <33 weeks) receiving enteral feeding for up to 8 weeks.[28] Patients received enteral formula containing 8 g/L scGOS/lcFOS (9:1) or a control formula without added fiber. There were no differences in tolerance overall between formulas, though the authors report an increase in tolerance for the most immature infants with the addition of the test article. No differences in growth parameters were reported, and no adverse events related to the test article were recorded.

While this is not an exhaustive review of the available literature surrounding addition of low/non-digestible carbohydrates to enteral formulas, it is nonetheless representative of the available literature. No reports which reported negative effects of enteral formula supplemented with such carbohydrates was found. However, we are aware of Tarleton 2013, in which the authors discuss comorbidities which could complicate use of fiber in enteral feeding. The authors observe that two medical disorders, namely bowel ischemia and severe dysmotility, could result in adverse events for patients receiving enteral formula supplemented with low digestible carbohydrates. However, we would note that enteral feeding patients are nearly always under the direct supervision of a physician who would be aware of these medical conditions, if present, and would prescribe enteral formulas without such supplementation. Taken as a whole, the available literature clearly indicates that use of low-digestible carbohydrates, such as 2'-FL, is GRAS for its intended purpose of use in enteral formulas.

FDA Question-16. On page 33, you state, "The addition of Amyris's 2'-FL in infant formula will enable the infant formula to more closely approximate the composition of human milk." Please provide a clarification for this statement given that human milk is a complex biological fluid containing over 150 human milk oligosaccharides, not just 2'-FL.

Response-16. This statement was not intended to imply that addition of 2'-FL to traditional infant formula simulates human milk. It was merely intended to convey that 2'-FL is one (of many) components of human milk and therefore addition of 2'-FL to infant formula is an incremental step towards human milk.

FDA Question-17. On page 47, you state, "Although no tolerance data are available from clinical studies, the levels of intake are not expected to result in any tolerance issues." This statement implies that clinical studies performed with 2'-FL do not provide any tolerance data. Please provide a clarification for this statement.

Response-17. This statement was intended to convey that no maximum tolerance (*i.e.,* a NOAEL) was established by these studies. Clearly, these studies indicate that 2'-FL is well tolerated at the levels indicated in the studies.

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[28] Modi et al. (2010) A randomized, double-blind, controlled trial of the effect of prebiotic oligosaccharides on enteral tolerance in preterm infants. *Pediatr Res* 68:440-445.

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FDA Question-18. Please provide an updated literature search on the safety of 2'-FL. Please indicate search terms utilized, databases searched, and the date through which the search was conducted.

Response-18. An updated literature search was conducted on October 12, 2021 which was intended to cover the period of time between submission of Amyris' GRAS Notice (January 2020) and submission of the attached responses. The following databases were searched: Google Scholar, Pubmed Central, Toxline, and ChemID*plus* Advanced. The following search terms were used, alone or together: "2'-FL", "2'-fucosyllactose", "fucosyllactose", "human milk oligosaccharide", "HMO", "toxicology", "clinical trial", and "randomized controlled trial". Relevant studies identified by the above-described search are summarized below.

Phipps *et al.* 2020 describes *in vitro* genotoxicity and subchronic studies in neonatal rats using a mixture of two HMOs: 2'-FL and lacto-N-fucopentaose.[29] The test article in both genotoxicity tests and the 90-day study was a mixture of 59% LNFP, 32% 2'-FL, and 9% other carbohydrates. This mixture was negative for genotoxicity in a bacterial reverse mutation assay and *in vitro* mammalian micronucleus assay. For the 90-day study, neonate Sprague Dawley rats were fed by gavage either vehicle, 1,000, 3,000, or 5,000 mg/kg bw/day of the test article with another group receiving 5,000 mg/kg bw/day of the reference control (oligofructose). Dosing began at day 7 of life and continued for 90 days. No test article related adverse effects were noted on clinical observations, body weight, food consumption, clinical pathology, and organ weights at any dose. The authors established that the highest dose tested (5,000 mg/kg bw/day) was the NOAEL for the study, equating to a 2'-FL dose of 1,600 mg/kg bw/day.

