Lane Toxicology Consulting, LLC
4423 Snowcap Ln, Broomfield, CO 80023
201/452-5816 (Phone) | 303/284-3030 (Fax)

September 14, 2020

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

RECEIVED
SEP 16 2020
OFFICE OF FOOD ADDITIVE SAFETY

Dear Dr. Gaynor:

Re: GRAS Notice for RG-I-enriched carrot fiber

In accordance with 21 CFR §170.203 through 170.255, NutriLeads, B.V., as the notifier, through me as its agent, hereby provides notice that the addition of RG-I-enriched carrot fiber to conventional food and beverage products is exempt from the premarket approval requirement of the federal Food, Drug, and Cosmetic Act because NutriLeads has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, one copy of the dossier setting forth the basis for NutriLeads' GRAS conclusion, as well as a consensus opinion of an independent panel of experts, and one signed copy of the conclusion from each member of an independent Expert Panel are provided. Additionally, an electronic copy (on CD) containing the GRAS dossier, all data and information supporting the company's conclusion, and the signed statements of the independent Expert Panel is enclosed. The enclosed electronic files were scanned for viruses using Microsoft Defender prior to submission and are thus certified as being virus-free.

Should you have any questions or concerns regarding this GRAS notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

Richard W. Lane, PhD
Lane Toxicology Consulting, LLC
Email: richardlanephd@gmail.com
Tel: 201-452-3816

SAFETY EVALUATION DOSSIER SUPPORTING A GENERALLY RECOGNIZED AS SAFE (GRAS) CONCLUSION FOR RG-I-ENRICHED CARROT FIBER

PREPARED FOR:

NutriLeads B.V.
Bronland 12N
6708 WH Wageningen, Netherlands

PREPARED BY AND CONTACT FOR TECHNICAL AND OTHER INFORMATION:

Richard W Lane, PhD
Lane Toxicology Consulting, LLC
4423 Snowcap Ln
Broomfield, CO 80023

September 14, 2020

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1. SIGNED STATEMENTS AND CERTIFICATION

This GRAS Notice is hereby submitted in accordance with Title 21 of the U.S. Code of Federal Regulations (CFR), Chapter I, Subchapter B, Part 170, Subpart E, to inform the Agency that the proposed uses of RG-I-enriched carrot fiber described herein are considered to be generally recognized as safe (GRAS).

A. Name and Address of Notifier

NutriLeads B.V., through its agent Lane Toxicology Consulting, LLC, hereby notifies the U.S. Food and Drug Administration (FDA) that the use of RG-I-enriched carrot fiber described below and which meets the specifications described herein, is exempt from the pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act because NutriLeads B.V. has determined that such uses are GRAS through scientific procedures.

Ruud Albers, Ph.D.
NutriLeads, B.V.
Bronland 12N
6708 WH Wageningen, Netherlands

B. Name of GRAS Substance

The subject of this GRAS Notice is RG-I-enriched carrot fiber. The name is abbreviated as "cRG-I" in this Notice.

C. Intended Use

RG-I-Enriched carrot fiber is proposed for use in multiple food categories (described below) at levels ranging from <1-22%, depending on the product. The physical or technical effect of cRG-I is to add a source of nutrients (i.e., fiber), in accordance with 21 CFR §170.3(o)(20) in selected conventional foods and beverages for which no standard of identity exists.

D. Basis for GRAS Conclusion

Regulatory Framework

The regulatory framework for determining whether a substance can be considered GRAS in accordance with section 201(s) (21 U.S.C. § 321(s)) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 et. Seq.) is set forth at 21 CFR §170.30, which states:

General recognition of safety may be based only on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food.

General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies which may be corroborated by unpublished studies and other data and information.

GRAS Conclusion

The basis for the GRAS conclusion for the intended uses of cRG-I is through scientific procedures in accordance with 21 CFR §170.30(a) and (b).

E. Availability of Information

Data and information that serve as the basis for the GRAS conclusion that are not appended to this Notice will be sent to FDA upon request or are available for FDA's review and copying at reasonable times from the Notifier or from Lane Toxicology Consulting, Broomfield, CO.

F. Certification of Completion

I hereby certify that, to the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes favorable information, as well as unfavorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the intended uses of cRG-I.

September 14, 2020

Date

Richard W Lane, PhD
President
Lane Toxicology Consulting, LLC
Broomfield, CO 80023
richardlanephd@gmail.com; 201-452-5816
Agent for NutriLeads, B.V.

2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

This section of the GRAS notice fulfills the requirements of 21 CFR §170.230 by providing information in regard to the GRAS material identity, method of manufacture, specifications, and physical or technical effect including product characteristics and analytical data.

The subject material is derived from the pectin fraction of carrot fiber. It consists mainly of the highly branched rhamnogalacturonan-I (RG-I) portion of the pectin molecules. The subject material is made in powder form containing not less than 75% (w/w) carbohydrates, of which at least 70% (w/w) is dietary fiber.

A. Trade or Common Name

The subject of this GRAS Notice is the enriched RG-I fraction of carrot pectin. It is denominated herein as "RG-I-enriched carrot fiber," also referred to as "cRG-I" (for carrot RG-I).

In external studies, the working denomination "NLXXX" was used for specific batches of cRG-I.

B. Chemical Name

Not applicable.

C. CAS Registry Number

Not applicable.

D. Molecular and Structural Formula

Not applicable.

RG-I-Enriched carrot fiber is derived from natural pectin, a plant-derived heteropolysaccharide. The stereochemistry of cRG-I is determined by the stereochemistry of these polymers, which are in turn determined by the stereospecificity of the enzymes involved in their biosynthesis. Due to the nature of raw material, cRG-I does not consist of a single, defined molecular structure with distinctive stereochemistry, but represents a mixture of rhamnogalacturonan-rich polysaccharides of complex arrangement. The complexity of cRG-I reflects its origin and there is no CAS number or defined structural formula assigned to this material.

E. Identity

RG-I-Enriched carrot fiber is obtained from the edible part of carrots after the juice is removed. A portion of that fiber, pectins, gums, and related polysaccharide substances, are mucilaginous polymers of sugar acids that hold plant cells together (Jackson, 2008). They commonly occur as complex branched chains. Pectin molecules are high-molecular-weight polysaccharides consisting of a backbone of two main covalently linked repeating structural units: the linear homogalacturonan ("HG") subunit and the highly branched rhamnogalacturonan-I ("RG-I") subunit. The HG subunits make up 65% of the pectin molecule and the RG-I subunits constitute approximately 20% to 35% (Mohnen, 1999; Mohnen, 2008). Two minor (<10%) units also occur: xylogalacturonan ("XGA") and rhamnogalacturonan-II ("RG-II") (Harholt *et al.*, 2010). The overall structure of a generalized pectin molecule is shown in this figure from Harholt *et al.* (2010):

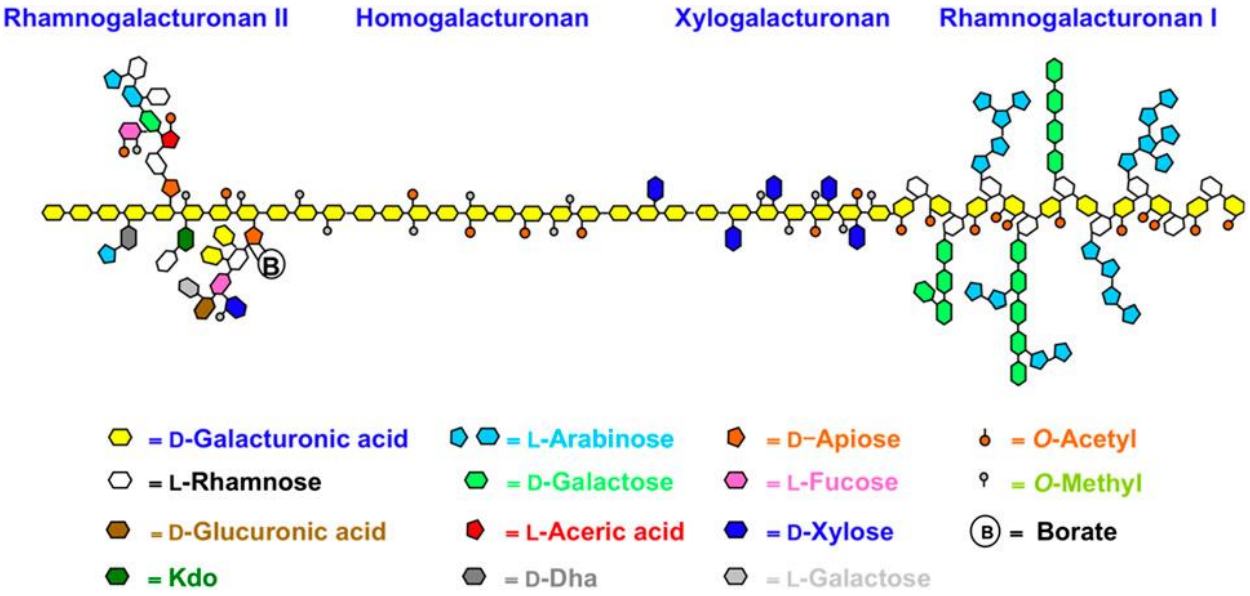

Figure 1. Schematic of pectin's structure. Pectin consists of four different types of connected polysaccharides with a schematic of their structures shown above. The HG and RG-I units are more abundant than the other components.
Kdo = 3-Deoxy-D-manno-2-octulosonic acid; D-Dha = 3-deoxy-D-lyxo-2-heptulosaric acid.

The HG unit consists of α-1,4-linked D-galacturonic acid monomers whereas the RG-I subunit has a backbone of the repeating disaccharide [-α-1,4-D-galacturonic acid-α-1,2-L-rhamnose-] (Harholt *et al.*, 2010). Depending on the plant species, the rhamnose residues in the RG-I backbone are substituted with β-1,4-D-galactan, branched arabinan α-1,5-linked L-arabinofuranose units with additional L-arabinofuranose side-chains or arabinogalactans (Coenen *et al.*, 2007).

The subject material is a hydrolysate of pectin from carrot pomace. It is produced by utilizing pectinase enzymes that partially hydrolyze the linear homogalacturonan backbone of pectin, including the XGA and RG-II regions, while retaining the branched RG-I structure which has a backbone of repeating disaccharide units of L-rhamnose and D-galacturonic acid (Harholt *et al.*, 2010). Subsequent ultrafiltration with a molecular weight cut off of >10 kDa eliminates smaller size molecules, including RG-II which has a molecular weight of approximately 4.8 kDa (Yapo, 2011). RG-II is unlikely to remain intact after enzyme treatment. The subject material, cRG-I, therefore consists mainly of the highly branched RG-I region of pectin.

RG-I-Enriched carrot fiber is characterized by its soluble dietary fiber content, the molecular weight pattern of the polymers and its monomer composition. The fiber fraction consists of RG-I polymers that are composed of uronic acids (max 35% w/w), rhamnose, arabinose and galactose, characteristic monosaccharides of the RG-I region (see Figure 1). Its molecular weight is between 10 and 1,000 kDa (Figures 5-8). Monosaccharides that are characteristic of the XGA and RG-II regions (xylose, fucose; Doco *et al.*, 1996) are present in very low amounts (<1%, see Table 3)

F. Production Process

The starting material for the production of cRG-I is carrot pomace, the carrot shavings produced from cut-and-peel carrot processing and the carrot juice industry (Arscott and Tanumihardjo, 2010). Carrot pomace is sourced externally from reputable suppliers such as GreenField (Carrot Fiber M20). The raw material meets strict specifications, undergoes analysis for contaminants (metals, pesticides, etc.), and its quality is appropriate for food use (see Product Data Sheet in Appendix I for composition). Carrot pomace is dispersed in water at a level of approximately 7-10% solids for processing.

The RG-I moiety is released from the insoluble carrot matrix material by a commercial, food-grade GRAS enzyme preparation (Pectinex® Ultra Mash, a blend of pectinases produced by submerged fermentation of selected strains of *Aspergillus aculeatus* and *Aspergillus niger*)[1] (see FDA, undated; FDA, 1985; supplier's certification in Appendix I). The enzymatic reaction uses two enzymes, pectin lyase (EC 4.2.2.10) and polygalacturonase (EC 3.2.1.15), to hydrolyze the pectin fraction of the carrot pomace. Together, the enzymes cleave α-(1,4)-glycosidic bonds, thereby degrading the HG backbone of the pectin molecule, while other glycosidic bonds, the α-(1,6) bonds in the branched sections remain unaffected.

Specifically, the pectin lyase ((1→ 4)-6-O-methyl-alpha-D-galacturonan lyase) cleaves (1→4)-alpha-D-galacturonan methyl ester bonds via β-elimination, resulting in oligosaccharides with 4-deoxy-6-

[1] Pectinase from *Aspergillus aculeatus* is covered under GRAS petition 5G0297. The FDA accepted and filed the petition on April 12, 1985 (FDA, 1985 and in Appendix I). Pectinase from *Aspergillus niger* is covered under GRAS Notice (GRN) 089 (FDA, 2001). Pectinase from *Aspergillus niger* is also covered by an FDA opinion letter (FDA, undated).

O-methyl-alpha-D-galact-4-enuronosyl groups at their non-reducing ends. Polygalacturonase is a pectin hydrolase that hydrolyzes the O-glycosyl bonds in pectin's polygalacturonan network, resulting in alpha-1,4-polygalacturonic residues. The combination of both enzymes in Pectinex® allows the efficient degradation of the linear sections while not substantially affecting the branched region of RG-I that is the major component of cRG-I. The reactions catalyzed by pectin lyase are presented in Figure 2:

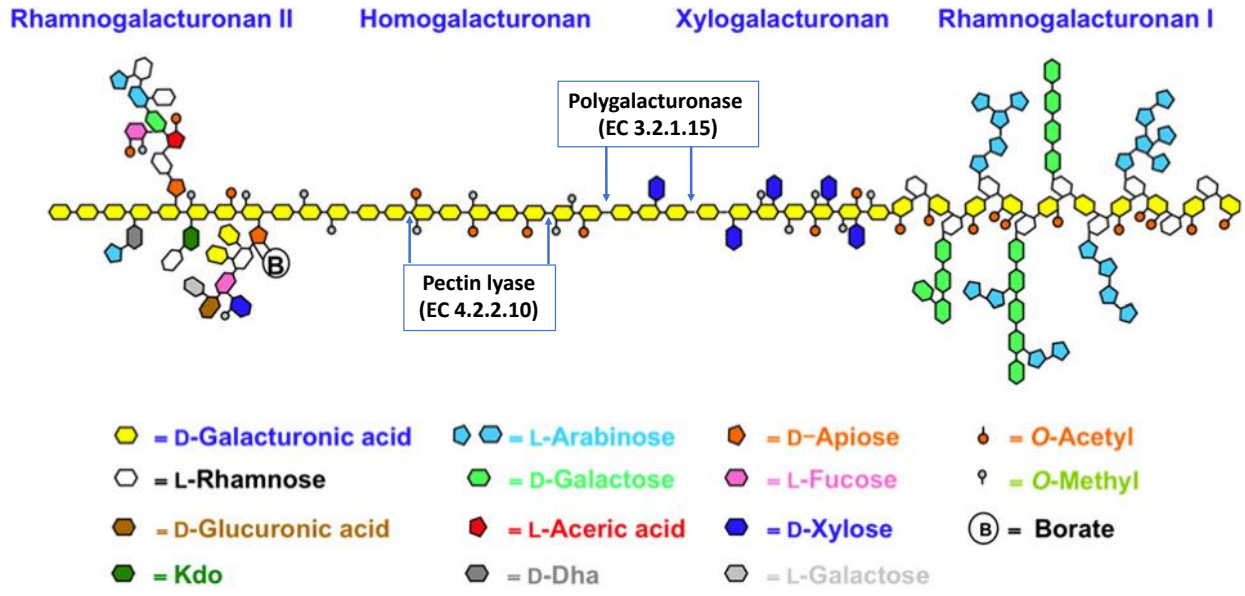

Figure 2: Activity of the enzymes used to manufacture cRG-I. Schematic structure of pectin from Harholt *et al.* (2010). See Figure 1 for abbreviations.

The enzyme mixture is added to the pomace mixture and hydrolysis is carried out for approximately 1.5-2 h at 44-46°C to allow for sufficient hydrolysis of the linear portion to pectin. This process isolates the branched section of the pectin, i.e., RG-I. The target molecule, RG-I, is water-soluble and subsequently recovered from the aqueous fraction.

Soluble and insoluble fractions are separated by decanting or a similar process. Approximately 45% of the dry matter of the starting material, the RG-I moiety together with remaining endogenous sugars, i.e. glucose, fructose, sucrose and enzymatic degradation products (other small sugars and oligosaccharides), are in the decanted aqueous phase. About 55% of the total solids of the starting material, mainly cellulosic matter, remains after decanting.

The recovered aqueous phase undergoes thermal treatment at 90°C for 30 sec to inactivate the enzymes (Duvetter *et al.*, 2009; Gonzalez and Rosso, 2011).

To remove the remaining insoluble material that might affect color and turbidity of final food products, the heat-treated aqueous phase is further clarified by centrifugation.

Rhamnogalacturonan-I, a high-molecular weight polysaccharide, is separated from low-molecular weight sugars and other small molecules by ultrafiltration. A polyethersulfone membrane (such as ST PES 10,000 DA Sanitary UF Membrane, Snyder Filtration; Appendix I) (21 CFR §177.2440) with a molecular weight cut-off of 10 kDa is used. Rhamnogalacturonan-I is contained in the filtration retentate and represents approximately 6% of the initial solids in the carrot pomace.

The retentate is concentrated by evaporation. The concentrated retentate is pasteurized at 90°C for 30 sec to ensure good microbial quality of the final product. The concentrated cRG-I extract is dried, passed through a sifter, and packaged in paper bags with a food-grade plastic liner (Appendix I) or other container suitable for holding a food ingredient.

A flow scheme showing the main manufacturing steps of cRG-I is given in Figure 3.

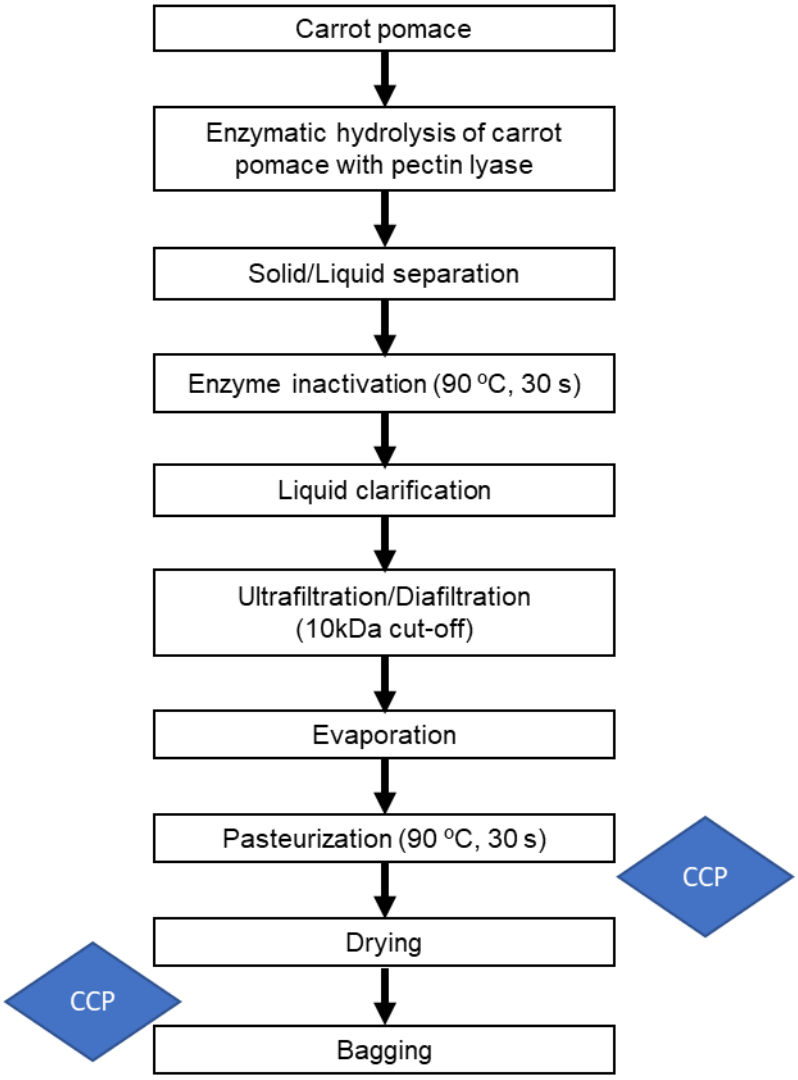

Figure 3. Manufacturing flow scheme of cRG-I indicating critical control points (CCP).