Leung *et al.* 2020 describes a double-blind, controlled study in which 461 children 1-2.5 years were randomly assigned to either 1) control (standard milk formula), 2) milk formula containing immunoglobulins, lactoferrin, TGF-b, 2'-FL and milk fat, 3) milk formula containing lower levels of bioactive proteins that formula 2, or 4) milk formula containing 2'-FL at the same level as formula #2.[30] The authors state that the components found in formula #2 are present at levels comparable to human milk but do not report specific levels. All interventions lasted for 6 months, and no adverse events related to the formulas were reported for any group.

In Vandenplas *et al.* 2020, 215 infants who were fed only infant formula who were ≤ 14 days old were randomized into control (formula with 0.8g/100 mL scGOS/lcFOS 9:1) or formula containing 0.8g/100 mL scGOS/lcFOS (9:1), 0.1g/100 mL 2'-FL, milk fat (unspecified amount) and

---

[29] Phipps KR, et al. (2020) Genotoxicity and neonatal subchronic toxicity assessment of a novel mixture of the himan-identical milk oligosaccharides lacto-N-fucopentose I and 2'-fucosyllactose. *J of Applied Toxicol.* 41(4): 632-49.

[30] Leung TF, et al. (2020) A randomized controlled trial of different young child formulas on upper repiratory and gastrointestinal tract infections in Chinese toddlers. *Pediatr Allergy Immunol.* 31(7):745-54.

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3'-galactosylactose (unspecified amount) until 17 weeks of age.[31] 215 infants were enrolled in the trial and were assessed for growth, gastrointestinal symptoms, and safety monthly. There were no significant differences in weight gain, length, or head circumference when comparing treatment groups. Further, there were no significant differences in reports of adverse events or gastrointestinal tolerance between treatment groups. The authors conclude that the results of the study support that formulas containing 2'-FL support adequate growth and are well-tolerated.

Finally, Ramirez-Farias *et al.* 2021 enrolled 48 infants (0-60 days old) with suspected food protein allergy, persistent feeding intolerance, or other conditions indicating use of extensively hydrolyzed formula (eHF) was appropriate for a two-month trial.[32] Due to the nature of the underlying conditions of the infants enrolled in this study, no control group or randomization was employed. 36 of the 48 participants completed the study. The study reported a statistically significant improvement in weight for age Z-score (i.e. weight gain) at day 60 of the trial when compared to day 1. Further, all participants who presented with persistent symptoms at day 1 (diarrhea, constipation, vomiting, etc.) either remained the same, improved, or completely resolved by day 60. Adverse events were reported in 15 (32%) of enrolled infants, and all were minor (dermatitis, gastrointestinal reflux, spit-up, etc.) and deemed to be not related to treatment. The authors concluded that the results of the study demonstrate that 2'-FL was safe and well-tolerated by the target population.

These studies, together with the previously submitted information, clearly indicate that 2'-FL is safe and well tolerated for the intended target populations.

FDA Question-19. You provide several appendices, but there is limited mention in the narrative of these appendices. We request that you provide additional context to these in the notice, in addition to the following:

FDA Question-19a. Appendix D (pages 28-39, chromatographic results) appears to show the results of analyses of standards and possibly a mixed standard (page 37 "oligo-mix"). The analysis of a sample of the notified ingredient is not shown. Please address.

Response-19a. All of the chromatographic results in Appendix D contain results of the analyses of standards for peak identification. The sample chromatographic results are attached in with the following document name:

---

[31] Vandenplas Y, et al. (2020) A partly fermented infant formula with postbiotics including 3'-GL, specific oligosaccharides, 2'-FL, and milk fat supports adequate growth, is safe and well-tolerated in healthy term infants: a double-blind, randomized, controlled, multi-country trial. *Nutrients*. 12:3560; doi:10.3390/nu12113560.

[32] Ramirez-Farias C., Baggs GE, and Marriage BJ. (2021) Growth, tolerance, and compliance of infants fed an extensively hydrolyzed infant formula with added 2'-FL fucosyllactose (2'-FL) human milk oligosaccharide. *Nutrients*. 13:186; doi.org/10.3390/nu13010186.

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Lot Number	Document Name
Lot H8163*	H8163 PP material.pdf
Lot H8452	Lot H8452 Prep 1.pdf
Lot H8561	Lot H8561 Prep 1.pdf
Lot H8750	Lot H8750 Prep 1.pdf
Lot H8781	Lot H8781 Prep 1.pdf

*Initial pilot plant manufacturing lot

FDA Question-19b. The peaks for 2'-FL (8.859 min, page 29) and 2'-FL in Oligomix (8.867 min, page 37) are unresolved. Please discuss if this was also observed in analyses of the 2'-FL ingredient and the identity of the co-purified component(s).

Response-19b. The chromatogram provided on page 29 is for a 2'-FL standard prepared in diluent. The chromatogram provided on page 37, is a mix of different carbohydrate standards with the same 2'-FL standard from page 29 spiked into the solution. The 2'-FL standard used for peak identification was injected at a high concentration causing poor peak shape of the 2'-FL peak in the chromatograms provided on page 29 and 37, however there are no unresolved peaks with the 2'-FL. Following the validated method, SOP00830, the samples and standards are analyzed at lower concentrations which corrects for the poor peak shape. In the analysis of the 2'-FL ingredient, there are no unresolved peaks at the 2'-FL retention time and the 2'-FL has a good peak shape. The regulatory sample chromatograms are provided in the response to question 19a.

FDA Question-19c. Please consider lowering the arsenic and mercury specifications (pages 41-44, Appendix E) to reflect results of batch analyses.

Response-19c. Amyris has considered revising the specification for arsenic and mercury per FDA's suggestion. The current specification for Arsenic is $\leq$0.2 mg/kg and the batch analyses report ND <0.005 mg/kg. Amyris has reviewed the manufacturing process and agree that Amyris can revise the limit for arsenic in the specification to $\leq$0.15 mg/kg. This update is reflected in Table 5b and 6. The current specification for Mercury is $\leq$0.1 mg/kg and the batch analyses report ND <0.002 mg/kg. Amyris is comfortable keeping the specification for Mercury at <0.1 mg/kg.

FDA Question-19d. Please consider lowering the specification for cobalt (≤ 0.03 mg/kg, pages 41-44, Appendix E), to reflect the results reported (<0.01 mg/kg).

Response-19d. Amyris has considered revising the specification for cobalt per FDA's suggestion. The batch analyses report cobalt is not detected in the finished product at <0.01 mg/kg. Amyris

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has reviewed the manufacturing process and are comfortable keeping the limit for cobalt in the specification at ≤0.03 mg/kg. This update is reflected in Table 5b and 6.

FDA Question-19e. Appendix G provides Amino Acid Content Analysis (pages 51-59, Appendix G). Ala, Glu, Phe, Pro, Thr were determined to be present at levels from <0.01-0.11% (Method AOAC 982.30). Please address how you use these analyses to support your safety determination.

Response-19e. A protein specification is proposed to consider residual levels and to close the mass balance. This amino acid analysis was performed as part of the product characterization. Achieving mass balance and complete product characterization supports the safety of the product.

FDA Question-20. As a general comment, although the GRAS Panel statement mentions medical foods (page 85, Appendix J), we did not consider the foods described for tube or enteral feeding for ages 11 and above to be medical foods.

Response-20. Amyris agrees with the FDA that the foods described for tube or enteral feeding for ages 11 and above are not to be considered as medical foods.

The responses provided herein aims to completely address the FDA questions. Please do not hesitate to contact the undersigned for any further information.

Sincerely,

[redacted]