The first critical control point (CCP), pasteurization at 90°C, reduces potential living vegetative organisms in the product before drying. It results in an increased shelf life for the end product. It is a critical control point because it is the last heat treatment with log-reduction of microorganisms possible before drying.

The second CCP involves filtering the dried product before bagging. This step ensures that no foreign bodies are in the product. The powder passes through a sifter and is bagged in paper bags with a food-grade plastic liner or other suitable packaging. In addition, the filtering improves the quality and solubility of the product. This step also involves metal detection to uncover any metal particles that might pass through the filter.

After production, cRG-I is analyzed and only released when it complies with the specifications.

Appendix I contains product data sheets and additional information for the starting materials and processing aids used in the production process.

G. Product Characteristics

RG-I-Enriched carrot fiber is an off-white to beige powder with a bland taste and no appreciable odor.

H. Product Composition

RG-I-Enriched carrot fiber is a carrot-derived pectin fraction obtained after enzymatic hydrolysis. It contains at least 75% carbohydrates. The most abundant polymer-bound sugars are uronic acids (max 35% w/w), rhamnose, arabinose and galactose. These monomers are typical constituents of pectin. Minor amounts of other sugars (glucose, mannose, xylose, fucose) are also present in the final product. The only other material present in any notable amount is protein (<6%).

Analytical Methods and Contracted Laboratories
The composition and quality parameters of cRG-I were analyzed by two accredited third-party laboratories employing standard procedures and validated in-house methods. Both laboratories comply with regulations for Food Grade facilities, e.g., ISO 22000, and have HACCP programs in place. The quality certificates and accreditation documents for the third-party laboratories are available upon request.

Five independently manufactured batches, produced at two different facilities by different contract manufacturers, were analyzed.

Dietary fiber analyses were initially conducted following AOAC 991.43, but this method showed low reproducibility and high variation in the results. Therefore, additional analyses were performed following AOAC 2011.25, which proved to be a more reliable and accurate method to determine dietary fiber (Table 1). Some certificates of analysis will show one or both methods of analyzing dietary fiber content. The values in the summary of the analytical results in Table 1, below, were obtained using the latter method.

The results of the complete analytical and microbiological analyses of five batches of cRG-I are summarized in Tables 1-4. For the certificates of analysis, please refer to Appendix II.

Table 1. Analysis of the composition of five representative batches of cRG-I.

	Method	Units	Internal Specification	Batch number				
Batch number Manufacturing site				**NL91** NIZO	**NL100** NIZO	**NL176** MTL	**NL189** MTL	**NL204** MTL
Basic composition								
Appearance	Visual		Off white to beige powder	complies	complies	complies	complies	complies
Protein (N*6.25)	Kjeldahl	% (w/w)	Max 6	4.8	5.8	1.9	1.5	2.4
Fat	Soxhlet	% (w/w)	Max 2	1.18	1.18	< 0.2	0.27	< 0.2*
Salt	Calculated	% (w/w)	Max 3	0.35	0.43	1.0	2.5	0.53
Total free sugars (as glucose)	Luff Schoorl (method HEC6A)	% (w/w)		11.3	14.7	8.39	6.71	13.1
Total carbohydrates*	Calculated	% (w/w)	Min 80	80.0	80.21	86.96	85.39	85.66
Total dietary fiber	AOAC 2011.25	% (w/w)	Min 70	71.4	70.7	81.3	81	79.7
Insoluble fiber	AOAC 2011.25	% (w/w)	Max 3.5	3.5	0.9	< 0.4	< 0.4	< 0.4
Ash	500-550^{o}C	% (w/w)	Max 10	5.31	6.11	5.53	7.13	5.05
Moisture	103^{o}C, 4h	% (w/w)	Max 10	9.14	6.7	5.41	5.71	6.69
Minerals and ions								
Sodium	ICP-MS	mg/kg		1,400	1,700	4,000	10,000	2,100
Magnesium	ICP-MS	mg/kg		1,300	1,400	1,100	1,700	n.a.
Potassium	ICP-MS	mg/kg		15,000	20,000	14,000	14,000	n.a.
Calcium	ICP-MS	mg/kg		9,900	8,400	7,600	9,700	7,400
Phosphorous	ICP-MS	mg/kg		690	930	180	140	n.a.
Water activity								
a_w		[-]	< 0.6	n.a.	0.430	0.318	0.213	0.247

Heavy metals								
Arsenic	ICP-MS	mg/kg	Max 0.1	< 0.1	< 0.1	< 0.02	< 0.02	< 0.02
Cadmium	ICP-MS	mg/kg	Max 1.0	0.29	0.27	0.22	<0.01	0.21
Copper	ICP-MS	mg/kg		1.8	1.9	1.6	1.1	1.9
Mercury	ICP-MS	mg/kg	Max 0.1	< 0.005	< 0.005	< 0.001	< 0.001	0.0014
Lead	ICP-MS	mg/kg	Max 1.5	0.34	0.38	1.5	0.76	0.91
Zinc	ICP-MS	mg/kg		18	17	15	0.7	15

* for calculation of total carbohydrates, a fat content of 0.2 was assumed.

n.a. not assessed

Table 2. Microbial analysis of five representative batches of cRG-I.

	Method	Units	Internal Specification	Batch number				
Batch number Manufacturing site				**NL91** NIZO	**NL100** NIZO	**NL176** MTL	**NL189** MTL	**NL204** MTL
Microbiological analysis								
Total plate counts	ISO 4833-1	cfu/g	< 10,000	3100	2400	1300	7300	650
Yeasts	ISO 7954 (1987)	cfu/g	< 100	< 10	< 10	< 10	< 10	< 10
Molds	ISO 7954 (1987)	cfu/g	< 100	< 10	< 10	< 10	< 10	220
Enterobacteriaceae	ISO 21528-2	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10
Salmonella spp.	ISO[a,b,c]	cfu/25g or cfu/50g	n. d.	n. d.	n. d.	n.d.	n. d.	n. d.
Escherichia coli	ISO 16649-2	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10

n. d. not detected

[a] ISO 6579 - (cfu/50g)

[b] ISO 6579 (GEN 25/05-11/08) - (cfu/25g)

[c] TRA 02/08-03/0 Equal AFNOR TRA 02/08-03/01 – (cfu/50g).

Monosaccharide Composition of cRG-I

In addition to understanding the quality of cRG-I, the monosaccharide composition is of value to confirm the material obtained from the manufacturing process is the same as that described in the literature.

Generally, analysis of sugars in polysaccharides is achieved by using chromatographic measurements after depolymerization using an acid hydrolysis reaction. However, application of this method to polysaccharides with uronic acids (sugars in which the terminal carbon's hydroxyl group has been oxidized to a carboxylic acid), such as pectin, presents problems because uronic acid, once released after hydrolysis, forms lactones irreproducibly (Blake and Richards, 1968). Also, uronic acids are often part of very acid resistant glycosidic linkage (De Ruiter *et al*., 1992). To overcome these issues it is generally necessary to use another depolymerization method. For cRG-I the monosaccharide composition was obtained employing a methanolysis method described by De Ruiter *et al.* (1992).

The RG-I region of pectin is characterized by high amounts of rhamnose, arabinose and galactose (see Figure 1). The monomer distribution of cRG-I generated after hydrolysis, shown in Table 3 and illustrated by Figure 4, confirms the characteristic composition of RG-I. In addition to the monosaccharides that are characteristic for the RG-I region in pectin, minor amounts of other monosaccharides were also identified. These probably originate from other parts of the pectin molecule and/or from non-pectin polysaccharides present in the raw material.

Table 3. Relative monosaccharide content of five batches of cRG-I after methanolysis.

Fraction	Sugar	NL91	NL100	NL176	NL189	NL204
Monosaccharides characteristic for RG-I from pectin	Rhamnose	11.3%	9.0%	13.4%	14.6%	14.1%
	Arabinose	23.2%	17.9%	21.1%	25.7%	21.7%
	Galactose	17.0%	13.8%	15.8%	22.0%	17.9%
	Uronic acids	24.1%	28.6%	28.9%	28.7%	28.0%
	Total RG-I sugars	**75.6%**	**69.3%**	**79.2%**	**91.0%**	**81.7%**
Other monosaccharides	Glucose	3.5%	5.1%	1.8%	1.6%	4.5%
	Fucose	0.6%	0.4%	0.8%	0.9%	0.8%
	Xylose	0.5%	0.4%	0.4%	0.5%	0.5%
	Mannose	0.6%	0.4%	0%	0%	0%
Total identified monosaccharides		**80.8%**	**75.6%**	**82.2%**	**94.0%**	**87.5%**

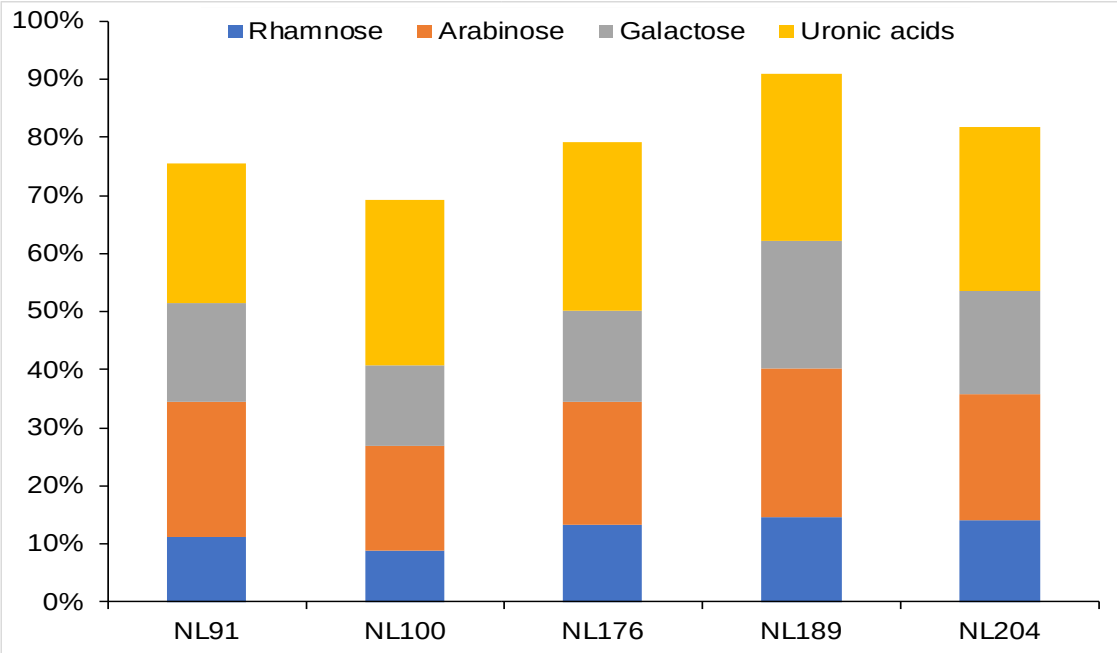

Figure 4. Relative distribution of monosaccharides that are characteristic for the RG-I region in pectin derived after methanolysis.

The total sugars associated with cRG-I in Table 1 closely aligns with the fiber analysis (Table 3) for each batch.

Manufacturing refinements (e.g., improving the ultrafiltration process) has resulted in variations in the percentage of RG-I-specific monosaccharides. The completion of the ultrafiltration process can be difficult to determine during production. Batches apparently lower in RG-I actually have more small sugars (<10 kDa, but mainly glucose) from the enzymatic hydrolysis process in the final product and consequently percentage-wise slightly less RG-I monosaccharides. Together, these explain some of the variability in total RG-I-associated sugars seen in Table 3.

Additionally, the identified monomers can provide further evidence on the branched structure of cRG-I. A report by Houben *et al.* (2011) compared cell wall composition and structures between broccoli, tomato and carrot. The authors describe a calculation to estimate branched RG-I content based on sugar monomer concentrations by summing the arabinose and galactose moieties and dividing this by the rhamnose concentration. The following table uses the values in Table 1 to calculate the branched ratio.

Table 4. Calculation of branched RG-I ratio according to method of Houben *et al.* (2011).

Sugar	NL91	NL100	NL176	NL189	NL204
Rhamnose	11.3%	9.0%	13.4%	14.6%	14.1%
Arabinose	23.2%	17.9%	21.1%	25.7%	21.7%
Galactose	17.0%	13.8%	15.8%	22.0%	17.9%
Branched RG-I ratio	**35.6**	**35.2**	**27.5**	**32.7**	**28.1**

These ratios are almost three times higher than described by Houben *et al.* (2011), who reported a ratio of 11.8 for the water-soluble pectin fraction from carrots. This clearly indicates the enrichment of RG-I.

Mass Balance

Using the information provided on protein, fat, total carbohydrates, ash and moisture contents (found in Table 1), a mass balance was calculated.

Table 5. Mass balance for cRG-I.

	Unit	NL91	NL100	NL176	NL189	NL204
Protein	% (w/w)	4.8	5.8	1.9	1.5	2.4
Fat	% (w/w)	1.18	1.18	0.2	0.27	0.2
Total free sugars (as glucose)	% (w/w)	11.3	14.7	8.39	6.71	13.1
Total dietary fiber	% (w/w)	71.4	70.7	81.3	81	80
Ash	% (w/w)	5.31	6.11	5.53	7.13	5.05
Moisture	% (w/w)	9.14	6.7	5.41	5.71	6.69
Mass balance	**% (w/w)**	**103.1**	**105.2**	**102.7**	**102.3**	**107.4**

The data demonstrate that cRG-I can be consistently manufactured within the specifications set by NutriLeads B.V. The calculated mass balance indicates that the chemical composition of cRG-I is assessed sufficiently with the analytical methods employed.

I. Specifications

RG-I-Enriched carrot fiber is a carrot-derived pectin extract in powder form containing no less than 75% (w/w) carbohydrates, of which at least 70% (w/w) is dietary fiber (see Table 1). The fiber fraction consists of polymers that are composed of uronic acids (max 35% w/w), rhamnose, arabinose and galactose (Table 4). Low amounts of additional monosaccharides (xylose, fucose,

mannose, glucose) are also present, as is some protein (Tables 1 and 3). The specifications of cRG-I are provided in Table 6.

Table 6. Specifications for cRG-I.

	Specification	Unit	Assay
Appearance	Off-white to beige	(color)	Visual
Appearance	Powder		Visual
Dispersibility	Water soluble		
a_w	Max 0.6		Resistive Electrolytic Hygrometer (REH)
Composition			
Moisture	Max 10	% w/w	103°C, 3h
Total carbohydrates	Min 75	% w/w	Calculated
Total dietary fiber content	Min 70	% w/w	In accordance with AOAC 2011.25.
Protein content	Max 6	% w/w	Kjeldahl, f=6.25
Total ash content	Max 10	% w/w	500-550°C
Heavy metals			
Arsenic	Max 0.1	mg/kg	ICP-MS
Mercury	Max 0.1	mg/kg	ICP-MS
Cadmium	Max 1.0	mg/kg	ICP-MS
Lead	Max 1.5	mg/kg	ICP-MS
Microbiology			
Total plate counts	Max 10,000	cfu/g	ISO 4833-1
Yeasts	Max 100	cfu/g	ISO 7954 (1987)
Molds	Max 100	cfu/g	ISO 7954 (1987)
Salmonella spp.	Not detected	/25g	TRA 02/08-03/0 Equal AFNOR TRA 02/08-03/01; ISO 6579 (GEN 25/05-11/08)
Escherichia coli	< 10	cfu/g	In accordance with ISO 16649-2 E. coli
Monosaccharide profile after hydrolysis			
Uronic acids	< 35	% w/w	Skalar Colorimetry
Rhamnose	6-19	% w/w	Methanolysis, HPAEC-PAD
Arabinose	14-30	% w/w	Methanolysis, HPAEC-PAD
Galactose	9-26	% w/w	Methanolysis, HPAEC-PAD
Shelf life		2 years from production date	

J. Stability

When stored under appropriate conditions and free from microbial contamination, foods with a low water activity (a_w<0.6) are expected to be microbiologically stable (Rahman and Labuza, 2007). RG-I-Enriched carrot fiber has a low a_w (<0.6) (see Table 4), therefore it can be expected that microbial growth is unlikely to occur.

To demonstrate the microbial, organoleptic and physical stability of cRG-I as well as the stability of the polymer pattern, a series of experiments was performed. Stability studies were conducted at 25°C and 60% relative humidity (RH) as well as at 40°C and 75% RH for the five batches that were characterized in detail for this Notice. These conditions are considered normal (25°C/60% RH) and accelerated (40°C/75% RH). The testing followed international guidelines (ICH, 2003). The study (storage and all analyses) was executed by an accredited laboratory. The analytical methods are described in the ICH guidelines.

Design of the Stability Study

The five batches used for the stability study were manufactured at different times. The first and second batches (NL91 and NL100) had been stored for about one year at ambient temperature prior to initiation of the study.

Immediately before initiation, a sufficient number of aliquots of each batch was packed in thermo-sealed, light-, oxygen- and moisture-proof aluminum foil bags and shipped to the study site for storage and analyses. The storage and sampling scheme is given in Table 7.

Table 7. Sampling scheme for stability assays of cRG-I.

Storage condition	Sampling and analysis						
25°C/60% RH	Initial analysis		3 months	6 months	1 year	2 years*	3 years*
40°C/75% RH	Initial analysis	1 month	3 months	6 months	1 year		

* storage/analyses still ongoing

Analyses of the stored samples:

At each time point and condition, samples were visually inspected for color and appearance and evaluated organoleptically for odor. Compliance with NutriLeads' microbial specifications was tested immediately after manufacturing and again at initiation of the stability studies.

Molecular weight distribution is a key characteristic to determine the stability of polysaccharides like the RG-I that is included in cRG-I. Since validated and published methods for the determination of the molecular weight distribution of pectin fractions do not exist, the testing laboratory developed a

method specifically for cRG-I and similar materials. The method description and validation report for this method is available upon request.

The molecular weight distribution was analyzed at each time point and compared with the initial values of the respective batch. Because molecular weight patterns must be analyzed at the same time to limit variability, the samples were withdrawn from storage at the specified time points and stored at -18°C until testing.

Five batches of cRG-I (NL91, NL100, NL176, NL189, NL204) were subjected to microbial and organoleptic analyses after storage at standard (25°C, 60% RH) and accelerated (40°C, 75% RH) conditions, following international recommendations (ICH, 2003). Due to handling errors by the contract laboratory, some samples were destroyed, which left three complete sets of data (NL91, NL100, NL189) and two partial sets (NL176, NL204) (see Table 8). The available data are summarized in Table 8 and Table 9.