Fernando Garcia
Vice President, Global Regulatory Affairs

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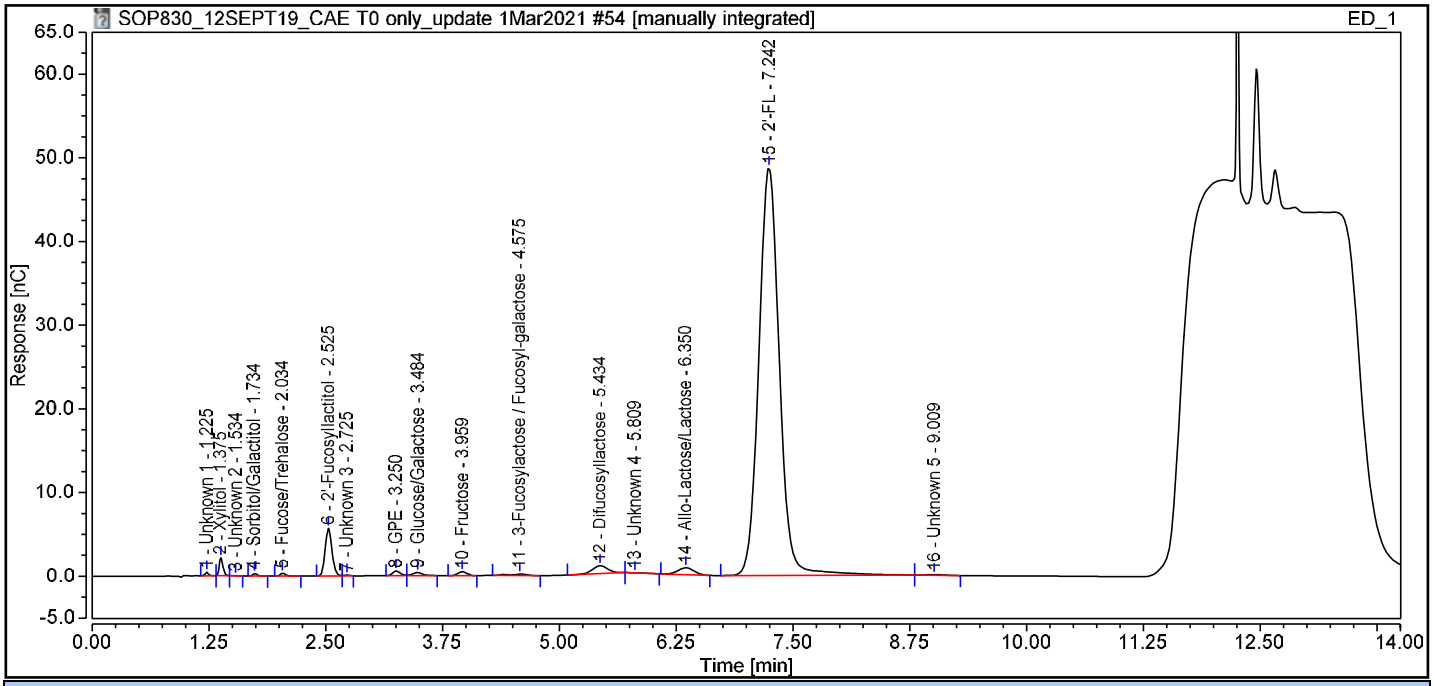
Chromatogram and Results

Injection Details

Injection Name:	**Lot H8452 Prep 1**	*Run Time (min):*	**14.00**
Vial Number:	**BB2**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars-regulatory**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Oct/19 11:44**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