Table 8. Microbial, organoleptic and physical stability analyses of five batches of cRG-I at 25°C/60% RH and at 40°C/75% RH.

	At production	Initial		1 month		3 months		6 months		12 months	
Batch		25°C 60% RH	40°C 70% RH	25°C 60% RH	40°C 70% RH	25°C 60% RH	40°C 70% RH	25°C 60% RH	40°C 70% RH	25°C 60% RH	40°C 70% RH
NL91	✓	✓	✓	ND	✓	✓	✓	✓	✓	✓	✓
NL100	✓	✓	✓	ND	✓	✓	✓	✓	✓	✓	✓
NL176	✓	✓	✓	ND	Missing	✓	✓	✓	✓	✓	✓
NL189	✓	✓	✓	ND	ND	✓	✓	✓	✓	✓	✓
NL204	✓	✓	✓	ND	Missing	Missing	✓	✓	Pending	✓	Pending

✓ data available, ND- not determined by design, Missing due to handling errors by the contract laboratory

Table 9. Polymer pattern stability analyses of five batches of cRG-I at 25°C/60% RH and 40°C/75% RH.

	Initial		1 month		3 months		6 months		12 months	
Batch	25°C 60% RH	40°C 70% RH	25°C 60% RH	40°C 70% RH	25°C 60% RH	40°C 70% RH	25°C 60% RH	40°C 70% RH	25°C 60% RH	40°C 70% RH
NL91	✓	✓	ND	✓	✓	✓	✓	✓	✓	✓
NL100	✓	✓	ND	✓	✓	✓	✓	✓	✓	✓
NL176	Missing	Missing	ND	Missing	Missing	Missing	Missing	Missing	ND	ND
NL189	Pending	Pending	ND	Pending	Pending	Pending	Pending	Pending	Pending	Pending
NL204	Missing	Missing	ND	Missing	Pending	Pending	Pending	Pending	Pending	Pending

✓ Data available, ND- Not determined by design, Missing due to handling errors by the contract laboratory

Results of the Stability Studies

Summaries of the microbial analyses for standard and accelerated conditions are given in Table 10 and Table 11. Results indicate that there is some variability in total plate counts and for *Bacillus cereus* and *E. coli*. This is probably due to sampling and/or analytical variability as they were seen at only one time point and one set of conditions. Importantly, there is no coherent indication for microbial growth over the time period involved.

Comparison of the microbial analyses immediately after manufacturing and at initiation of the stability study did not reveal relevant changes (all certificates of analysis can be provided). Importantly, all analyses confirm the stability of the product up to the latest time points tested. These observations prove the microbial, physical, organoleptic and chemical stability of cRG-I for at least one year under normal storage conditions. Given the results of the tests performed under accelerated conditions, NutriLeads B.V. extrapolates the stability under normal conditions to a shelf life of two years.

Table 10. Microbial analysis of five batches of cRG-I at standard conditions (25°C/60% RH).

			NL91				NL100			
Analysis	**Internal Specification**	**Unit**	**Initial**	**3 months**	**6 months**	**1 year**	**Initial**	**3 months**	**6 months**	**1 year**
Total aerobic count	10,000	cfu/g	3100	1500	2800	2000	6000	2400	5000	6000
Yeasts	100	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Molds	100	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae	< 10/g	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Salmonella spp	n.d./25 g	cfu/50g	n. d.	n. d.	n. d.	n. d.	-	n. d.	n. d.	n. d.
Escherichia coli	< 10/g	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Bacillus cereus		cfu/g	1100	300	1000	500	1200	1000	900	900
Thermophilic aerobic spore-forming count 55°C		cfu/g	< 10	90	< 10	30	< 10	< 10	< 10	< 10
Thermophilic anaerobic spore-forming count 55°C		cfu/g	< 10	<10	< 10	< 10	< 10	< 10	< 10	< 10

			NL176				NL189			
Analysis	**Internal Specification**	**Unit**	**Initial**	**3 months**	**6 months**	**1 year**	**Initial**	**3 months**	**6 months**	**1 year**
Total aerobic count	10,000	cfu/g	-	780	1500	2700	2600	1900	1100	1000
Yeasts	100	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Molds	100	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae	< 10/g	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Salmonella spp	n.d./25 g	cfu/50g	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.
Escherichia coli	< 10/g	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 100	< 10
Bacillus cereus		cfu/g	1300	1500	2000	< 1100	< 100	200	< 100	< 100
Thermophilic aerobic spore-forming count 55°C		cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Thermophilic anaerobic spore-forming count 55°C		cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10

n.d. not detected; - not tested or pending

Table 10, continued.

			NL204			
Analysis	**Internal Specification**	**Unit**	**Initial**	**3 months**	**6 months**	**1 year**
Total aerobic count	10,000	cfu/g	420	-	20,000	2200
Yeasts	100	cfu/g	< 10	-	< 10	<10
Molds	100	cfu/g	80	-	130	30
Enterobacteriaceae	< 10/g	cfu/g	< 10	-	< 10	<10
Salmonella spp	n.d./25 g	cfu/50g	-	-	-	-
Escherichia coli	< 10/g	cfu/g	< 10	-	< 10	<10
Bacillus cereus		cfu/g	200	-	< 100	100
Thermophilic aerobic spore-forming count 55°C		cfu/g	< 10	-	< 10	<10
Thermophilic anaerobic spore-forming count 55°C		cfu/g	< 10	-	< 10	<10

n.d. not detected; - not tested or pending

Table 11. Microbial analysis of five batches of cRG-I at accelerated conditions (40°C/75% RH).

			NL91					NL100				
Analysis	**Internal Specification**	**Unit**	**Initial**	**1 month**	**3 months**	**6 months**	**1 year**	**Initial**	**1 month**	**3 months**	**6 months**	**1 year**
Total aerobic count	10,000	cfu/g	3100	2800	3200	780	700	6000	2800	3900	2800	<1000
Yeasts	100	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Molds	100	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae	< 10/g	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Salmonella spp	n.d./25 g	cfu/50g	n. d.	n. d.	n. d.	n. d.	n. d.	-	n. d.	n. d.	n. d.	n. d.
Escherichia coli	< 10/g	cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Bacillus cereus		cfu/g	1100	400	600	600	200	1200	900	600	1300	900
Thermophilic aerobic spore-forming count 55°C		cfu/g	-	< 10	< 10	< 10	< 10	< 10	< 10	10	< 10	< 10
Thermophilic anaerobic spore-forming count 55°C		cfu/g	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10

			NL176					NL189				
Analysis	**Internal Specification**	**Unit**	**Initial**	**1 month**	**3 months**	**6 months**	**1 year**	**Initial**	**1 month**	**3 months**	**6 months**	**1 year**
Total aerobic count	10,000	cfu/g	-	-	2800	2100	3700	2600	-	2300	1500	800
Yeasts	100	cfu/g	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10
Molds	100	cfu/g	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10
Enterobacteriaceae	< 10/g	cfu/g	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10
Salmonella spp	n.d./25 g	cfu/50g	-	-	-	-	n. d.	n.d.	-	n. d.	n. d.	n. d.
Escherichia coli	< 10/g	cfu/g	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10
Bacillus cereus		cfu/g	1300	-	1500	900	1200	< 100	-	200	100	<100
Thermophilic aerobic spore-forming count 55°C		cfu/g	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10
Thermophilic anaerobic spore-forming count 55°C		cfu/g	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10

n.d. not detected - not tested or pending

Table 11, continued.

			NL204				
Analysis	**Internal Specification**	**Unit**	**Initial**	**1 month**	**3 months**	**6 months**	**1 year**
Total aerobic count	10,000	cfu/g	420	-	190	-	-
Yeasts	100	cfu/g	< 10	-	< 10	-	-
Molds	100	cfu/g	80	-	< 10	-	-
Enterobacteriaceae	< 10/g	cfu/g	< 10	-	< 10	-	-
Salmonella spp	n.d./25 g	cfu/50g	-	-	n. d.	-	-
Escherichia coli	< 10/g	cfu/g	< 10	-	< 10	-	-
Bacillus cereus		cfu/g	200	-	< 100	-	-
Thermophilic aerobic spore-forming count 55°C		cfu/g	< 10	-	< 10	-	-
Thermophilic anaerobic spore-forming count 55°C		cfu/g	< 10	-	< 10	-	-

n.d. not detected - not tested or pending

Stability of cRG-I

The stability of the polymeric structure of cRG-I was assessed for two batches by analyzing their molecular weight patterns by chromatography after storage at standard (25°C, 60% RH) and accelerated (40°C, 75% RH) conditions. Results for batches NL91 and NL100 are presented in Appendix II.

The following method (report from the performing laboratory is too large for inclusion; available upon request) was used. High Performance Size Exclusion Chromatography (HPSEC) was performed using three TosoH Bioscience TSKgel Super AW columns (4000, 3000 and 2500; 150 × 6 mm) in series preceded with a TSKgel guard column Super AW-L (35 × 4.6 mm). Samples (10 µL) were injected and eluted at 55°C using 0.2 M sodium nitrate as eluent (pH set to 2.5 with nitric acid). Sodium nitrate is known to be effective in lowering interactions of charged molecules with the mobile phase. The low pH was used in order to have mainly galacturonic acid instead of (negatively charged) galacturonate moieties in the samples. Refractive index detection was applied. Pullulan and disaccharide standards of known molecular mass were used for calibration. A run time of 30 min was applied.

Normally the elution profile is plotted against time to show the molecular weight profile. For comparison of the same batch over time, as done here, it is typical to plot the signal over time cumulatively to show any degradation, which would be reflected in a shift of the cumulative plot.

The chromatograms did not reveal any relevant changes in molecular mass distribution at standard and accelerated storage conditions, confirming the stability of the molecular weight pattern of RG-I. As an example, chromatograms for NL91 are shown in Figures 5 to 8.

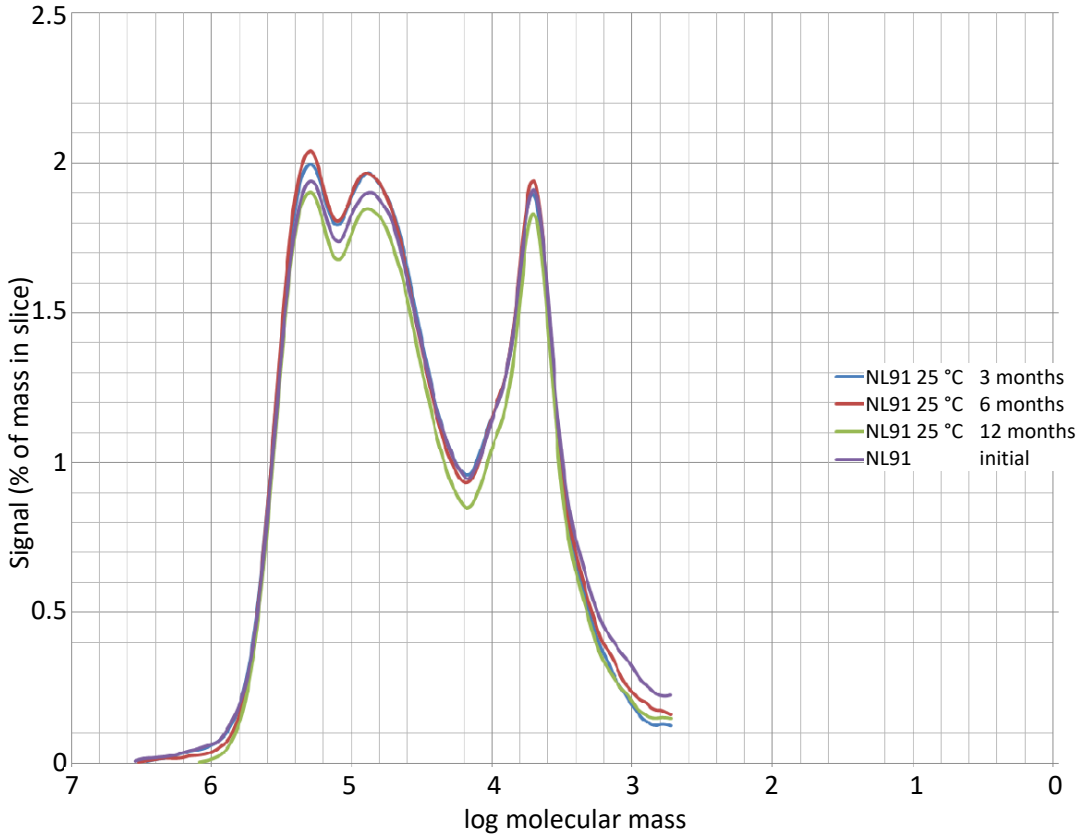

Figure 5. Differential molecular mass distribution for NL91 stored at standard conditions.

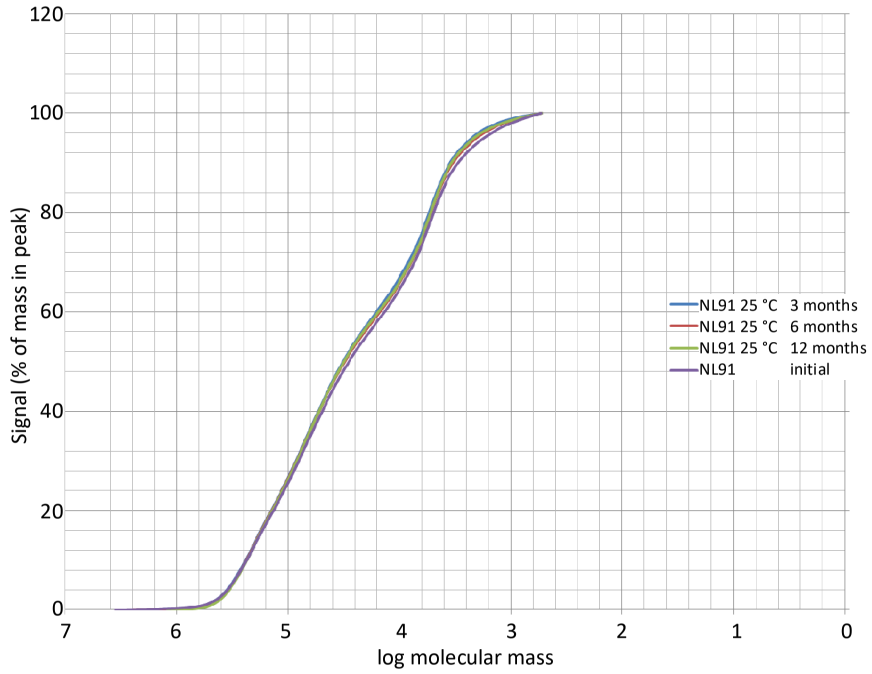

Figure 6. Cumulative molecular mass distribution for NL91 stored at standard conditions.

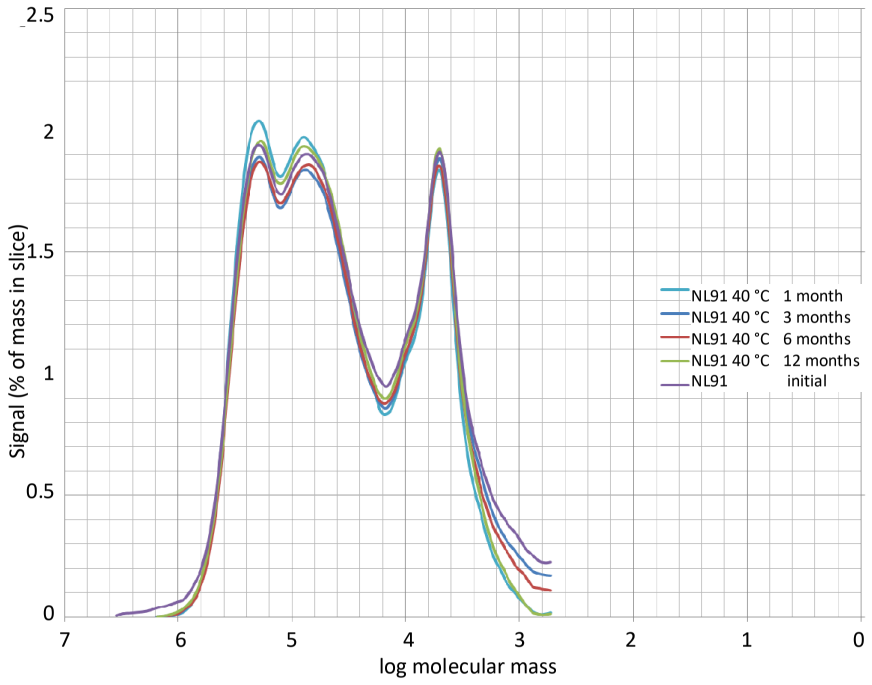

Figure 7. Differential molecular mass distribution for NL91 stored at accelerated conditions.

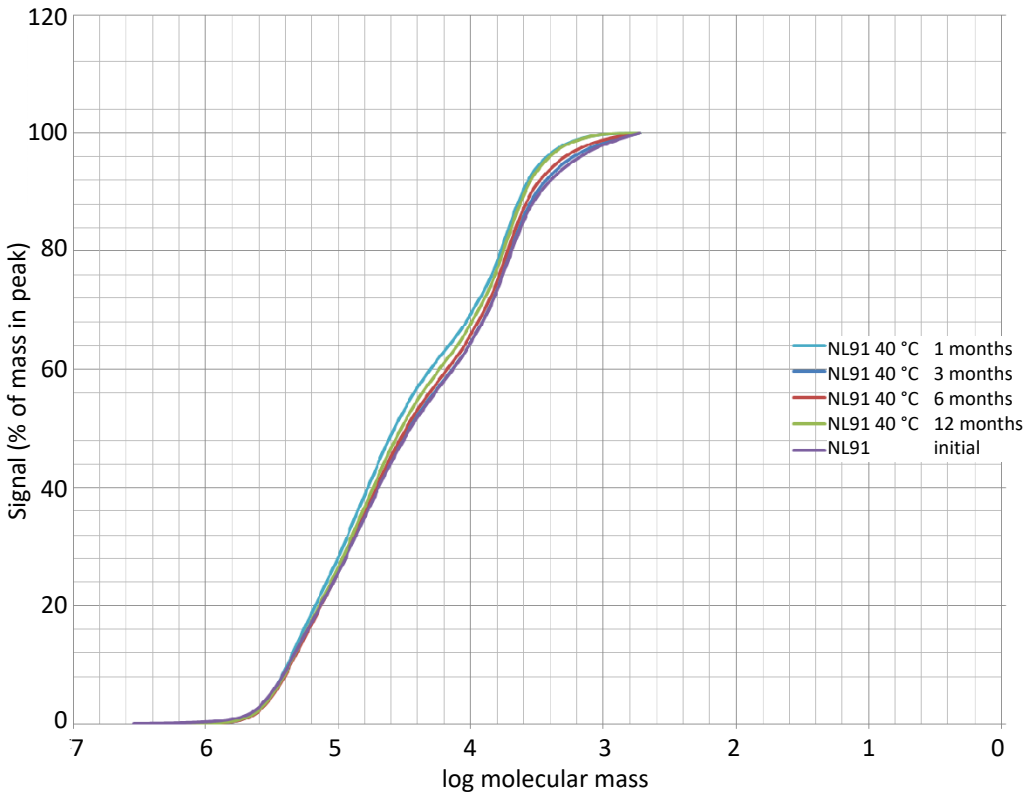

Figure 8. Cumulative molecular mass distribution for NL91 stored at accelerated conditions.