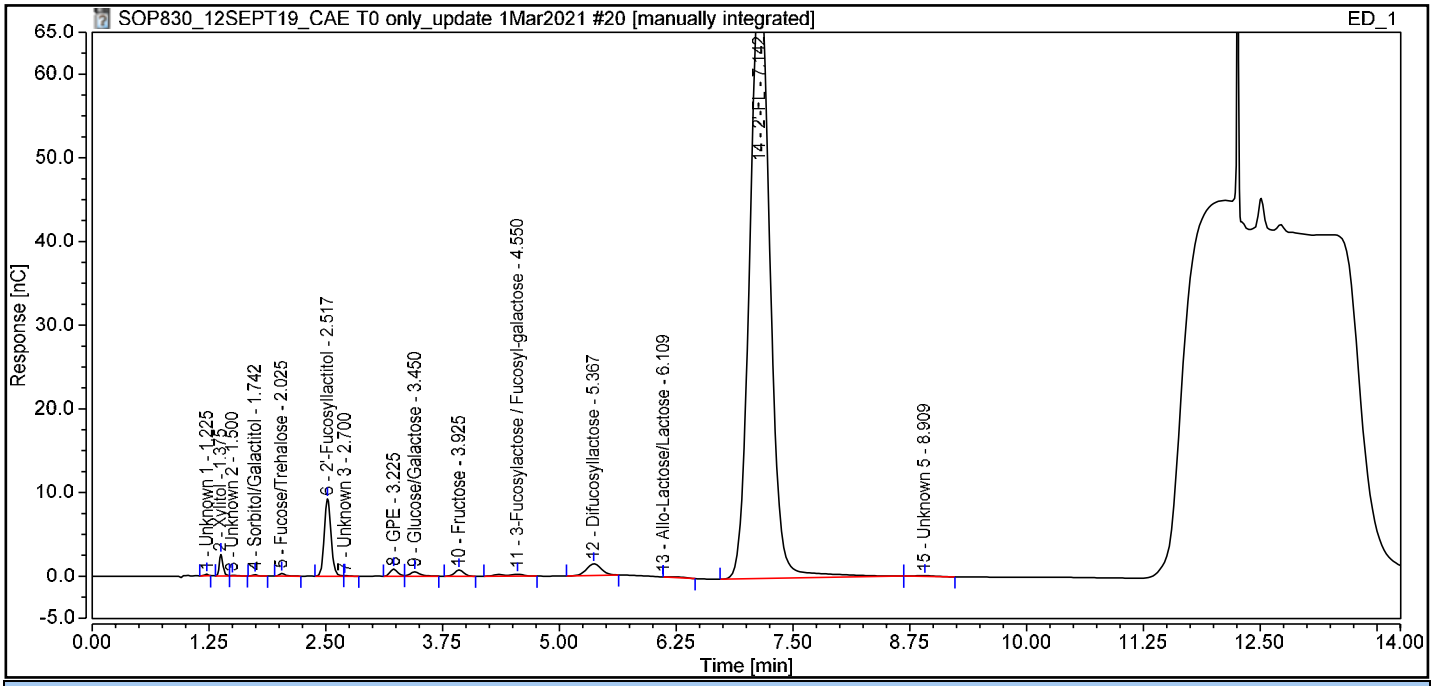
No.	Peak Name	Retention Time min	Rel. Ret.Time. (2'-FL)	Area nC*min	Height nC	Relative Area %	Relative Height %
1	Unknown 1	1.225	0.17	0.020	0.435	0.15	0.71
2	Xylitol	1.375	0.19	0.100	2.165	0.73	3.54
3	Unknown 2	1.534	0.21	0.002	0.030	0.02	0.05
4	Sorbitol/Galactitol	1.734	0.24	0.017	0.303	0.13	0.50
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	Fucose/Trehalose	2.034	0.28	0.024	0.341	0.18	0.56
6	2'-Fucosyllactitol	2.525	0.35	0.481	5.701	3.54	9.31
7	Unknown 3	2.725	0.38	0.006	0.082	0.05	0.13
8	GPE	3.250	0.45	0.055	0.572	0.41	0.93
9	Glucose/Galactose	3.484	0.48	0.045	0.375	0.33	0.61
10	Fructose	3.959	0.55	0.054	0.467	0.40	0.76
11	3-Fucosylactose / Fucosyl	4.575	0.63	0.039	0.168	0.29	0.28
12	Difucosyllactose	5.434	0.75	0.184	0.943	1.36	1.54
13	Unknown 4	5.809	0.80	0.004	0.021	0.03	0.03
14	Allo-Lactose/Lactose	6.350	0.88	0.163	0.824	1.20	1.35
15	2'-FL	7.242	1.00	12.381	48.720	91.09	79.60
16	Unknown 5	9.009	1.24	0.015	0.062	0.11	0.10
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:				**13.592**	**61.209**	**100.00**	**100.00**

Chromatogram and Results

Injection Details

Injection Name:	**Lot H8561 Prep 1**	*Run Time (min):*	**14.00**
Vial Number:	**BA1**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars-regulatory**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**22/Oct/19 19:35**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

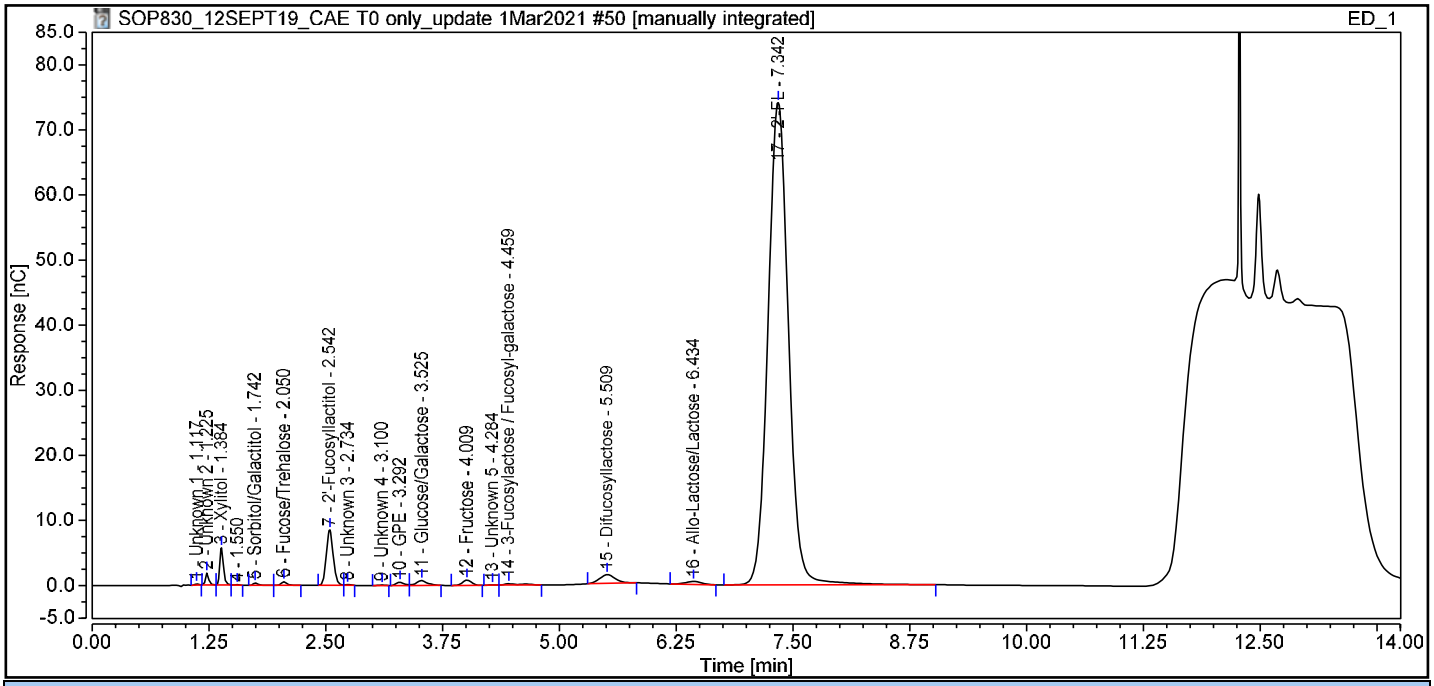
No.	Peak Name	Retention Time min	Rel. Ret.Time. (2'-FL)	Area nC*min	Height nC	Relative Area %	Relative Height %
1	Unknown 1	1.225	0.17	0.010	0.201	0.05	0.23
2	Xylitol	1.375	0.19	0.117	2.539	0.59	2.84
3	Unknown 2	1.500	0.21	0.006	0.080	0.03	0.09
4	Sorbitol/Galactitol	1.742	0.24	0.010	0.176	0.05	0.20
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	Fucose/Trehalose	2.025	0.28	0.023	0.324	0.12	0.36
6	2'-Fucosyllactitol	2.517	0.35	0.776	9.236	3.93	10.33
7	Unknown 3	2.700	0.38	0.005	0.077	0.03	0.09
8	GPE	3.225	0.45	0.083	0.867	0.42	0.97
9	Glucose/Galactose	3.450	0.48	0.066	0.535	0.33	0.60
10	Fructose	3.925	0.55	0.086	0.746	0.43	0.83
11	3-Fucosylactose / Fucosyl	4.550	0.64	0.063	0.228	0.32	0.26
12	Difucosyllactose	5.367	0.75	0.257	1.393	1.30	1.56
n.a.	Unknown 4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
13	Allo-Lactose/Lactose	6.109	0.86	0.015	0.000	0.08	0.00
14	2'-FL	7.142	1.00	18.199	72.919	92.20	81.55
15	Unknown 5	8.909	1.25	0.023	0.093	0.12	0.10
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:				**19.738**	**89.414**	**100.00**	**100.00**