Summary of the Stability Results

The data demonstrate that cRG-I is stable under the conditions of the tests, as would be expected for a polysaccharide in a dry, powder form.

The microbial analysis of five batches did not reveal relevant microbial growth in any of the samples analyzed at different time points after storage at either 25°C/65% RH or 40°C/70% RH. In one case (NL204, 25°C/65% RH) aerobic plate counts were higher after six months of storage when compared with the initial counts; this was deemed to be an isolated finding which can most likely be attributed to sample handling. The few other microbiology results that deviate from initial findings or from specifications were random and it is concluded that they are due to sampling error or incorrect laboratory methodology.

The organoleptic and physical analysis showed no changes in any of the samples. All samples were stable with respect to their molecular weight distribution, meaning no degradation of the polysaccharide was observed. The samples always complied with the specifications and did not cause concerns about the stability of cRG-I.

Together, the data prove the microbial, physical, organoleptic and chemical stability of cRG-I for at least one year under normal storage conditions. Due to the results of the tests performed under accelerated conditions, which showed no significant changes in the tested batches, NutriLeads B.V. extrapolates the stability under normal conditions to a shelf life of two years. The studies are continuing to investigate timeframes of up to two and three years.

K. Nutrition Information

Table 12 shows the nutrition profile of cRG-I based on mean data in Table 1. The energy content is based on energy values provided by FAO/INFOODS guidelines (FAO/INFOODS, 2012). Some nutrient values were derived from the composition of dehydrated carrots (USDA, 2019).

Table 12. Nutrition profile of cRG-I.

	Amount per 100 g	Amount per serving*	% Daily Value per serving
Calories	216 kcal	8 kcal	
Total fat	1 g	<1 g	<1%
Saturated fat**	<1 g	<1 g	<1%
Trans fat**	0 g	0 g	
Cholesterol**	0 g	0 g	<1%
Sodium	384 mg	14 mg	
Total carbohydrates	88 g	3 g	1%
Dietary fiber	77 g	3 g	10%
Total sugars	11 g	<1 g	
Added sugars	0 g	0 g	<1%
Protein	3 g	<1 g	<1%
Vitamin D**	0 mcg	0 mcg	<1%
Calcium	860 mg	31 mg	2%
Iron**	4 mg	<1 mg	1%
Potassium	1575 mg	58 mg	1%

*Serving size 3.66 g

**Estimated from the composition of dehydrated carrots

L. Physical or Technical Effect

RG-I-Enriched carrot fiber is intended for addition to conventional foods for which no standard of identity exists as a source of nutrients, primarily fiber (21 CFR §170.3(o)(20)).

3. DIETARY EXPOSURE

This section of the GRAS Notice fulfills requirements of 21 CFR §170.235 in regard to the dietary exposure of cRG-I as a result of its intended uses and use levels in a variety of foods.

A. Basic Considerations

The source of the cRG-I is carrot, a staple food throughout the world, including the U.S., with a long history of safe use (described below). Carrot fiber, a major component of carrot pomace, the starting material for cRG-I, has been determined to be GRAS (GRN 0116) by a panel of experts and FDA has not objected to that determination (FDA, 2003). Pectin, a major component of carrot fiber, is a GRAS substance with no limitation in use other than current Good Manufacturing Practices (21 CFR §184.1588). Pectin is recognized as a dietary fiber (FDA, 2018).

Dietary fiber is considered a "nutrient of public health concern" because low intakes are associated with potential health risks (Dietary Guidelines Advisory Committee, 2015). Yet most Americans do not consume the recommended amount of daily dietary fiber (HHS and USDA, 2015; Quagliani and Felt-Gunderson, 2017). The Institute of Medicine of The National Academies (IOM) currently recommends an Adequate Intake (AI) level of 38 and 25 g fiber/day for adult males and females (ages 19-50 years), respectively (IOM, 2005). According to 2009-2010 NHANES data, mean daily dietary fiber intake is 16.2 g (Quagliani and Felt-Gunderson, 2017).

A tolerable upper intake level for dietary fiber has not been defined by the Institute of Medicine (IOM, 2005; Turner and Lupton, 2011) and addition of fiber to foods has not been seen to pose any public health consequences. On the contrary, since dietary fiber intake is inadequate in many populations, providing a convenient source of dietary fiber can be of public health interest.

RG-I Enriched carrot fiber will also serve as a source of dietary fiber (Van den Abbeele, 2020), and the addition of cRG-I into new food categories can help fill a nutrient gap.

B. History of Carrot Consumption

Carrots (*Daucus carota* L.) were among the vegetables eaten by early civilizations in Sumer and Egypt, around 3000 B.C. (McGee, 1984). Traces of carrot have been discovered at archeological sites such as pre-historic lake dwellings in Switzerland; carrot was included in the listing of vegetables in the Babylonian royal gardens in the 8th century B.C. (Davidson, 1999). It is possible that some of these early references could refer to carrot's use as an aromatic herb rather than root vegetable (Davidson, 1999) but it is difficult to think that people would not be eating it for nutritional needs.

Original carrots were purple and yellow, initially described in Iran and northern Arabia in the 10th century (Arscott and Tanumihardjo, 2010). An Arab writer, Ibn Al-Awam, produced the first description of the modern carrot in Andalusia in the early 12th century. Carrots reached Western Europe in the 14th century and Britain in the l5th. The violet/purple carrot was grown in Italy by the early 1300s (Schneider, 2001). Carrots spread to the Middle East, North Africa, and China by the mid-15th century (Arscott and Tanumihardjo, 2010).

Yellow carrots were preferred in northern Europe until the development of orange carrots in The Netherlands in the 18th century. White carrots were noted in Europe and red carrots are thought to have originated in China around this time (Arscott and Tanumihardjo, 2010).

The Dutch were leaders in the improvement of carrot varieties. The early descriptions were all of two types, purple/red and pale yellow/white. The orange carrot first appeared in Dutch paintings in the 17th century and soon dominated carrot production. Cultivated carrots were brought to the New World before 1565, likely by the Spanish, and the roots were adopted by native Americans (Davidson, 1999). Carrots were introduced to the Jamestown Colony in 1607-9 (Trager, 1995).

Orange carrots have mainly supplanted the other colors in the West, but purple and yellow carrots persist in some areas of Turkey, India, and China and red carrots in Japan. Thorough documentations of the domestication and historical development of carrots have been published (from Arscott and Tanumihardjo, 2010).

C. Consumption of Carrots in the United States

Carrots are one of the more popular vegetables in the U.S. and fresh-market carrot consumption has been increasing over the past few decades (Lucier and Lin, 2007). These authors from the United States Department of Agriculture (USDA) show, using disappearance data, that *per capita* consumption of carrots in the U.S. is roughly 12 lb/person/yr (Lucier and Lin, 2007). This is approximately 15 g/person/day, or 0.25 g/kg/day.

The U.S. EPA, using more recent NHANES two-day consumption data, estimated *per capita* carrot intake to be 0.15 g/kg/day for the entire U.S. population (EPA, 2018), or roughly 9 g/person/day (about one large carrot per week). The EPA has broken down carrot intake by age group as well (Table 13).

Table 13. *Per capita* 2-day average intake of carrots based on 2005–2010 NHANES data.

Age group	N	% consuming once in the 2-d period	Mean (g/kg-day)	Standard Error
1 to <2 years	728	55	0.47	0.03
2 to <3 years	751	48	0.43	0.05
3 to <6 years	1,418	44	0.38	0.06
6 to <11 years	2,292	43	0.24	0.04
11 to <16 years	2,551	35	0.11	0.02
16 to <21 years	2,191	35	0.08	0.01
21 to <30 years	2,082	45	0.10	0.01
30 to <40 years	2,282	47	0.12	0.01
40 to <50 years	2,378	47	0.12	0.01
50 to <60 years	2,103	52	0.13	0.01
60 to <70 years	2,214	54	0.14	0.01
70 to <80 years	1,578	57	0.14	0.01
80+ years	915	57	0.15	0.01
Whole population	24,673	46	0.15	<0.005

g/kg-day, edible portion, uncooked weight

Ref: Table 9-5 in *Update for Chapter 9 of the Exposure Factors Handbook*. (EPA, 2018)

D. Consumption of Carrot Fiber and cRG-I

Carrot fiber is of interest to food processors due to the large quantities of remains created in the cut-and-peel carrot and carrot juice industries. Carrot pomace is the wet carrot shavings produced from carrot processing, which is subsequently dried to form a powder (Arscott and Tanumihardjo, 2010). Carrot fiber has properties, depending on how the pomace is processed, that make it attractive as a food ingredient (Arscott and Tanumihardjo, 2010).

A noted above, carrot fiber had been consumed as a component of carrots for centuries. Carrots contain approximately 3% total dietary fiber, with the reported range from 2.4% to 6.4% (Arscott and Tanumihardjo, 2010). Using the higher estimate of carrot consumption from the USDA (shown above, 15 g/person/day), the U.S. population, on a *per capita* basis, consumes approximately 0.45 g carrot fiber/person/day from fresh carrots or approximately 0.0075 g/kg bw/day (based on a 60-kg person).

The soluble fiber portion of dietary carrot fiber consist of fermentable hemicellulose and pectin (Marlett, 1992) and constitute 8% to 50% of total fiber (Arscott and. Tanumihardjo, 2010). Thus, *per capita* consumption of pectin from carrots might be as high as 0.004 g/kg bw/day (assuming 50% of total fiber is pectin), or approximately 0.22 g/person/day. Based on intakes provided in Table 13, carrot intake ranges over seven-fold by age, which means carrot pectin would range from 0.0012 g/kg bw/day for young adults 16- <21 years of age to 0.007 g/kg bw/day for toddlers 1-2 years old (assuming 3% fiber content of which 50% is soluble fiber pectin).

Carrot fiber itself has been used as a food ingredient in multiple food categories in the U.S. for over a decade (GRN 0116; FDA, 2003). Likewise, pectins have been used as ingredients in a broad range of food applications for decades. Data indicating how much of these ingredients are consumed by persons in the U.S. cannot be found in public databases. Older estimates of intake are that people consuming a normal Western diet ingest around 4-5 g of pectins each day and worldwide annual consumption is estimated at around 45 million kg (Willats *et al.*, 2006).

Pectins are a constituent of cell walls of all green land plants (SCOGS, 1977). Thus, the consumption of plant foods means that humans have always consumed pectin in their diet (SCOGS, 1977).

The RG-I structure is an intrinsic element of pectin. Humans have been exposed to some small amount of it through microbial breakdown of pectin in the intestines. Free RG-I is a constituent of a broad range of processed fruit and vegetable products, albeit at low levels (Schols *et al.*, 1990). Pectinolytic enzymes like pectin lyases and polygalacturonases are commonly used to manufacture juices and wine to remove cloudiness, to increase the release of antioxidants from the matrices of fruits and vegetables, and to increase the efficiency of the juicing or homogenization process. Additionally, while most pectin is retained in the pomace as an integral part of the plant cell wall, a small part of branched structures like RG-I is released into foods and beverages due to non-enzymatic hydrolysis of the pectin backbone. Thus, fruit and vegetable juices and purees may contain RG-I structures similar to those present in cRG-I (Schols *et al.*, 1990). Wines also contain RG-I structures resulting from cell wall degradation through fermentation (Jackson, 2014).

Since Americans do not consume a sufficient amount of fiber and since there are no health concerns with fiber in general or pectin specifically, NutriLeads, B.V. proposes levels for cRG-I in several food categories that could allow fiber nutrient claims to be made. A product must contain between 10% and 19% of the daily reference value (DRV) per reference amount customarily consumed (RACC) to make a "good source of fiber" claim (21 CFR §101.54) in those categories for which nutrient claims can be made. The DRV for dietary fiber is 28 g in adults and children ≥4 years of age (including pregnant and lactating women) and 14 g in children 1 to 3 years of age (21 CFR §101.9).

E. Target Population

The target population is the general population, excluding infants younger than six months of age, who do not consume products intended for addition of cRG-I.

F. Intended Uses and Use Levels

RG-I-Enriched carrot fiber is proposed for use in various food categories at levels providing an additional or alternative source of dietary fiber at 10% of the DRV per RACC. Proposed food uses of cRG-I include non-alcoholic beverages and beverage bases, breakfast cereals, dairy product analogs, grain products and pastas, baby food, milk products, processed fruit juices, processed vegetable juices, snack foods, and soup. Proposed food categories and use levels are provided in Table 14.

In the assessment, cRG-I was assumed to be the sole source of dietary fiber added to target foods. Therefore, cRG-I was assumed to provide 2.8 g dietary fiber per RACC in conventional foods and 1.4 g dietary fiber per RACC in foods for infants and young children 1 through 3 years of age (baby foods). RACCs for each of the intended food uses are based on 21 CFR §101.12 (FDA, 2019c). Resulting maximum fiber use levels from cRG-I range from <1 to 20 g/100g of the food or beverage. Use levels expressed on a g/100 g basis were applied in the intake assessment.

Table 14. Individual proposed food uses and use levels of cRG-I in the U.S. to make a fiber claim and the resulting cRG-I levels

Food Category (21 CFR §170.3 – U.S. FDA, 2019a)	Food Uses[a]	Fiber Level (g/serving)	RACC[b] (g or mL)	Maximum Fiber Level (g/100 g)	Maximum cRG-I Level (g/100 g)
Beverages and Beverage Bases, Nonalcoholic	Energy Drinks	2.8	360	0.78	0.98
	Enhanced, Flavored, Carbonated, or Fortified Water Beverages	2.8	360	0.78	0.98
	Non-Milk-Based Meal Replacement, Protein, and Nutritional Beverages	2.8	240	1.17	1.46
	Soft Drinks (including Regular and Diet)	2.8	360	0.78	0.98
	Sport or Electrolyte Drinks, Fluid Replacement Drinks	2.8	360	0.78	0.98
Breakfast Cereals	Hot Breakfast Cereals (e.g. Oatmeal, Grits), including Instant and Regular	2.8	240 (or 1 cup prepared)[c]	1.17 (prepared)	1.46
	Ready-to-Eat Breakfast Cereals				
	Puffed Cereals	2.8	15	18.67	23.34
	High-Fiber Cereals	2.8	40	7.00	8.75
	Biscuit-Type Cereals	2.8	60	4.67	5.84
Dairy Product Analogs	Non-Dairy Milk	2.8	240	1.17	1.46
	Non-Dairy Cream	2.8	15	18.67	23.34
	Non-Dairy Yogurts	2.8	170	1.65	2.06
	Non-dairy Ice Creams	2.8	160 (or 2/3 cup)[c]	1.75	2.19
Grain Products and Pastas	Cereal and Granola Bars	2.8	40	7.00	8.75
	Energy Bars, Protein Bars, and Meal Replacement Bars	2.8	40	7.00	8.75

Table 14. Individual proposed food uses and use levels of cRG-I in the U.S. to make a fiber claim and the resulting cRG-I levels

Food Category (21 CFR §170.3 – U.S. FDA, 2019a)	Food Uses[a]	Fiber Level (g/serving)	RACC[b] (g or mL)	Maximum Fiber Level (g/100 g)	Maximum cRG-I Level (g/100 g)
Baby Food	Baby Food: Cereals				
	Dry Instant	1.4	15	9.33	11.66
	Prepared, Ready-to-Serve	1.4	110	1.27	1.59
	Other Cereal and Grain Products, Dry Ready-to-Eat Cereals, Cookies, Teething Biscuits, and Toasts	1.4	7 to 20	20.00	25.00
	Baby Food: Dinners, Desserts, Fruits, Vegetables, Or Soups				
	Dinners, Desserts, Fruits, Vegetables, Or Soups Dry Mix Type[d]	1.4	15	9.33	11.66
	Dinners, Desserts, Fruits, Vegetables, Or Soups Junior Type and Strained Type, Ready-to-Serve	1.4	110	1.27	1.59
	Dinners, Stews or Soups for Young Children, Ready-to-Serve	1.4	170	0.82	1.03
	Fruits for Young Children, Ready-to-serve	1.4	125	1.12	1.40
	Vegetables for Young Children, Ready-to-serve	1.4	70	2.00	2.50
	Baby Food: Juice	1.4	120	1.17	1.46
Milk Products	Dry Milks	2.8	240 (prepared)	1.17	1.46
	Evaporated or Condensed Milk	2.8	30	9.33	11.66
	Fermented Milks, Plain or Flavored	2.8	240	1.17	1.46
	Flavored Milk, Milk Drinks, and Mixes, Milk Shakes	2.8	240	1.17	1.46
	Milk-Based Meal Replacement and Nutritional Beverages	2.8	240	1.17	1.46
	Plain or Flavored Yogurt	2.8	170	1.65	2.06
	Yogurt Drinks	2.8	93 to 207[e]	3.01	3.76
Processed Fruits and Fruit Juices	Fruit Drinks and Ades including Smoothies	2.8	240	1.17	1.46
	Fruit Juices and Nectars	2.8	240	1.17	1.46
Processed Vegetables and Vegetable Juices	Vegetable Juices, Nectars and Blends	2.8	240	1.17	1.46
Snack Foods	Snack Foods (Potato Chips, Popcorn, Pretzels and Corn-based Savory Snacks)	2.8	30	9.33	11.66
Soups and Soup Mixes	Soups (Prepared and Canned)[f]	2.8	245 (prepared)	1.14	1.43

Table 14. Individual proposed food uses and use levels of cRG-I in the U.S. to make a fiber claim and the resulting cRG-I levels

Food Category (21 CFR §170.3 – U.S. FDA, 2019a)	Food Uses[a]	Fiber Level (g/serving)	RACC[b] (g or mL)	Maximum Fiber Level (g/100 g)	Maximum cRG-I Level (g/100 g)

CFR = Code of Federal Regulations; RACC = Reference Amounts Customarily Consumed per Eating Occasion; U.S. = United States.

[a] cRG-I is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

[b] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2019b). RACCs are included for reference, however the assessment was conducted based on use levels expressed per liter. When a range of values is reported for a proposed food use, particular foods within that food use may differ with respect to their RACC.

[c] Calculated based on food item density using unit converter (https://www.aqua-calc.com/calculate/food-volume-to-weight).

[d] No food codes were identified for this category in the NHANES 2015-2016 database.

[e] RACC has not been established for yogurt drinks; however, an approximate serving size was established based on products currently on the U.S. market.

[f] Food codes with meat products were included in the intake estimate; however, cRG-I is not intended for use in meat products. Inclusion of meat products in these food-use categories is not expected to appreciably affect the intake calculations.

G. Estimated Daily Intake of RG-I-Enriched Carrot Fiber

Available Data and Methods

An estimate of daily intake (EDI) for cRG-I was determined by Intertek based on proposed food uses of cRG-I and use levels providing dietary fiber at 10% of the DRV per RACC for each individual food use in conjunction with food consumption data included in the U.S. National Center for Health Statistics' (NCHS) National Health and Nutrition Examination Survey (NHANES) 2015-2016. Individual food codes selected for inclusion in each proposed use category are provided in Appendix III.

Calculations for the mean and 90th percentile *per capita* and consumer-only intakes of dietary fiber from cRG-I were performed for all proposed food uses and the percentages of consumers were determined. Similar calculations were used to estimate the intake of dietary fiber from cRG-I resulting from each individual proposed food use, including the calculations of percent consumers. Corresponding intakes of cRG-I were then calculated based on the mean level of total dietary fiber in cRG-I (76.5%, w/w) determined from analyses of five batches of the ingredient.