Chromatogram and Results

Injection Details

Injection Name:	**Lot H8750 Prep 1**	*Run Time (min):*	**14.00**
Vial Number:	**BA8**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars-regulatory**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**25/Oct/19 10:43**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

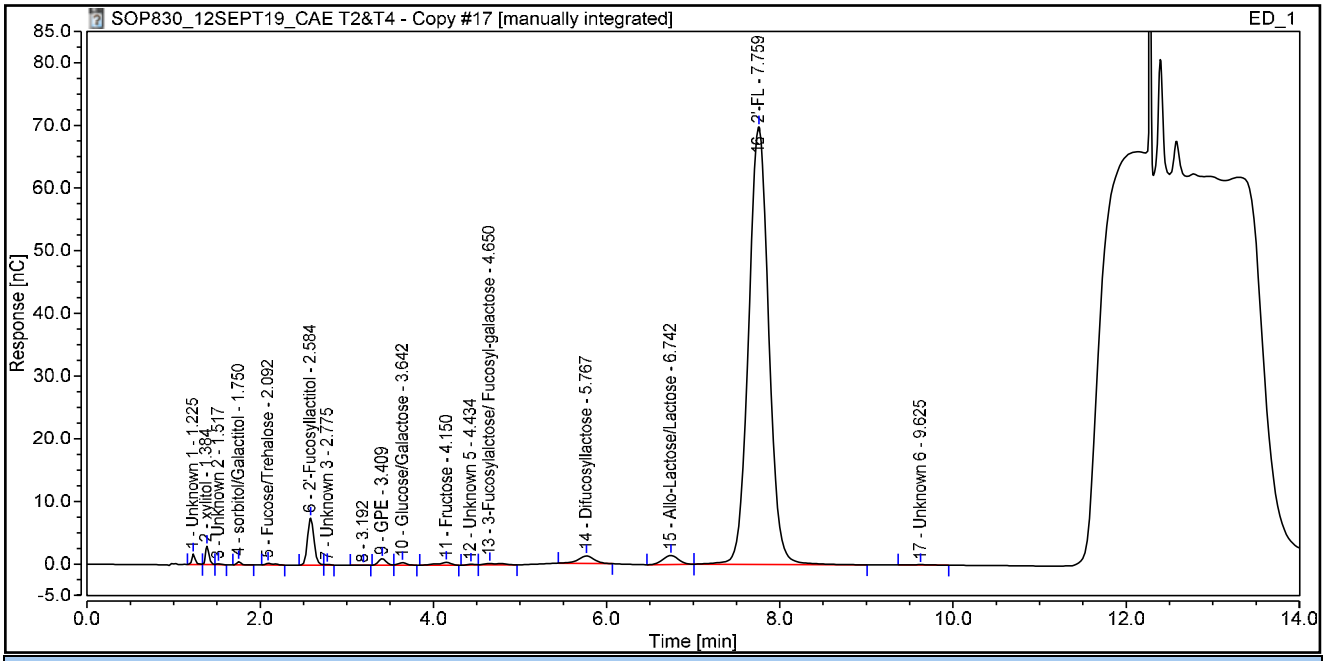
No.	Peak Name	Retention Time min	Rel. Ret.Time. (2'-FL)	Area nC*min	Height nC	Relative Area %	Relative Height %
1	Unknown 1	1.117	0.15	0.009	0.204	0.04	0.21
2	Unknown 2	1.225	0.17	0.074	1.782	0.36	1.86
3	Xylitol	1.384	0.19	0.269	5.693	1.30	5.95
4		1.550	0.21	0.003	0.058	0.01	0.06
5	Sorbitol/Galactitol	1.742	0.24	0.022	0.371	0.11	0.39
n.a.	Mannitol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
6	Fucose/Trehalose	2.050	0.28	0.039	0.581	0.19	0.61
7	2'-Fucosyllactitol	2.542	0.35	0.727	8.544	3.52	8.93
8	Unknown 3	2.734	0.37	0.009	0.118	0.04	0.12
9	Unknown 4	3.100	0.42	0.006	0.073	0.03	0.08
10	GPE	3.292	0.45	0.051	0.510	0.25	0.53
11	Glucose/Galactose	3.525	0.48	0.105	0.769	0.51	0.80
12	Fructose	4.009	0.55	0.095	0.812	0.46	0.85
13	Unknown 5	4.284	0.58	0.003	0.040	0.02	0.04
14	3-Fucosylactose / Fucosyl	4.459	0.61	0.045	0.188	0.22	0.20
15	Difucosyllactose	5.509	0.75	0.257	1.333	1.25	1.39
16	Allo-Lactose/Lactose	6.434	0.88	0.091	0.464	0.44	0.49
17	2'-FL	7.342	1.00	18.817	74.088	91.25	77.48
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:				**20.622**	**95.628**	**100.00**	**100.00**