The NHANES (NCHS, 2020) is a complex, multistage probability sample designed to be representative of the civilian U.S. population. The survey collects two days of food intake data, in addition to nutrition, demographic, and health information. Intertek used statistically weighted values from the survey in the analyses. The statistical weights compensate for variable probabilities of selection, adjust for non-response, and provide intake estimates that are representative of the U.S. population and the selected age-gender subgroups. The statistical modeling software used for this analysis was DaDiet, (Version 17.04).

Intertek estimated the daily intake on a *per capita* and per "user" basis. In this analysis, a "user" is anyone who reported consuming at least one category of food in which it is proposed to use cRG-I on either of the survey days, i.e. USDA's "user" definition. Each individual who reported consuming a cRG-I food on either of the survey days was identified, and that individual's responses for both survey days were used. Because cRG-I is likely to be consumed over a lifetime, it is appropriate to average exposures over a longer period than one day. Therefore, Intertek used each respondent's food consumption averaged over the two days of the NHANES. A 2-day average typically overestimates lifetime average daily intake especially for foods eaten infrequently; however, only two nonconsecutive days' worth of food consumption data are available in the most recent NHANES database. It is well known that food consumption data collected over longer periods of time, e.g., 14 days as in Market Research Corporation of America consumer surveys, yield estimates of daily intake that may be significantly lower than 2-day averages (Lambe *et al.*, 2000). Therefore, actual consumer exposures is expected to be lower than these estimates.

Estimated Daily Intake

The EDI of cRG-I was calculated by multiplying each NHANES respondents' 2-day average food intake by the use levels described in Table 14, above, as adjusted. Mean and 90th percentile daily intakes, as g of cRG-I/day, were estimated for the proposed uses. Each individual's intake of cRG-I was divided by his/her bodyweight to provide the *per capita* and per user intakes on a bodyweight basis.

The EDI of fiber from proposed uses of cRG-I among users in the total U.S. population, assuming the maximum proposed use level for each food category, is not more than 8.5 g/day at the mean intake and 16.2 g/day at the 90th percentile of intake. This is equivalent to 141 mg/kg bw/day and 282 mg/kg bw/day for mean and 90th percentile intake for users-only of these foods.

The corresponding EDI of cRG-I among users in the total U.S. population from all proposed uses, assuming the maximum proposed use level for each food category is not more than 11 g/day at the mean intake and 21 g/day at the 90th percentile of intake (equivalent to 184 and 369 mg/kg/day, respectively). A further breakout of estimated cRG-I intakes by age/sex subgroups is shown below in Tables 15 and 16.

Table 15. Summary of the estimated daily intake of cRG-I from proposed food uses in the U.S. by population group (based on 2015-2016 NHANES data)

Population Group	Age Group (Years)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants and Young Children	0 to 2	5.7	13.2	87.0	565	6.6	13.9
Children	3 to 11	10.5	18.3	99.8	1,174	10.5	18.3
Female Teenagers	12 to 19	10.4	18.5	99.0	470	10.5	18.5
Male Teenagers	12 to 19	12.2	22.0	98.0	486	12.5	22.1
Female Adults	20 and up	9.8	18.9	96.9	2,139	10.1	19.0
Male Adults	20 and up	12.0	23.3	96.0	1,911	12.5	23.8
Total Population	All ages	10.7	20.9	96.7	6,745	11.0	21.1

n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

Table 16. Summary of the estimated daily per kilogram body weight intake of cRG-I from proposed food uses in the U.S. by population group (based on 2015-2016 NHANES data)

Population Group	Age Group (Years)	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants and Young Children	0 to 2	490	1,124	86.9	560	563	1,137
Children	3 to 11	401	728	99.8	1,169	403	728
Female Teenagers	12 to 19	180	359	99.0	462	182	359
Male Teenagers	12 to 19	190	341	98.0	485	193	345
Female Adults	20 and up	135	261	96.9	2,125	139	263
Male Adults	20 and up	137	267	96.0	1,887	144	271
Total Population	All Ages	186	386	96.7	6,688	184	369

bw = body weight; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

On a bodyweight basis, the highest per user mean and 90th percentile intake estimates are among infants and young children up to 24 months of age at 563 mg/kg bw/day and 1137 mg/kg bw/day, respectively. This is 6.6 and 13.9 g of cRG-I per child each day (Table 15), or approximately 5 g and 10.5 g (mean and 90th percentile intakes) of fiber each day. An AI has not been established for infants up to 12 months of age, but an AI of 19 g/day has been recommended for children 1-3 years (IOM, 2005). Although the 90th percentile estimates for infants and children are approximately 3-fold higher than that of the total population EDI, on a per person basis they are within the IOM's AIs for dietary fiber of 19-31 g/day (IOM, 2005). The range of exposures among remaining population subgroups is similar to the total population EDI, for both mean and 90th percentile estimates.

As noted in the EDI report (Intertek, 2020, Appendix III), the above estimates based on 2-day average intakes may be conservative and may not necessarily represent long-term intakes because

(1) they may not capture infrequent consumers of foods proposed to contain RG-I-enriched carrot fiber, (2) assume that subjects who consumed cRG-I-containing products on both survey days actually consume these cRG-I-containing products every day of the year, and (3) do not adjust for potential day-to-day variation in cRG-I intake. A 2-day average typically overestimates long-term (chronic) daily intake and does not necessarily represent long-term intakes (Lambe *et al.*, 2000).

Further, as cRG-I is a new ingredient with limited production and is expected to cost more than other sources of fiber, it is not expected to appear in a broad range of mainstream products. Thus, intake will rarely be near the EDIs shown above.

H. Combined Intake from the Proposed Uses and Other Sources

Data about the content of the RG-I portion of pectins in commercial foods and beverages, as well as pectins in their natural matrix in fruits and vegetables, are scarce. RG-I-Enriched carrot fiber is a new product and not available in other foods. Therefore, the NutriLeads B.V. did not calculate a combined intake.

I. Exposure to Undesirable Substances

RG-I-Enriched carrot fiber may contain low levels of certain heavy metals. The Notifier has set the specifications for the respective contaminants to levels which will ensure that all requirements of *Food Chemicals Codex* (FCC) 9th edition for pectin (USP, 2014) are met for the proposed use levels described herein. Internal specifications control for heavy metals, including some not listed in the FCC monograph on pectin. Other potential contaminants (e.g., pesticides) are minimized through quality control practices of the incoming raw carrot pomace. Pesticides are not included in specifications for cRG-I but have been analyzed in carrot pomace (results can be found in Appendix I).

Levels of β-carotene and other low-molecular weight molecules from carrots are reduced during the ultrafiltration step in the production of cRG-I. Residual levels of β-carotene have been measured and are shown in Table 17.

Table 17. Levels of β-carotene in cRG-I (µg/100 g)

	Batch number	NL91	NL100	NL176	NL189	NL204
Analyte	**Method**					
Beta-carotene	EN 12823-2:2000	27.2	351	329	702	52.4

At these levels, intake of β-carotene would not exceed 0.2 mg/person/day which is far below a level that has been reported to cause effects in humans (see Narrative).

J. Precautions and Restrictions of Use

Not intended for consumption by infants under the age of 6 months.

4. SELF-LIMITING LEVELS OF USE

This section of the GRAS Notice fulfills requirements of 21 CFR §170.240 by providing information about any self-limiting characteristics of RG-I-enriched carrot fiber use.

RG-I-Enriched carrot fiber is a dietary fiber and is incorporated into specific food products at specified levels to enhance the fiber content of those foods. The use in foods is considered to be self-limiting for technological reasons as it may negatively impact the product texture and/or flavor profile, either of which could affect consumer acceptance. These technical limitations will limit use levels in foods or consumption of the foods, thereby limiting consumption of cRG-I.

5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

This section of the GRAS Notice fulfills the requirements of 21 CFR §170.245 by commenting on evidence of a substantial history of consumption of the notified substance for food use by a significant number of consumers prior to January 1, 1958.

General recognition of safety for cRG-I is established through scientific procedures. Therefore, information regarding experience based on common use of the notified substance in food prior 1958 is not applicable.

The historical consumption of carrots and carrot products is discussed in Part 3 as supporting information.

6. BASIS FOR CONCLUSION OF GRAS STATUS FOR RG-I-ENRICHED CARROT FIBER (NARRATIVE)

This section of the GRAS Notice fulfills the requirements of 21 CFR §170.250 by providing a narrative in regard to the generally available and accepted scientific data, information, methods, or principles that are relied on to establish safety.

A. Introduction

The safety evaluation of cRG-I is based on a large number of reliable, original, peer-reviewed publications and published expert reviews concerning carrots, dietary fiber and pectins, as well as toxicology studies in animals and clinical humans with cRG-I. The safety evaluation of cRG-I is also based upon its manufacture (standard processes used by the food industry), composition (fiber, sugars, proteins and salts), specifications, and estimated intake.

The following subparts of this Notice provide a description of the intestinal fermentation, toxicological studies, allergenicity and clinical studies on carrots, fiber and pectins that support the safe use of cRG-I for its intended uses. This viewpoint is substantiated by fermentation and toxicology studies with cRG-I. In addition, the conclusions reached by an independent panel of qualified experts are presented in Appendix IV and are considered to be accurate by the Notifier.

B. Regulatory Status of Carrot Fiber and Related Products

The principal components of cRG-I – fiber, sugars, proteins and salts – are nutrients that are part of the normal human diet and already determined to be safe for consumption.

Pectins, from which cRG-I is derived, are affirmed as GRAS (21 CFR §184.1588) and used as a gelling, thickening and stabilizing agents, (USP, 2014). They are approved for general use in foods worldwide (JECFA, 2014).

Carrot fiber, a main component of carrot pomace from which cRG-I is derived (Sharma *et al.*, 2012), has been determined to be GRAS by a panel of qualified experts (GRN 0116; FDA, 2003). Citrus is another source of pectins in the diet and dried citrus pulp and citrus flour are GRAS (GRN 0487; FDA, 2014 and GRN 0599; FDA, 2016), as are dried orange pulp (GRN 0154; FDA, 2004) and orange pomace (GRN 0719; FDA, 2017).

C. Safety of Carrots and Carrot Fiber

There are no known detrimental effects associated with typical consumption of carrots. Carrots are encouraged to be consumed as part of a normal diet because they are rich in vitamins and fiber.

Eating an excessive amount of carrots (>250 g/day, about three large carrots), though, can result in skin discoloration and elevated liver enzymes from hypercarotenemia and hypervitaminosis A due to β-carotene and vitamin A (Sansone and Sansone, 2012; Priyadarshani, 2018). This is not a result of fiber and not relevant to the ingestion of cRG-I as β-carotene and other low-molecular-weight molecules are greatly reduced during the ultrafiltration step of the manufacturing process (discussed above).

The safety of carrot fiber has been established to be GRAS and has received a 'No Questions" letter from the FDA (GRN 0116, 2003). The basis of the safety determination by qualified experts was: (1) substantial similarity to the fiber portion of fresh carrot; (2) common knowledge of the historical consumption of fresh carrot including its fiber portion, and; (3)and the estimated daily intake of carrot and carrot fiber associated with the proposed uses, which was negligible.

D. Safety of Pectins

A report by the Select Committee on GRAS Substances (SCOGS, 1977) on pectins concluded that "There is no evidence in the available information on pectin and pectinates, including amidated pectins, that demonstrates or suggests reasonable grounds to suspect a hazard to the public when they are used at levels that are now current or might reasonably be expected in the future." Pectins are affirmed as GRAS by the U.S. FDA (21 CFR §184.1588) for use in foods in general with no limitation other than current Good Manufacturing Practices.

JECFA determined that the appropriate group ADI for pectins was "not specified" (JECFA, 1981). That has not changed over subsequent re-evaluations (JECFA, 2015). In 2014 it reconsidered non-amidated pectin as part of a proposed use in infant formula (JECFA, 2014). While no overt toxicological effects were found in the new data presented, decreased food intake and body weight gain were a concern at the use levels proposed. An addendum to the toxicological monograph was made focusing on use in infant formulas (JECFA, 2015). No action was taken on this proposed use and more data were requested.

The safety of pectins has been thoroughly reviewed recently by the EFSA ANS Panel (2017). The Panel found no evidence of adverse effects and concluded that there is no safety concern for the use of pectins as food additives for the general population for the reported uses and use levels of pectins and that there is no need for a numeric ADI. These conclusions and the studies upon which they are based are publicly available.

E. Safety of RG-I-Enriched Carrot Fiber

Pectins are soluble dietary fiber (FDA, 2018) and therefore are non-digestible and not absorbed from the intestine (Saito *et al.*, 2005). Likewise, RG-I is also non-digestible (Wu *et al.*, 2019; Van den Abbeele *et al.*, 2020). As RG-I is a large polysaccharide derived from pectins with a molecular mass greater than 10 kDa (Figures 5 & 6), it will not be absorbed from the intestine. Since both pectin and RG-I are unabsorbed and RG-I pectic fraction makes up as much as 35% of pectin (Nahm *et al.*, 2020), safety studies on pectin are relevant to the safety of cRG-I. In addition, NutriLeads B.V. has carried out *in vitro* fermentation, genotoxicity, and toxicity studies with its cRG-I to support a safety review.

As dietary fiber is by definition not hydrolyzed by human enzymes (Saito *et al.*, 2005), the indigestibility of pectins means there is no direct uptake of the material itself into the body (EFSA ANS Panel, 2017). Further, in their "Re-evaluation of pectin (E 440i) and amidated pectin (E 440ii) as food additives," the EFSA ANS Panel (2017) stated that pectin is stable in human saliva and simulated gastric juice and found evidence of only low digestion in the upper parts of the digestive tract which was linked to bacteria present in the terminal ileum. Rather, pectin is partially fermented by the microflora in the gastrointestinal tract to oligogalacturonic acids, which are then further metabolized to short-chain fatty acids (SCFA), such as acetate, propionate and butyrate (JECFA, 2014).

Literature Review of Digestion and Fermentation of Pectins, RG-I and cRG-I
To collect information on whether pectin derivatives like cRG-I are handled in the intestines similarly to pectin, literature searches were performed in the PubMed database (PubMed, 2020) through August 12, 2020. The objectives were to find published information on digestion or fermentation of pectins and pectin-derived oligosaccharides by the human intestinal microbiome. Relevant publications are summarized below.

Khodaei *et al.* (2016) studied the fermentation of galactose-rich oligosaccharides derived from enzymatically hydrolyzed potato RG-I in an *in vitro* simulation system. Fermentation of potato RG-I in a continuous system with a selection of immobilized human fecal microbiota showed an increase of beneficial bacterial species, suggesting a prebiotic effect. Compared to fructooligosaccharides, less butyrate but more acetate was generated by fermentation of potato RG-I. The total amounts of SCFA remained in the range of the fructooligosaccharides. Under the experimental conditions, about 80% of RG-I and derived oligosaccharides remained undigested.

In vitro fermentation properties of oligosaccharides derived from orange peel pectin in a mixed fecal bacterial culture over 24 h were assessed by Manderson *et al.* (2005). Pectic oligosaccharides were able to increase *Bifidobacteria* and *Eubacterium rectale*. These results were an indication that pectic oligosaccharides can have a beneficial effect on fecal microflora.

Bang *et al.* (2018) investigated the prebiotic effect of pectin on microbiota derived from three healthy donors. While the microbial composition of the donor samples differed, pectin fermentation resulted in the increase of similar bacterial populations in the microbiota from all three. Moreover, the authors noted rapidly increased acetate production, rising concentrations of butyrate after 6 h of pectin fermentation, and an almost quantitative degradation of pectin after 18 h.

Tingirikari (2018) extensively reviewed microbiota-accessible pectic poly- and oligosaccharides in the human intestine and confirmed the inability of human enzymes to degrade pectin or its oligosaccharides as well as their prebiotic effect on favorable intestinal bacteria.

Chung *et al.* (2019) investigated the influence of arabinoxylan-oligosaccharides, pectin and inulin on the diversity of human colonic microbiota under *in vitro* conditions. The authors showed strong differences at the species level, with some species thriving on single substrates, while others increased significantly on mixed substrate. They reasoned that in order to promote intestinal microbiome diversity, complex non-digestible substrates and mixtures should be employed. Moreover, the authors showed a significant correlation between propionate formation and percentage of *Bacteroides* in the microbiome.

Wilkowska *et al.* (2019) reported the prebiotic effect of pectin-derived oligosaccharides derived from mild acid or enzymatic hydrolysis of apple pomace. They confirmed the increase of *Bifidobacteria* and inhibition of *Enterobacteriaceae*, and revealed a reduced adhesion of the bacteria to intestinal cells.

An *et al.* (2019) compared young adults to elderly adults and investigated the effect of pectin supplementation on fecal microbiota composition, SCFAs and exhaled volatile organic compounds (VOCs) using a randomized, double-blind, placebo-controlled parallel design approved by a medical ethics committee. Fifty-two young adults and 48 elderly adults consumed 15 g/day sugar beet pectin or maltodextrin for four weeks. Young and elderly adults showed similar fecal SCFA exhaled VOC profiles and fecal microbiota profiles with just five genera significantly different in relative abundance. Pectin supplementation did not significantly alter fecal microbiota, SCFA or exhaled VOC profiles in elderly or young adults. In neither of the two age groups were there any effects of pectin supplementation on fecal microbiota, SCFA, and exhaled VOC profiles.

In summary, the reports indicate that pectins and pectin-derived oligosaccharides are not digested by enzymes within the human intestine, are fermented by colonic microbiota, fermentation results in the production of SCFA, and *Bifidobacteria* and *Bacterioides* usually show enhanced growth while *Enterobacteriaceae* levels are reduced. However, it can be difficult to detect these effects in clinical studies.

In Vitro Fermentation Studies with cRG-I

To confirm that cRG-I is also subject to colonic metabolism, NutriLeads B.V. conducted *in vitro* fermentation experiments using human fecal microbiota (Van den Abbeele *et al.*, 2020). The results

confirm that cRG-I is fermented by the human microbiota and exerts a prebiotic effect as it increases the abundance of *Bifidobacteria* and triggers the production of acetate, propionate and butyrate. In addition, the production of (detrimental) branched SCFA and ammonia from protein fermentation is reduced. The fermentation of cRG-I led to the generation of SCFA at levels equivalent to inulin but with reduced production of gas. Gas production often limits the intake of inulin and other fermentable fibers as they cause bloating and intestinal discomfort.

These studies support a conclusion that cRG-I is handled by the human intestine similar to pectins and other oligosaccharides. In this regard the way cRG-I is handled by the human digestive tract is substantially equivalent to pectins, a GRAS substance, and other oligosaccharides which have "No Objection" letters to their GRAS status from FDA.

Literature Review of the Toxicology of Pectin, Pectin Subunits, and cRG-I

A literature search on the toxic potential of pectin-derived RG-I and carrot was performed on the PubMed database (PubMed, 2020) through August 12, 2020. The search for toxicity studies related to pectin or rhamnogalacturonan revealed 71 citations of which one was deemed potentially relevant. No publications that directly addressed the safety of carrot-derived pectin or rhamnogalacturonan were identified.

The one publication, by Garthoff *et al.* (2010), addressed the safety of pectin-derived acidic oligosaccharides (pAOS). Pectin-derived acidic oligosaccharides are from the linear sections of the pectin structure and are composed of 62% galacturonic acid oligomers. Their molecular weight is below 3,800 kDa and their methylation degree is about 50%. Pectin-derived acidic oligosaccharides are non-digestible carbohydrates that attracted interest as potential substitutes for acid human milk oligosaccharides, to be used as an ingredient in infant formulas and medical nutrition.

Although pAOS is derived from pectin and are not digestible, they have limited relevance to cRG-I because they are shorter, linear and composed of acidic saccharides, while cRG-I is larger, branched and consists primarily of neutral monosaccharides like rhamnogalacturonans, arabinans and galactans. Nonetheless, the publication is included here to demonstrate the safety of other pectin subunits.

The genotoxic potential of pAOS was evaluated (Garthoff *et al.*, 2010). Pectin-derived acidic oligosaccharides were not mutagenic in the Ames test. Positive results were obtained in the chromosome aberration test only at highly cytotoxic concentrations. The effects obtained in the mouse lymphoma test were equivocal. Pectin-derived acidic oligosaccharides were considered not genotoxic when tested in a rat micronucleus test *in vivo*. Garthoff *et al.* (2010) also performed a subchronic dietary study of pAOS in rats, preceded by a 4-week parental and *in utero* exposure phase. Administration of pAOS did not affect parental health nor pup characteristics. In the subchronic study slight diffuse hyperplasia of the epithelial layer of the urinary bladder was noted in the high-dose group, which was assumed to result from concurrently elevated urinary sodium levels due to

high sodium in pAOS and elevated urinary pH. When pAOS was administered concomitantly with NH_4Cl in a satellite study, an acidifying agent, hyperplasia was completely abrogated demonstrating that the effect was due to the ion, not the pAOS. A no-observed adverse effect level (NOAEL) of 2.5% pAOS in the diet was identified, which corresponds to 1.7 g/kg bw/day.

The EFSA "Compendium on Botanicals" (EFSA, 2012) was consulted but no relevant entries were identified.

EFSA provides a literature review on the toxicity of pectins in its "Re-evaluation of pectin (E 440i) and amidated pectin (E 440ii) as food additives" (EFSA ANS Panel, 2017). The EFSA Panel considered the acute toxicity of pectins to be low, as no LD_{50} values were identified. Few published genotoxicity studies were found by the EFSA Panel; nonetheless, based on what has been published and what was available to the Panel, no *in vitro* or *in vivo* studies were identified that raised any concern. The review of short-term and subchronic oral toxicity studies did not reveal adverse effects for pectin up to 13,500 mg/kg bw in rats, which was the highest amount tested (Til *et al.*, 1972). There was an effect on empty cecum weights but this was considered an adaptive response and not an adverse effect. From studies with pectin in drinking water at several concentrations, NOAELs of 3,366 mg/kg bw and 3,916 mg/kg bw were reported in male and female rats, respectively, corresponding to the highest administered concentration of pectin (article by Takagi *et al.*, (1997) is in Japanese with parts in English that were used by the EFSA Panel). The EFSA Panel further identified studies on chronic toxicity and carcinogenicity which established the highest amount tested, 5,000 mg/kg bw day, as the NOAEL for chronic toxicity (a study by Palmer *et al.*, 1974 which was unavailable but cited in Borzelleca *et al.*, 1996). The findings of numerous studies on pectin's possible promoting effect on cancer development, which were either inhibitory or showed no effect, were deemed not relevant to a safety determination (EFSA ANS Panel, 2017).

Pectins are GRAS substances in the U.S. and are consumed around the world. There are no reports indicating adverse effects in humans. Nonetheless, to be certain that nothing has been recently published a literature a review of pectin's effects on humans was performed on December 16, 2019, on the PubMed database and again on August 12, 2020 (PubMed, 2020). The objective was to identify studies performed with humans to investigate the potential toxicity of pectins. The literature review revealed only two nutritional/pharmacologic studies that report on unwanted side effects that are relevant.

In one study, up to 15 g pectin/day was administered for four weeks to men and women to ascertain its effect on mild hypercholesterolemia (Brouns *et al.*, 2012). No side effects were noted other than flatulence. In another study, men and women were given approximately 15 g pectin/day for three weeks to examine its effect on hunger, satiety and body weight (Howarth *et al.*, 2003); no beneficial or adverse effects were seen. Other studies found in the literature review used a mixture of pectin and other fibers and were deemed unsuitable for inclusion or did not mention anything about adverse or untoward effects.

A search on carrot-related toxicity yielded 44 citations, of which two articles were deemed applicable to a safety review of cRG-I.

Ma *et al.* (2016) studied the physicochemical properties and intestinal protective effects of ultra-micro ground insoluble dietary fiber from carrot pomace. The processing led to a substantial increase of the surface area of the fiber and improved the water-holding, swelling and oil-holding capacities. Moreover, it was shown that insoluble carrot dietary fiber has no toxicity for Caco-2 cells at a concentration of 10.0 mg/L. While carrot pomace is the raw material from which cRG-I is extracted, the findings reported by Ma *et al.* (2016) have limited relevance for cRG-I as the quality of the materials cannot be compared. Also, the insoluble fraction was studied whereas cRG-I is a soluble fraction of carrot pomace.

In a case report, Sansone and Sansone (2012) describe a 48-year-old male who complained to his primary care physician of abdominal discomfort and yellow/orange skin discoloration. Physical examination was normal except for some mild mid-abdominal discomfort (no observed skin color changes). Laboratory studies indicated elevated liver enzymes. Upon further questioning, the patient reported ingesting 3 kg of carrots per week to facilitate his dieting effort. The patient was diagnosed with constipation, hypercarotinemia, and possible vitamin A toxicity. Following the cessation of excessive carrot ingestion, liver enzymes normalized within a month. The lack of relevance of β-carotene toxicity to cRG-I has been discussed.

In summary, PubMed searches up to August 12, 2020, (PubMed, 2020) did not reveal any information suggesting genotoxicity, mutagenicity or toxicity of pectins, pectin fractions in general, or materials derived from carrot other than β-carotene which is considered a nutrient under normal circumstances.

Toxicology Studies with cRG-I

A series of toxicology studies with NutriLeads' cRG-I, listed below, was conducted at accredited laboratories following Good Laboratory Practices (GLP). These studies recently were published by Jonker *et al.*, 2020.

Table 18. Toxicology studies with NutriLeads' cRG-I.

Study	Guideline	GLP	Batch no.
Bacterial reverse mutation assay	OECD 471	Yes	NL100
Bacterial reverse mutation assay	OECD 471	Yes	NL176
In vitro micronucleus assay	OECD 487	Yes	NL176
In vitro mammalian cell gene mutation assay	OECD 490	Yes	NL176

Dose-range finding study	OECD 408	No	NL100
90-Day oral toxicity study in rats	OECD 408	Yes	NL100
Recovery/homogeneity of test article in rat chow	Laboratory-developed	No	NL100

Bacterial mutagenicity

To test the mutagenic potential of NutriLeads' cRG-I and its potential metabolites, a bacterial reverse mutation assay was performed in the presence and absence of a rat liver metabolizing system (S9 mix) according to the most recent guidelines (OECD Test No. 471, 1997) using *Escherichia coli* (*E. coli*) strain WP_2*uvrA* and *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 (Jonker *et al.*, 2020).

In an initial test with cRG-I (batch NL100), no relevant dose-related increases in the number of revertant (His^+) colonies were found in tester strains TA1535, TA98 and TA100 and no relevant dose-related increases in the number of revertant (Trp^+) colonies were seen in the tester strain WP_2*uvrA*, both in the absence and presence of S9-metabolic activation. Although for some strains elevated revertant frequencies were observed, these were all below the threshold for a clear positive mutagenic response. Unexpectedly, however, *Salmonella typhimurium* strain TA1537 showed reproducibly positive responses.

The responses observed in the Ames assay were thought to be attributable to the release of histidine into the culture medium, which is recognized as a confounding factor in bacterial reverse mutation tests involving enzymes (Thompson *et al.*, 2005; EFSA, 2014). Free amino acids released into the culture medium are known to enhance the growth of the test bacteria leading to additional spontaneous mutations (Thompson *et al.*, 2005; EFSA, 2014). Increases in spontaneous mutations resulting from the presence of histidine is often misinterpreted as a genotoxic response when in fact it is the amino acid content of the culture medium that is responsible for the overall increase in the bacterial growth and greater potential for mutations to occur.

To elucidate whether the above findings might be false-positives, two batches (NL100 and NL176, total protein content of 5.8% and 1.9%, respectively; Table 5) were tested for the presence of total (free and bound) histidine and tryptophan. These amino acids may interfere with the assays, which are based on the growth inhibition of the tester strains on histidine-depleted (*Salmonella typhimurium*) or tryptophan-depleted (*E. coli*) medium (Busch and Bryan, 1987). Indeed, it was found that batch NL100 contained low but measurable levels of histidine, which could have contributed to the effects seen in the first Ames test. Batch NL100 contained higher levels of histidine and tryptophan than batch NL176 (109 mg/100 g and 44 mg/100 g respectively versus 61 mg/100 g and <0.01 g/100g in NL176; values are totals of free and bound histidine and tryptophan). Since the concentration of histidine was higher in NL100 than in NL176, the assay was repeated with batch NL176.

At the same time, in order to prepare for the *in vitro* mammalian genotoxicity assays, it was also necessary to make sure that the false positive was not due to possible microbial contamination of NL100. Thus, measures were taken to guarantee sterility of the test article. While NL100 satisfied the microbial specifications for a food ingredient, the *in vitro* mammalian tests require even lower microbial activity. Sterile filtration was not possible as it might change the composition of the product. Therefore, an irradiated sample of batch NL176 was generated, which reduced microbial counts enough to enable testing in the mammalian assay. The irradiated batch NL176 was used for a follow-up mutagenicity experiment and performed at the same laboratory as the first experiments.

Chemical analysis of the irradiated batch of cRG-I found it to be very close to the non-irradiated batch of cRG-I confirming that irradiation did not change the chemical composition (Figure 9).

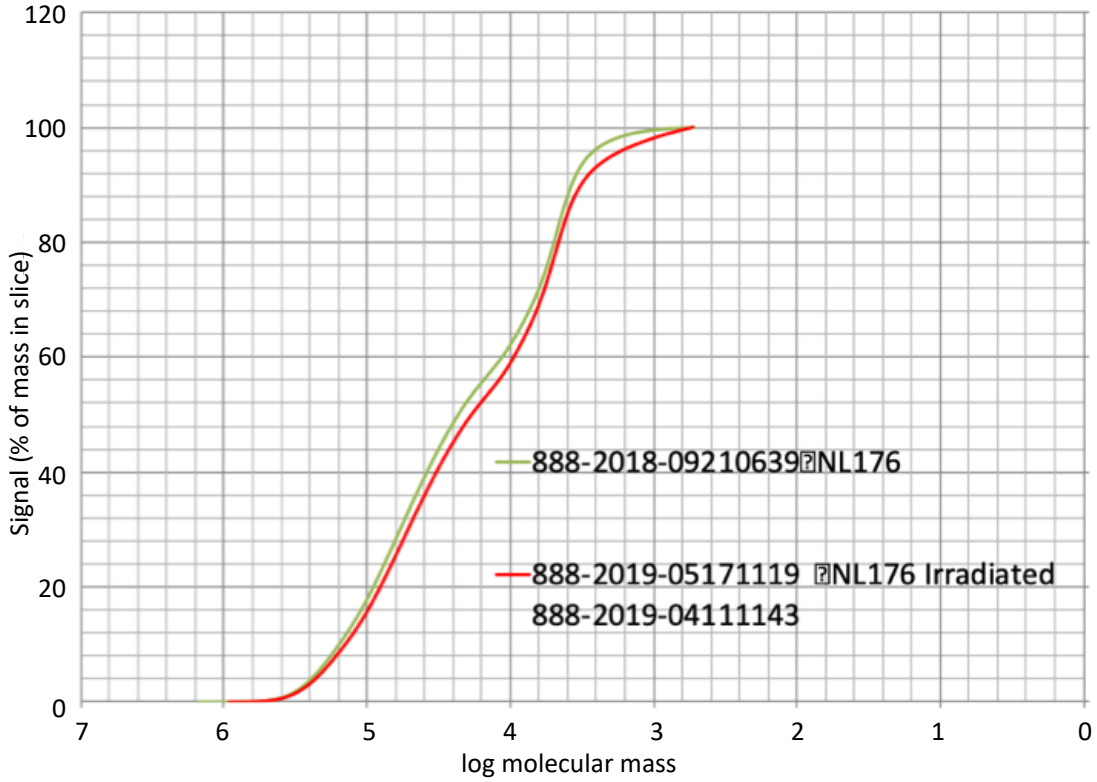

Figure 9. Molecular size distribution analysis (high performance size exclusion chromatography) comparing batch NL176 before and after irradiation.

According to Jonker *et al.* (2020), batch NL100 of cRG-I showed a positive response in the Ames test in strain TA1537, in the presence and absence of metabolic activation. The authors note that the criteria for a positive response in strains other than TA1537 were not met. It was apparent that the non-sterility of the sample may have interfered with the reliability of the outcome (non-scoreable plates at the highest tested concentration in TA1535 and TA98). A subsequent Ames test was conducted in the same laboratory as the initial test but with an irradiated batch of cRG-I (NL176). It was negative for induction of revertants in all strains including TA1537. The positive response in

the initial Ames test therefore could have been driven by the presence of microbial contamination. Automated scoring systems, which were used for both Ames tests, are not able to discriminate between genuine revertant bacterial colonies and small contaminating colonies on the plates. When present in test materials, histidine has been shown to induce positive responses in the Ames test even at low levels (Busch and Bryan, 1987). A study by Thompson *et al.* (2005) showed that effects on growth of revertant colonies by excess histidine in the Ames test did not occur until levels were at least 16μg/plate above normal trace levels. While the levels of free histidine derived from cRG-I were lower than this, at higher treatment concentrations the test formulations were a hazy suspension rather than a clear solution and particles of cRG-I deposited on the plate surface could have increased the local histidine concentration sufficiently to stimulate revertant colony growth (Thompson *et al.*, 2005). It is therefore conceivable that histidine presence may have had a growth-promoting effect on the *Salmonella typhimurium* strains particularly TA1537. Further, TA1537 has been shown to be more sensitive than other *Salmonella typhimurium* strains or the detection of frameshift and base pair substitution mutations in concurrent experiments with commonly used genotoxic positive controls (Skopek *et al.*, 1978). It is therefore conceivable that TA1537 could be more sensitive to changes in histidine than other strains in the same way that it has a greater overall sensitivity to mutagens. Thus, it is likely that microbial contamination combined with histidine presence (albeit both at low levels) in batch NL100 contributed to the positive response in TA1537 from the first Ames test.

Based on this series of testing, the authors concluded that cRG-I is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

In vitro mammalian genotoxicity

To further clarify the findings in bacteria, two mammalian genotoxicity assays were conducted, an *in vitro* micronucleus assay in human peripheral lymphocytes and an *in vitro* mouse lymphoma assay. Both studies followed OECD guidelines and were executed by an accredited, GLP-certified contract laboratory (Jonker *et al.*, 2020).

To test the genotoxic potential of NutriLeads' cRG-I (batch NL 176) and its metabolites, the frequency of micronucleated cells was determined in cultured human lymphocytes in the presence and absence of S9 in accordance with OECD guidance No 487 (OECD, 2016a).

The formation of micronuclei was studied under the following conditions:

Without S9:	3 h treatment + 24 h recovery
	24 h treatment + 0 h recovery
With S9:	3 h treatment + 24 h recovery

In the absence of noteworthy cytotoxicity or precipitate, the concentrations selected for the micronucleus analysis were 1250, 2500 and 5000 μg/mL for the three experimental conditions, with 5000 μg/mL being the highest recommended concentration.

Following the 3-h treatments with and without S9 and the 24-h treatment without S9, neither a statistically significant nor a concentration-related increase in the frequency of micronucleated/binucleated cells was noted at any of the analyzed concentrations in comparison to the corresponding vehicle control. Moreover, none of the analyzed concentrations showed frequencies of micronucleated/binucleated cells of each replicate culture above the corresponding vehicle control historical range. Hence, the results meet the criteria for a negative response.

Under the experimental conditions of the study, cRG-I did not induce any chromosome damage or damage to the cell division apparatus in cultured mammalian somatic cells using human lymphocytes, either in the presence or absence of a rat liver metabolizing system.

The potential of cRG-I (batch no NL 176) to induce mutations at the TK (thymidine kinase) locus in L5178Y $TK^{+/-}$ mouse lymphoma cells was tested (Jonker *et al.*, 2020). The test was performed in the absence and presence of S9. This *in vitro* mammalian cell gene mutation test (OECD, 2016b) is able to identify substances that cause base-pair mutations, frameshift mutations, small deletions, large deletions and rearrangements of the relevant chromosomes.

Since the test article was found to be freely soluble and non-cytotoxic in a preliminary test, the highest concentration selected for the main experiment was 5000 μg/mL, according to the criteria specified in the international guidelines for a substance of unknown or variable composition.

No cytotoxicity was observed at any of the tested concentrations, as shown by the absence of any noteworthy decrease in the adjusted relative total growth values.

In the presence of S9 metabolic activation, no increase in the mutation frequency was noted at any of the concentrations relative to the corresponding vehicle control and no dose response relationship was evident. These results meet the criteria of a negative response.

In the absence of S9, a dose-response relationship was observed by linear regression. However, considering that the induced mutation frequencies obtained in test article-treated cultures (up to 71 x 10^{-6}, at the highest tested concentration) were clearly below the global evaluation factor of 126 x 10^{-6} (OECD, 2016b), this dose-response relationship is considered irrelevant in terms of mutagenicity. As all values were within the laboratory's historical control range, these results are considered to meet the criteria of a negative response (Jonker *et al.*, 2020).

<u>Subchronic oral toxicity</u>

To identify potential toxicity and a NOAEL, a 90-day toxicity study was performed in WistarHan rats according to the most recent OECD guidance (OECD Test No. 408, 2018). The study was performed by an accredited laboratory under GLP (Jonker *et al.*, 2020).

A standard rodent diet was modified by substituting corn starch in the base formulation to contain the test article (batch NL 100) at three concentrations: 2.5%, 5%, and 10%; levels established based on a 14-day range-finding study. The test feed was analyzed for the concentration of the test article and its homogeneity in feed by an accredited laboratory. Analysis of diet preparations confirmed that the concentrations of cRG-I were in agreement with the target concentrations and that cRG-I was homogeneously distributed in the diet.

Ten male and 10 female animals were observed at each level. cRG-I was well tolerated even at the highest administered concentration. The overall mean daily intake of cRG-I calculated from the nominal dietary cRG-I concentrations and the feed consumption and body weight data was 1.8, 3.4 and 6.9 g/kg body weight/day for males and 1.9, 3.9 and 7.8 g/kg body weight/day for females of the 2.5%, 5% and 10% groups, respectively. Findings were minor, not considered adverse and attributed to the intake of a relatively high amount of fermentable fiber.

Increased cecum weights (full and empty) were observed at the middle and high concentration in males and at the high concentration in females. The relative differences from controls were up to +50% in males (full and empty), and +41% (full) or +26% (empty) in females. Microscopy revealed minimal lymphogranulocytic inflammatory cell infiltrate in the cecal mucosa in a few males and minimal hypertrophy of the mucosa in a few females.

Additionally, males exhibited a modest increase in water consumption (up to 16% in the middle and high concentration groups).

The cecal enlargement without corresponding histopathology represented an adaptive response to the ingestion of large amounts of poorly digestible, fermentable carbohydrates, which is generally considered of no toxicological importance. Cecal enlargement is a common finding in rats consuming large amounts of carbohydrates that are poorly digestible or slowly absorbed in the upper parts of the digestive tract (Bar *et al.*, 1995; De Groot, 1987; Delaney *et al.*, 2003; Garthoff *et al.*, 2010; Hodgkinson *et al.*, 1982; Jonker *et al.*, 2010a; Newberne *et al.*, 1988).