Chromatogram and Results

Injection Details

Injection Name:	**Lot H8781 Prep 1**	*Run Time (min):*	**14.00**
Vial Number:	**BD1**	*Injection Volume:*	**5.00**
Injection Type:	**Unknown**	*Channel:*	**ED_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**SOP00830-6**	*Bandwidth:*	**n.a.**
Processing Method:	**Radha 3 sugars - Copy**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**18/Oct/19 08:53**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Rel. Ret.Time. (2'-FL) min	Area nC*min	Height nC	Relative Area %	Relative Height %
1	Unknown 1	1.225	0.16	0.076	1.674	0.37	1.90
2	xylitol	1.384	0.18	0.136	2.916	0.66	3.32
3	Unknown 2	1.517	0.20	0.009	0.152	0.04	0.17
4	sorbitol/Galactitol	1.750	0.23	0.030	0.538	0.15	0.61
5	Fucose/Trehalose	2.092	0.27	0.029	0.286	0.14	0.32
6	2'-Fucosyllactitol	2.584	0.33	0.651	7.508	3.17	8.54
7	Unknown 3	2.775	0.36	0.007	0.091	0.03	0.10
8		3.192	0.41	0.002	0.025	0.01	0.03
9	GPE	3.409	0.44	0.108	1.059	0.53	1.21
10	Glucose/Galactose	3.642	0.47	0.046	0.401	0.22	0.46
n.a.	Unknown 4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
11	Fructose	4.150	0.53	0.083	0.474	0.40	0.54
12	Unknown 5	4.434	0.57	0.010	0.104	0.05	0.12
13	3-Fucosylalctose/ Fucosyl	4.650	0.60	0.049	0.184	0.24	0.21
14	Difucosyllactose	5.767	0.74	0.256	1.187	1.24	1.35
15	Allo-Lactose/Lactose	6.742	0.87	0.292	1.413	1.42	1.61
16	2'-FL	7.759	1.00	18.745	69.802	91.23	79.43
17	Unknown 6	9.625	1.24	0.017	0.063	0.08	0.07
n.a.	6'-galactosyllactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:				**20.548**	**87.879**	**100.00**	**100.00**

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Confidential Business Information

Amyris Response to FDA Deficiencies Letter – GRN 000987

Response Information Provided as Confidential Business Information

15 October 2021

FDA Question-3. On page 9, you state that "promoters and terminators used to express the genes are native to S. cerevisiae, and include but are not limited to, promotors of GAL1 and GAL10 proteins, and terminators of PGK1 and CYC1." Please describe the names of the additional native promoters and terminators used to generate the production strain.

(b) (4)

[redacted]

FDA Question-4a. We request additional information about the genes used to create the production strain: Define the gene that is derived from the assembled metagenome (page 9) and further describe why you believe that gene is not likely to be either toxigenic or allergenic, preferably at the genetic level.

(b) (4)

[redacted]

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FDA Question-4b. On page 10 you note "The genes used to create the production strain are found in Table 4." (You have described the taxonomy in Table 3). Please correct this reference and also add the source organism for each inserted gene. The enzymes and their technical effects are listed in Table 3 (page 11). Please clarify the role of dihydrofolate reductase in the production of 2'-FL.

(b) (4)

[redacted]

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