Enlargement of the ceca by dietary pectin was reported by Til *et al.* (1972) and likewise the effect was considered by the authors of the study to be an adaptive response rather than a toxic effect.

The increase in water consumption could be related to the cecal enlargement of treated rats from the high fiber intake. Increased water consumption can occur as a non-specific effect in rats fed high amounts of poorly digestible carbohydrates (De Groot *et al.*, 1995). Further, cRG-I (batch NL100) contains 2% potassium, which resulted in increased potassium intake by about 20% at the highest dose level (the basal diet contains about 0.95% potassium). In the absence of any evidence of pathological conditions known to increase water consumption (e.g. renal dysfunction), the higher water consumption of male rats fed cRG-I was regarded as a physiological, non-adverse response to high consumption of poorly digestible carbohydrates and/or potassium (Jonker *et al.*, 2020).

Functional tests showed lower forelimb grip strength values in males at 5% and 10% concentrations. These differences from controls were considered not to reflect impaired neuromuscular function as there were no corroborative clinical signs, changes in other functional measures in the same neuromuscular domain, or morphological alterations in neuronal tissues. Moreover, mean grip strength values in treated males remained in the laboratory's normal range for this endpoint.

The other end points examined showed no test article-related adverse effects.

In conclusion, ingestion of cRG-I by male and female Wistar Han rats for 13 weeks at dietary levels up to 10% (w/w) was well tolerated without any test article-related adverse effects. Therefore, the NOAEL of cRG-I under the conditions of this study was 10% (w/w) of the diet. This dietary level was equivalent to an overall mean daily intake of 6.9 g/kg bw/day in males and 7.8 g/kg bw/day in females (Jonker *et al*., 2020).

Summary of the toxicology studies with cRG-I

NutriLeads' cRG-I is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay. There is no detectable increase in mutation frequency in an *in vitro* mouse lymphoma TK assay nor any evidence of chromosome damage/ rearrangements in an *in vitro* micronucleus assay in human peripheral lymphocytes. RG-I-Enriched carrot fiber is therefore neither mutagenic nor genotoxic *in vitro*.

The 90-day subchronic oral toxicity study showed no adverse effects and supports the safety and tolerability of NutriLeads' cRG-I up to the highest dietary concentration tested (10%), corresponding to 6.9 and 7.8 g/kg bw/day in male and female rats, respectively.

Since the subchronic oral toxicity study did not reveal signs of test article-related toxicity nor was there evidence of genotoxicity, and because studies with pectins and pectin subunits have not shown toxicity, no further oral toxicity studies were conducted.

Allergenicity

Allergenicity to carrots, the source material for cRG-I, does occur but generally is not a major concern because of its rarity. Carrot is not listed as an allergen in the Food Allergen Labeling and Consumer Protection Act of 2004 (Public Law 108-282, Title II) nor is it a common allergen that is listed in Annex II of Regulation (EU) 1169/2011 (European Parliament, 2011). According to the Food Allergy Research and Resource Program at the University of Nebraska (FARRP), carrot is not listed as an allergen to be labeled anywhere in the world (FARRP, 2020).

Despite the considerations by expert groups described above, a literature search was performed to provide a scientific background to understand the allergenic potential of carrot. The literature search was performed on the PubMed database (2020) between 2010 and August 12, 2020, to collect

published information on carrot-related allergies. Since cRG-I contains protein, the objectives of the literature search were to find information on possible allergenic effects of carrots and whether the putative allergens might still be present in cRG-I. Relevant studies are summarized below.

Carrot allergy is described as a pollen-related allergy, mainly birch pollen (Markovic-Housley *et al.*, 2009; Beyer *et al.,* 2016). The most important allergenic protein in carrot is Dau c 1, a plant-defense-induced ribonuclease of the pathogenesis-related protein family 10 (PR-10), inducing IgE antibodies in 98% of carrot allergic patients (Markovic-Housley *et al.*, 2009). Other allergenic proteins are Dau c 4 (profilin), isoflavone reductase, nonspecific lipid-transfer protein and cross-reactive carbohydrates. Dau c 1 shares high homology with the Api g 1, an allergenic protein from celery and is related to the major birch-pollen antigen Bet v 1 (Bet v 1 is responsible for IgE binding in more than 95% of birch pollen-allergic patients).

Faeste *et al.* (2010) identified the isoforms of the carrot allergens Dau c 1, as well as Dau c 3 (non-specific lipid transfer protein), Dau c 4 and Dau c Cyp (cyclophilin). The authors describe Dau c 1 as heat-labile protein, which might degrade during extraction.

This assumption was supported by Lyons *et al.* (2018), who investigated possible dietary approaches for subjects with pollen-related food allergy. They identified four studies on the effect of heat processing on apple, celery, hazelnut and carrot and reported that while all individuals showed mild allergic reactions to raw carrot, there were no symptoms observed for cooked carrot. More detailed investigation of the major allergen, Dau c 1 (and isoforms), revealed a denaturing temperature of 43°C, further supporting the assumption that cooked carrot (and hence cRG-I) is not allergenic.

An article outside the 10-year scope of the search deserves to be mentioned. A study by Ballmer-Weber *et al.* (2001) confirmed the allergenicity of raw carrot by means of a double-blinded, placebo-controlled food challenge performed in central Europe. Allergic reactions to raw carrot affect up to 25% of food-allergic subjects in that region. Positive subjects had exclusively specific IgE antibodies to birch pollen-related carrot allergens, Dau c 1 being the major one. The lack of inhibition of IgE binding to Dau c 1 by birch allergens in a subgroup of patients might indicate a secondary immune response to new epitopes on the food allergen that are not cross-reactive with Bet v 1.

The only other source of protein that could become part of cRG-I is the enzyme preparation used to extract and cleave the pectin in carrot. This enzyme is GRAS, present in very small amounts and denatured by heating in the manufacturing process (discussed above). Nonetheless, the source is an *Aspergillus* species so it was included in the review. There were no reports on oral allergic reactions caused by the enzymes used for manufacture of cRG-I nor their production organisms.

In summary , there are no reports for allergic reactions to cooked carrot products. The main carrot allergen, Dau c 1, is heat labile and denatures at temperatures above 43°C (Lyons *et al.*, 2018). Since

the manufacturing process for cRG-I includes two pasteurization steps at 90°C, Dau c 1 and other possible allergic protein present in cRG-I are denatured and inactivated.

The total protein content in cRG-I is less than 6% (Table 5), including the enzyme preparation used to extract and cleave the pectin. When cRG-I is added to food at levels to produce nutrient claims, even if the carrot protein were still intact, the amount of allergenic protein would be low and therefore presents a low risk of causing an allergic reaction. Together, it is concluded that allergic reactions would be extremely uncommon in consumers of cRG-I and if any were to occur, they would be mild.

Based on this review, the Notifier considers cRG-I to be non-allergenic at the levels of intended use and deemed testing of cRG-I unnecessary.

F. Summary of Safety Assessment and GRAS Conclusion

The safety assessment of cRG-I is based upon its source – commonly consumed carrots – and pectin, a high-molecular weight carbohydrate present in virtually all plants as well as studies on cRG-I conducted by NutriLeads B.V. People have been consuming carrots for centuries without adverse effects. There are no toxic effects associated with carrots or carrot products other than with excessive intake for a prolonged period of time and are reversible upon cessation of consumption (Sansone and Sansone, 2012). Raw carrots can elicit allergic reactions in people allergic to birch pollen but is uncommon and no government requires labeling of carrots as an allergen.

People have been consuming pectins as a part of their diets without adverse effects for centuries. There are no toxic effects associated with pectins (EFSA ANS Panel, 2017). FDA considers pectins to be GRAS, permitting general use in foods with no limitation on use other than current Good Manufacturing Practices. JECFA has set an ADI of "not specified" for pectins. EFSA did not assign a numeric ADI.

The material from which cRG-I is derived, carrot pomace, is a carrot fiber, a material that has received a "No Objections" letter from FDA (GRN 0116) (FDA, 2003). RG-I-Enriched carrot fiber is a subunit of the pectin molecule and is composed of common monosaccharides. It is obtained after enzymatic hydrolysis of carrot pomace. Its identity has been confirmed by chemical analysis of its monosaccharides. Other constituents are common nutrients.

The safety assessment of cRG-I is based on information about its manufacture and composition. The manufacturing process uses methods that are commonly used in the food industry and are performed under GMP. The specifications for cRG-I are suitable for food-grade ingredients. The overall chemical composition is fiber, sugars, water and protein. The composition and microbial quality were analyzed for five representative batches and demonstrated that the manufacturing

process is capable of producing cRG-I consistently and according to specifications. Stability tests at standard and accelerated conditions were performed and support a shelf life of at least two years.

The process to manufacture cRG-I, especially the filtration step, greatly reduces low-molecular weight constituents (e.g., phytochemicals, such as carotenoids, anthocyanins and other phenolic compounds) and vitamins such as vitamin A (through *a*- and *β*-carotene), thiamin, riboflavin, and niacin (Arscott and. Tanumihardjo, 2010). No adverse effects from low molecular weight compounds can be expected. Further, levels of heavy metals are within accepted criteria for food ingredients. The manufacturing process has two pasteurization steps, so there is reasonable certainty that cRG-I is not allergenic as the most prominent allergen in carrot is heat labile.

The safety assessment of cRG-I is corroborated by qualified experts that pectin, the specific fiber from which cRG-I is derived, is affirmed as GRAS for use in foods in general at levels not to exceed current Good Manufacturing Practices (SCOGS, 1977; 21 CFR §184.1588). Likewise, experts outside the U.S. have come to the same conclusion about pectin's safety (JECFA 1981; JECFA, 2014; EFSA ANS Panel, 2017). The safety assessment of cRG-I is further corroborated on the basis of the finding that ingredients from other foods that are high in pectin are also GRAS (e.g., GRN 0154, 0487, 0599, 0719) (FDA, 2004; FDA, 2014; FDA 2016; FDA, 2017).

Published literature focusing on the digestibility and fermentation of materials similar to cRG-I, including pectin and oligosaccharides, demonstrate that these substances are undigested and unabsorbed, and fermented in the gut by the microflora. Likewise, published articles on these related materials did not identify any adverse effects.

The safety assessment of cRG-I considered *in vitro* fermentation studies with cRG-I (Van den Abbeele *et al.*, 2020), which demonstrate that it is undigested and unabsorbed by humans and then fermented in the gut by the colonic microflora to SCFAs.

RG-I-Enriched carrot fiber is proposed for use as a source of dietary fiber, a macronutrient that is necessary to be consumed to be added to a variety of foods commonly consumed for which no standard of identity exists. Based on these food categories and use levels ranging from <1-22%, daily intake estimates were derived from the NHANES 2015-2016 database (NCHS, 2020). The conservative EDI of cRG-I from all proposed uses, and assuming the maximum proposed use level for each food category, is 11.2 g/day at the mean intake and 21.2 g/day at the 90th percentile of intake among users in the total U.S. population, equivalent to 184 mg/kg/day and 369 mg/kg/day, respectively.

Furthermore, the EDI of the dietary portion of cRG-I was compared to the Dietary Reference Intake levels for dietary fiber. The AI levels for dietary fiber are 30-38 g/day and 21-26 g/day for adult males and females, respectively (IOM, 2005). The EDI of dietary fiber from cRG-I comes

close to the AI but even when using conservative upper estimates of intake for cRG-I, the EDI for the fiber portion of cRG-I by Americans is less than the AI for dietary fiber.

As there is no tolerable upper intake level of intake for dietary fiber (IOM, 2005) to which the EDI can be compared, any excursions above the AI are considered by qualified experts to be of minor safety concern.

The pivotal safety data for cRG-I includes both genetic toxicity and a 90-day subchronic feeding study (Jonker *et al* , 2020). The toxicological studies were performed following OECD guidelines under GLP. RG-I-Enriched carrot fiber is not genotoxic up to the highest tested concentration of 5000 µg/ml. A 90-day oral study in rats did not reveal adverse effects up to the highest level tested, 10% w/w of cRG-I in the diet. This dietary level was established as the NOAEL, corresponding to overall mean daily intakes of 6.9 and 7.8 g cRG-I/kg bw/day for males and females, respectively. This NOAEL provides a significant margin of safety when compared to the 90th percentile EDI of 0.369 g/kg bw/day, for an ingredient considered a macronutrient, where the concentration of the test article generally cannot be incorporated into the diet at sufficiently high levels to derive a 100-fold safety factor without resulting in nutritional imbalances which can lead to secondary consequences such as adverse physiological effect.

The safety studies reported in the recently published article by Jonker *et al.* (2020) and information described in this GRAS Notice satisfy the safety standard of reasonable certainty of no harm. No toxic effects were observed, even at the highest amounts administered to animals. It is fermented the same manner as pectin and other oligosaccharides. In addition, these data and information are known and accepted by a consensus of qualified experts in the general scientific community. The totality of published data on closely related materials (e.g., pectins, oligosaccharides) also supports the safety of cRG-I for human consumption. This not only assures that the intended uses of cRG-I described in this Notice are safe, but also comprises common knowledge that cRG-I is also generally recognized as safe under its proposed conditions of use.

Overall, NutriLeads B.V. concludes that cRG-I, produced in accordance with current Good Manufacturing Practices, is reasonably certain not to cause harm under its intended conditions of use and is generally recognized as safe for the intended uses by scientific procedures. This is supported by the fact that:

- the RG-I subunit of cRG-I is a pectic polysaccharide that naturally occurs in pectin, a GRAS material;
- cRG-I is derived from carrot fiber, also a GRAS material;
- cRG-I is not absorbed from the intestine and fermented to SCFA, natural constituents in the body;
- cRG-I is a natural component of commonly consumed carrots;
- cRG-I is made to a consistent quality, that it is subjected to heat treatments and it has a low protein level so there is minimal chance of allergenic reactions and

- cRG-I does not raise concerns in genotoxicity and subchronic toxicity studies.

NutriLeads B.V. is not aware of information that would be inconsistent with a conclusion that the proposed uses of cRG-I, meeting appropriate specifications and used according to current Good Manufacturing Practices, are GRAS.

None of the data and information in Parts 2 through 7 of the current GRAS Notice is considered to be exempt from disclosure under the Freedom of Information Act (FOIA; 5 U.S.C. 552).

G. Independent Expert Review and Conclusion

In order to assure that the common knowledge about the safety of cRG-I is generally accepted by a consensus of qualified experts, NutriLeads B.V. convened an Expert Panel of prominent experts in the field of food chemistry, food toxicology and food ingredient safety evaluation, composed of Ashley Roberts, Ph.D., (AR Toxicology, Inc.), Thomas Vollmuth, Ph.D. (Vollmuth and Associates, LLC) and James R. Coughlin, Ph.D. (Coughlin & Associates), to independently review this document and the data supporting it. The individuals comprising this Panel are qualified by scientific training and experience to evaluate the safety of substances intended to be added to food. They have critically evaluated the available information summarized in this document and have individually and collectively concluded that cRG-I, produced consistent with current Good Manufacturing Practices and meeting the specifications described herein, is safe under its intended conditions of use. The Panel further concluded that the proposed uses cRG-I satisfy the safety standard of reasonable certainty of no harm. Thus, the Panel concluded that cRG-I is not only safe, but generally recognized as safe (GRAS) for the intended conditions of use described herein. The Panel's GRAS opinion is included as an attachment to this document (see Appendix IV).

7. REFERENCES

Note: All references listed herein are considered *generally available*.

An, R, Wilms, E, Smolinska, A, Hermes, G, Masclee, A, de Vos, P, Schols, HA, van Schooten, FJ, Smidt, H, Jonkers, D, Zoetendal, EG, and Troost, FJ. 2019. Sugar beet pectin supplementation did not alter profiles of fecal microbiota and exhaled breath in healthy young adults and healthy elderly. *Nutrients*, **11**(9), 2193. https://doi.org/10.3390/nu11092193.

Arscott, SA and Tanumihardjo, SA. 2010. Carrots of many colors provide basic nutrition and bioavailable phytochemicals acting as a functional food. *Comp Rev Food Sci Food Safety*. **9**, 223-239. DOI: 10.1111/j.1541-4337.2009.00103.x.

Ballmer-Weber, BK, Wüthrich, B, Wangorsch, A, Fötisch, K, Altmann, F, and Vieths, S. 2001. Carrot allergy: double-blinded, placebo-controlled food challenge and identification of allergens. *J Allergy Clin Immunol.* **108**(2), 301-7. https://doi.org/10.1067/mai.2001.116430.

Bang, SJ, Kim, G, Lim, MY, Song, EJ, Jung, DH, Kum, JS, Nam, YD, Park, CS and Seo, DH. 2018. The influence of in vitro pectin fermentation on the human fecal microbiome. *AMB Express* **8**(1), 98. DOI: 10.1186/s13568-018-0629-9.

Bar, A, Til, HP and Timonen, M. 1995. Subchronic oral toxicity study with regular and enzymatically depolymerized sodium carboxymethylcellulose in rats. *Food Chem. Toxicol.* **33**, 909–917. https://doi.org/10.1016/0278-6915(95)00069-e.

Beyer, S, Franke, A, Simon, JC and Treudler, R. 2016. Measurement of health-related quality of life in adult patients with birch pollen-associated food allergy. *J Dtsch Dermatol Ges.* (J German Soc. Dermatol.) **14**, 397-404. DOI: 10.1111/ddg.12731.

Blake, JD and Richards, GN. 1968. Problems of lactonisation in the analysis of uronic acids. *Carbohydrate Research*, **8**, 275-281. DOI:10.1016/S0008-6215(00)82233-8.

Borzelleca**,** JF, Filer Jr., LJ, Kinoshita, FK, Gerrish, TC, Kuo, PK and La Du, BN. 1996. Evaluation of the safety of sodium pectate as a food ingredient. *Food Chem. Toxicol.* **34**, 21-25. https://doi.org/10.1016/0278-6915(95)00096-8.

Brouns, F, Theuwissen, E, Adama A, Bell, M, Berger, A, and Mensink, RP. 2012. Cholesterol-lowering properties of different pectin types in mildly hyper-cholesterolemic men and women. *Eur J Clin Nutr.* **66**(5), 591-599. DOI: 10.1038/ejcn.2011.208.

Busch, DB and Bryan, GT. 1987. Presence and measurement of sample histidine in the Ames test: quantification and possible elimination of a source of false-positive mutagenicity test results. *Environ. Mol. Mutagen.* **10**, 397–410. https://doi.org/10.1002/em.2850100409 .

Chung, WSF, Walker, AW, Vermeiren, J, Sheridan, PO, Bosscher, D, Garcia-Campayo, V, Parkhill, J, Flint, HJ and Duncan, SH. 2019. Impact of carbohydrate substrate complexity on the diversity of the human colonic microbiota. *FEMS Microbiol Ecol.* **95**. DOI: 10.1093/femsec/fiy201.

Coenen, GJ, Bakx, EJ, Verhoef, RP, Schols, HA and Voragen, AGJ. 2007. Identification of the connecting linkage between homo- or xylogalacturonan and rhamnogalacturonan type I. *Carbohydr Polym* **70**, 224-235. DOI: 10.1016/j.carbpol.2007.04.007.

Columbia University. Undated. Orange skin tone from too many carrots? https://goaskalice.columbia.edu/answered-questions/orange-skin-tone-too-many-carrots.

Davidson, A. 1999 *The Oxford Companion to Food.* Pp. 140-141. Oxford University Press, Oxford.

De Groot, A. 1987. Biological effects of low digestibility carbohydrates. In: Leegwater, DC, Feron, VJ, and Hermus, RJJ. (Eds.), Low Digestibility Carbohydrates, pp. 12–22. Pudoc Wageningen.

De Groot, AP, Lina, BAR, Hagenaars, AJ, Hollanders, VMH, Andringa, M and Feron, VJ. 1995. Effects of a dietary load of acid or base on changes induced by lactose in rats. *Food Chem. Toxicol.* **33**, 1–14. https://doi.org/10.1016/0278-6915(95)80241-x.

Delaney, B, Carlson, T, Frazer, S, Zheng, T, Hess, R, Ostergren, K, Kierzek, K, Haworth, J, Knutson, N, Junker, K and Jonker, D. 2003. Evaluation of the toxicity of concentrated barley β-glucan in a 28-day feeding study in Wistar rats. *Food Chem. Toxicol.* **41**, 477–487. https://doi.org/10.1016/s0278-6915(02)00298-3.

De Ruiter, GA, Schols, HA, Voragen, AGJ and Rombouts, FM. 1992. Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Anal. Biochem.* **207**, 176-185. https://doi.org/10.1016/0003-2697(92)90520-H.

Dietary Guidelines Advisory Committee. 2015. Scientific Report of the 2015 Dietary Guidelines Advisory Committee: Advisory Report to the Secretary of Health and Human Services and the Secretary of Agriculture. U.S. Department of Agriculture, Agricultural Research Service, Washington, DC.

Doco, T, Williams, P, Vidal, S, and Pellerin, P. 1997. Rhamnogalacturonan II, a dominant polysaccharide in juices produced by enzymic liquefaction of fruits and vegetables. *Carbohydrate Res.* **297**, 181-186. https://doi.org/10.1016/S0008-6215(96)00260-1.

Duvetter, T, Sila, DN, Van Buggenhout, S, Jolie, R, Van Loey, A and Hendrickx, M. 2009. Pectins in processed fruit and vegetables: Part I – Stability and catalytic activity of pectinases. *Compr. Rev. Food Sci. Food Saf.* **8**, 75-85.

EFSA (European Food Safety Authority). 2012. Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements. *EFSA Journal.* **10**(5): 2663. https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2012.2663.

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food). 2017. Mortensen A, Aguilar F, Crebelli R, Di Domenico A, Dusemund B, Frutos MJ, Galtier P, Gott D, Gundert-Remy U, Lambre C, Leblanc J-C, Lindtner O, Moldeus P, Mosesso P, Oskarsson A, Parent-Massin D, Stankovic I, Waalkens-Berendsen I, Wright M, Younes M, Tobback P, Ioannidou S, Tasiopoulou S and Woutersen RA. Scientific Opinion on the re-evaluation of pectin (E 440i) and amidated pectin (E 440ii) as food additives. *EFSA Journal.*;**15**(7):4866, 62 pp. https://doi.org/10.2903/j.efsa.2017.4866.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids) 2014. Explanatory Note for the Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on the Submission of a Dossier on Food Enzymes. EFSA Supporting Publications. 11. 10.2903/sp.efsa.2014.EN-689.

European Commission. 2005a. COMMISSION REGULATION (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Off J Eur Union*. L 338/1-338/26.

European Commission. 2005b. REGULATION (EC) NO 396/2005 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. *Off J Eur Union*. L 70/1-70/16.

European Commission. 2006. COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off J Eur Union*. L 364/5-364/24.

European Commission. 2017. Guidance document describing the food categories in Part E of Annex II to Regulation (EC) No 1333/2008 on Food Additives. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/fs_food-improvement-agents_guidance_1333-2008_annex-2.pdf

European Parliament, Council of the European Union. 2011. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. *Off. J.* L 304, 22.11.2011, p. 18–63.

EPA (U.S. Environmental Protection Agency). 2018. Exposure Factors Handbook Chapter 9 (Update): Intake of Fruits and Vegetables. U.S. EPA Office of Research and Development, Washington, DC, EPA/600/R-18/098F.

FAO/INFOODS. 2012. Guidelines for Converting Units, Denominators and Expressions Version 1.0. FAO, Rome.

Faeste, CK, Jonscher, KR, Sit, L, Klawitter, J, Lovberg, KE and Moen, LH. 2010. Differentiating cross-reacting allergens in the immunological analysis of celery (Apium graveolens) by mass spectrometry. *J AOAC Int.* **93**(2): 451-461.

FARRP (Food Allergy Research and Resource Program). 2020. https://farrp.unl.edu/IRChart.

FDA (Food and Drug Administration). 1985. Novo Laboratories, Inc.; Filing of Petition for Affirmation of GRAS Status. Notice. [Docket No. 85G-0103]. *Fed. Reg.* **50**, 14770-14771.

FDA (Food and Drug Administration). 2001. GRAS Notification No. 0089. Five enzyme preparations from *Aspergillus niger*: Carbohydrase enzyme preparation, catalase enzyme preparation, glucose oxidase enzyme preparation, pectinase enzyme preparation, and protease enzyme preparation. Submitted by Enzyme Technical Association.

FDA (Food and Drug Administration). 2003. GRAS Notification No. 0116. Carrot fiber. Submitted by Exponent on behalf of Wm. Bolthouse Farms, Inc.

FDA (Food and Drug Administration). 2004. GRAS Notification No. 0154. Dried orange pulp. Submitted by Hyman, Phelps & McNamara, P.C. on behalf of Fiberstar, Inc.

FDA (Food and Drug Administration). 2014. GRAS Notification No. 0487. Dried citrus pulp. Submitted by Hyman, Phelps & McNamara, P.C. on behalf of Fiberstar, Inc.

FDA (Food and Drug Administration). 2016. GRAS Notification No. 0599. Citrus flour. Submitted by ToxStrategies, Inc. on behalf of Cargill, Inc.

FDA (Food and Drug Administration). 2017. GRAS Notification No. 0719. Orange pomace and enzyme-treated orange pomace. Submitted by PepsiCo, Inc.

FDA (Food and Drug Administration). 2018. Review of the Scientific Evidence on the Physiological Effects of Certain Non-Digestible Carbohydrates. Available at https://www.fda.gov/media/113659/download

FDA (Food and Drug Administration). 2019a. Part 170 – Food additives. Section §170.3 – Definitions. In *U.S. Code of Federal Regulations. Title 21: Food and Drugs*. Available at: https://www.govinfo.gov/app/collection/cfr/2019/title21.

FDA (Food and Drug Administration). 2019b. Part 101 – Food labeling. Section §101.54 – Nutrient content claims for "good source," "high," "more," and "high potency." In *U.S. Code of Federal Regulations. Title 21: Food and Drugs*. Available at: https://www.govinfo.gov/app/collection/cfr/2019/title21.

FDA (Food and Drug Administration). 2019c. Part 101 – Food labeling. Section §101.12 – Reference amounts customarily consumed per eating occasion. In *U.S. Code of Federal Regulations. Title 21: Food and Drugs*. Available at: https://www.govinfo.gov/app/collection/cfr/2019/title21.

FDA (Food and Drug Administration). Undated. Enzyme Preparations Used in Food (Partial List). Available at https://www.fda.gov/food/generally-recognized-safe-gras/enzyme-preparations-used-food-partial-list.

Garthoff, JA, Heemskerk, S, Hempenius, RA, Lina, BA, Krul, CA, Koeman, JH and Speijers, GJ. 2010. Safety evaluation of pectin-derived acidic oligosaccharides (pAOS): Genotoxicity and sub-chronic studies. *Regul Toxicol Pharmacol.* **57**(1): 31- 42. DOI: 10.1016/j.yrtph.2009.12.004.

Gonzalez, SL and Rosso, ND. 2011. Determination of pectin methylesterase activity in commercial pectinases and study of the inactivation kinetics through two potentiometric procedures. *Cienc. Tecnol. Aliment., Campinas*. **31**(2): 412-417.

Harholt, J, Suttangkakul, A and Vibe Scheller, H. 2010. Biosynthesis of Pectin. *Plant Physiol.* **153**, 384–395. DOI: 10.1104/pp.110.156588.

HHS (U.S. Department of Health and Human Services) and USDA (U.S. Department of Agriculture). 2015. *2015-2020 Dietary Guidelines for Americans*, Eighth Edition. Available at https://health.gov/dietaryguidelines/2015/resources/2015-2020_Dietary_Guidelines.pdf

Hodgkinson, A, Davis, D, Fourman, J, Robertson, WG and Roe, FJC. 1982. A comparison of the effects of lactose and of two chemically modified waxy maize starches on mineral metabolism in the rat. *Food Chem. Toxicol.* **20**, 371–382. https://doi.org/10.1016/s0278-6915(82)80101-4.

Houben, K, Jolie, RP, Fraeye, I, Van Loey, AM and Hendrickx, ME. (2011). Comparative study of the cell wall composition of broccoli, carrot, and tomato: Structural characterization of the extractable pectins and hemicelluloses. *Carbohydr Res.* **346**(9): 1105- 1111. DOI: 10.1016/j.carres.2011.04.014.

Howarth, NC, Saltzman, E, McCrory, MA, Greenberg AS, Dwyer J, Ausman, L, Kramer DG, and Roberts, SB. 2003. Fermentable and nonfermentable fiber supplements did not alter hunger, satiety or body weight in a pilot study of men and women consuming self-selected diets. *J Nutr.* **133**(10):3141-4. DOI: 10.1093/jn/133.10.3141

ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use). 2003. ICH Harmonised Tripartite Guideline Q1A(R2): Stability testing of new drug substances and products. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

IOM (Institute of Medicine). 2005. *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids*. Washington, DC: The National Academies Press.

Jackson, RS. 2008. Chemical constituents of grapes and wine. In *Wine Science: Principles and Applications* (Third Edition), pp. 270-331.

Jackson, RS. 2014. Chemical constituents of grapes and wine. In *Wine Science: Principles and Applications* (Fourth Edition), pp. 347-426.

JECFA (Joint FAO/WHO Expert Committee on Food Additives). 1981. *Evaluation of Certain Food Additives*. Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization Technical Report Series 669. Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives). 2014. *Evaluation of Certain Food Additives*. Seventy-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization Technical Report Series 990. Geneva.

JECFA (Joint FAO/WHO Expert Committee on Food Additives). 2015. Safety evaluation of certain food additives. Prepared by the Seventy-ninth meeting of the Joint FAO/ WHO Expert Committee on Food Additives (JECFA). WHO food additives series 70. Geneva.

Jonker, D, Kuper, CF, Maquet, V, Nollevaux, G and Gautier, S. 2010. Subchronic (13-week) oral toxicity study in rats with fungal chitin-glucan from *Aspergillus niger*. *Food Chem. Toxicol.* **48**, 2695–2701. https://doi.org/10.1016/j.fct.2010.06.042.

Jonker, D, Fowler, P, Albers, R, Tzoumaki, MV, van het Hof, KH, and Aparicio-Vergara, M. 2020. Safety assessment of rhamnogalacturonan-enriched carrot pectin fraction: 90-Day oral toxicity study in rats and *in vitro* genotoxicity studies. *Food Chem. Toxicol.* **139**, 111243. https://doi.org/10.1016/j.fct.2020.111243.

Khodaei, N, Fernandez, B, Fliss, I and Karboune, S. 2016. Digestibility and prebiotic properties of potato rhamnogalacturonan I polysaccharide and its galactose-rich oligosaccharides/ oligomers. *Carbohydr Polym.* **136**: 1074-1084. DOI: 10.1016/j.carbpol.2015.09.106.

Lambe, J, Kearney, J, Leclercq, C, Zunft, H, De Henauw, S, Labmerg-Allardt, C, Dunne, A, and Gibney, M. 2000. The influence of survey duration on estimates of food intakes and its relevance for public health nutrition and food safety issues. *Eur. J. Clin. Nutr.* **54**: 166-173. DOI: 10.1038/sj.ejcn.1600915.

Lucier, G and Lin, B-W. 2007. Factors affecting carrot consumption in the United States. Outlook Report from the Economic Research Service. *VGS319-01.* USDA.

Lyons, SA, Dijk, AMV, Knulst, AC, Alquati, E, Le, TM and Os-Medendorp, HV. 2018. Dietary interventions in pollen-related food allergy. *Nutrients.* **10**(10). DOI: 10.3390/nu10101520.

Ma, S, Ren, B, Diao, Z, Chen, Y, Qiao, Q and Liu, X. 2016. Physicochemical properties and intestinal protective effect of ultra-micro ground insoluble dietary fibre from carrot pomace. *Food Funct.* **7**(9): 3902-3909. DOI: 10.1039/C6FO00665E.

Manderson, K, Pinart, M, Tuohy, KM, Grace, WE, Hotchkiss, AT, Widmer, W, Yadhav, MP, Gibson, GR and Rastall, RA. 2005. In vitro determination of prebiotic properties of oligosaccharides derived from an orange juice manufacturing by-product stream. *Applied Environ Microbiol.* **71**(12): 8383-8389. DOI: 10.1128/aem.71.12.8383-8389.2005.

Markovic-Housley, Z, Basle, A, Padavattan, S, Maderegger, B, Schirmer, T and Hoffmann-Sommergruber, K. 2009. Structure of the major carrot allergen Dau c 1. *Acta Crystallogr. D Biol Crystallogr.* **65**(Pt 11): 1206-1212. Doi: 10.1107/s0907444909034854.

Marlett, JA. 1992. Content and composition of dietary fiber in 117 frequently consumed foods. *J Am Diet Assoc.* **92**(2): 175-186.

McGee, H. 1984. *On Food and Cooking.* Collier Books**,** New York.

Mohnen, D. 1999. Biosynthesis of pectins and galactomannans. *Compr. Nat. Prod. Chem.* **3**, 497-527. DOI: 10.1016/B978-0-08-091283-7.00099-0.

Mohnen, D. 2008. Pectin structure and biosynthesis. *Current Opinion Plant Biol.* **11**, 266-277. https://doi.org/10.1016/j.pbi.2008.03.006.

Nahm, MH, Yu, J, Vlach, J, and Bar-Peled, M. 2020. A common food glycan, pectin, shares an antigen with *Streptococcus pneumoniae* capsule. *mSphere.* **5,** e00074-20. https://doi.org/10.1128/mSphere.00074-20.

NCHS (National Center for Health Statistics). 2020. National Health and Examination Survey. NHANES 2015-2016 Overview. Available at: https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/overview.aspx?BeginYear=2015

Newberne, PM, Conner, MW and Estes, P. 1988. The influence of food additives and related materials on lower bowel structure and function. *Toxicol. Pathol.* **16**, 184–197. https://doi.org/10.1177/019262338801600211.

OECD (Organization for Economic Cooperation and Development). 1997. Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264071247-en.

OECD (Organization for Economic Cooperation and Development). 2016a. Test No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264264861-en.

OECD (Organization for Economic Cooperation and Development). 2016b. Test No. 490: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264264908-en.

OECD (Organization for Economic Cooperation and Development). 2018. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264070707-en.

PubMed. 2020. U.S. National Library of Medicine. https://www.ncbi.nlm.nih.gov/pubmed/

Priyadarshani, AMB. 2018. Insights of hypercarotenaemia: A brief review. *Clin Nutr ESPEN.* **23**: 19-24. https://doi.org/10.1016/j.clnesp.2017.12.002.

Quagliani, D and Felt-Gunderson, P. 2017. Closing America's Fiber Intake Gap - Communication Strategies from a Food and Fiber Summit. *Am J Lifestyle Med.* 11(1): 80–85. DOI: 10.1177/1559827615588079.

Rahman, MS and Labuza, TP. 2007. Water activity and food preservation. In *Handbook of Food Preservation*. Second Ed. Rahman, MS, CRC Press. pp. 447-476.

Saito, D, Nakaji S, Fukuda, S, Shimoyama, T, Sakamoto, J and Sugawara, K. 2005. Comparison of the amount of pectin in the human terminal ileum with the amount of orally administered pectin. *Nutrition*. **21**(9): 914-919. DOI: 10.1016/j.nut.2005.01.005 .

Sansone, RA and Sansone, LA. 2012. Carrot man: A case of excessive beta-carotene ingestion. *Int J Eat Disord*. **45**:816–818. https://doi.org/10.1002/eat.22015.

Schneider, E. 2001. *Vegetables from Amaranth to Zucchini: The Essential Reference*. Harper Collins, New York.

Schols, HA, Posthumus, MA and Voragen, AGJ. 1990. Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydr Res*. **206**, 117-129. DOI: 10.1016/0008-6215(90)84011-I.

SCOGS (Select Committee on GRAS Substances). 1977. *Evaluation of the Health Aspects of Pectin and Pectinates as Food Ingredients*. Life Sciences Research Office, Bethesda, MD.

Sharma, KD, Karki, S, Thakur, NS and Attri, S. 2012. Chemical composition, functional properties and processing of carrot-a review. *J. Food Sci. Technol.* **49**(1), 22–32. https://doi.org/10.1007/s13197-011-0310-7.

The University of Manchester. 2006. http://research.bmh.manchester.ac.uk/informall/allergenic-food/?FoodId=14.

Thompson, C, Morley, P, Kirkland, D and Proudlock R. 2005. Modified bacterial mutation test procedures for evaluation of peptides and amino acid-containing material. *Mutagenesis*. **20**(5), 345-350. DOI: 10.1093/mutage/gei045.

Til, HP, Seinen, W and de Groot, AP. 1972. Sub-chronic (90-day) toxicity study with two samples of pectin (Melange A2 and C2) in rats. Unpublished TNO Rapport Nr. R 3843. Cited in the EFSA ANS Panel report.

Tingirikari, JMR. 2018. Microbiota-accessible pectic poly- and oligosaccharides in gut health. *Food Funct*. **9**(10), 5059-5073. DOI: 10.1039/c8fo01296b.

Trager, J. 1995. *The Food Chronology*. Henry Holt & Company, New York.

USDA (U.S. Department of Agriculture). 2015. National Nutrient Database for Standard Reference. Release 28. For nutrient β-Carotene. Available at https://ods.od.nih.gov/pubs/usdandb/VitA-betaCarotene-Content.pdf.

USDA (U.S. Department of Agriculture). 2019. Food Data Central. Available at https://fdc.nal.usda.gov/fdc-app.html#/food-details/170500/nutrients.

USP (The United States Pharmacopeial Convention). 2014. *Food Chemicals Codex*. Ninth Edition. Rockville, MD.

Van den Abbeele, P, Verstrepen, L, Ghyselinck, J, Albers, R, Marzorati, M and Mercenier, A. 2020. A novel non-digestible, carrot-derived polysaccharide (cRG-I) selectively modulates the human gut microbiota while promoting gut barrier integrity: An integrated in vitro approach. *Nutrients*. **12**, 1917. DOI:10.3390/nu12071917.

Wilkowska, A, Nowak, A, Antczak-Chrobot, A, Motyl, I, Czyzowska, A and Paliwoda, A. 2019. Structurally different pectic oligosaccharides produced from apple pomace and their biological activity in vitro. *Foods*. **8**(9). DOI: 10.3390/foods8090365.

Willats, WGT, Knox, JP, and Mikkelsen, JD. 2006. Pectin: new insights into an old polymer are starting to gel. *Trends in Food Sci. Technol.* **17**, 97–104.

Wu, D, Zheng, J, Mao, G, Hu, W, Ye, X, Linhardt, RJ, and Chen, S. 2019. Rethinking the impact of RG-I mainly from fruits and vegetables on dietary health. *Crit. Rev. Food Sci. Nutr.* DOI: 10.1080/10408398.2019.1672037.

Yapo, BM. 2011. Pectin rhamnogalacturonan II: On the "small stem with four branches" in the primary cell walls of plants. *Int. J. Carbohydr. Chem.* **2011**, 1–11. https://doi.org/10.1155/2011/964521.