Fermentalg

13 April 2021

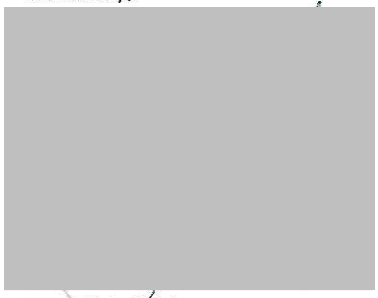
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

Re: Phycocyanin-Rich *Galdieria sulphuraria* Extract

Dear Sir or Madam:

Accompanying this letter is a Notice pursuant to regulations of the Food and Drug Administration found at 21 CFR Part 170 setting forth the basis for the conclusion reached by the submitter, Fermentalg, that phycocyanin-rich *Galdieria sulphuraria* extract is Generally Recognized as Safe (GRAS) under the intended conditions of use described in the Notice.

Sincerely,



Hywel Griffiths
Chief Scientist
Fermentalg
Email: hgriffiths@fermentalg.com

GRAS NOTICE FOR A PHYCOCYANIN-RICH *GALDIERIA SULPHURARIA* EXTRACT

SUBMITTED TO:
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 USA

SUBMITTED BY:
Fermentalg
4 Rue Rivière, 33500 Libourne
France

DATE:
13 April 2021

GRAS Notice for a Phycocyanin-Rich *Galdieria sulphuraria* Extract

TABLE OF CONTENTS

List of Appendices

List of Figures and Tables

GRAS Notice for a Phycocyanin-Rich *Galdieria sulphuraria* Extract

Part 1. §170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Fermentalg hereby informs the United States (U.S.) Food and Drug Administration (FDA) that the intended uses of a phycocyanin-rich *Galdieria sulphuraria* extract, as manufactured by Fermentalg, in various conventional food and beverage products as described in Section 1.3 below, are not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Fermentalg's view that these notified uses of phycocyanin-rich *G. sulphuraria* extract are Generally Recognized as Safe (GRAS). In addition, as a responsible official of Fermentalg, the undersigned hereby certifies that all data and information presented in this notice represent a complete and balanced submission that is representative of the generally available literature. Fermentalg considered all unfavorable as well as favorable information that is publicly available and/or known to Fermentalg and that is pertinent to the evaluation of the safety and GRAS status of the phycocyanin-rich *G. sulphuraria* extract as a food ingredient for addition to conventional food products, as described herein.

Signed,

13th April 2021

Hywel Griffiths
Chief Scientist
Fermentalg

Date

1.1 Name and Address of Notifier

Fermentalg
4 Rue Rivière, 33500 Libourne
France

1.2 Common Name of Notified Substance

Phycocyanin-rich *Galdieria sulphuraria* extract.

1.3 Conditions of Use

Fermentalg intends to market the phycocyanin-rich *G. sulphuraria* extract as an ingredient in conventional food products in the U.S. Similar to the C-phycocyanin-rich extract of the cyanobacterium *Arthrospira maxima* or *Arthrospira platensis* (also known as *Spirulina maxima* or *Spirulina platensis*) [GRAS Notice (GRN 424)], phycocyanin-rich *G. sulphuraria* extract is intended for use as an ingredient in all foods except infant formulas and foods under the jurisdiction of the United States Department of Agriculture (USDA) at levels up to a maximum of 250 mg/serving (Desert Lake Technologies LLC, 2012). The intended use of phycocyanin-rich *G. sulphuraria* extract, as described in this GRAS Notice, is not as a color additive. Fermentalg recognizes that phycocyanin-rich *G. sulphuraria* extract may impart a color to food, and therefore, a color additive petition will follow.

1.4 Basis for GRAS

Pursuant to 21 CFR §170.30 (a)(b) (U.S. FDA, 2019), Fermentalg has concluded that the intended uses of the phycocyanin-rich *G. sulphuraria* extract as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Fermentalg
4 Rue Rivière, 33500 Libourne
France

Should the FDA have any questions or additional information requests regarding this Notification, Fermentalg will supply these data and information upon request.

1.6 Freedom of Information Act, 5 U.S.C. 552

It is Fermentalg's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the Freedom of Information Act, 5 U.S.C. 552.

Part 2. §170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

Galdieria (Cyanidophyceae, Rhodophyta), including *G. sulphuraria*, is a genus of unicellular algae that grows in acidic hot springs and other highly acidic environments (Moon *et al.*, 2014). *G. sulphuraria* can grow in the dark, utilizes a wide range of carbon sources (Gross and Scharrenberger, 1995), and is known to be capable of producing significant quantities of phycocyanin under certain growth conditions. Graziani *et al***.** (2013) reported protein concentrations ranging from 27 to 33% for the dried *G. sulphuraria* biomass, which was comparable to other microalgae proposed as protein sources to be integrated into the diet (Spolaore *et al.,* 2006). Furthermore, depending on the cultivation process (autotrophic or heterotrophic conditions), varying levels of vitamins, carotenoids, chlorophylls, and phycobiliproteins were detected. Phycobiliproteins identified in the biomass included allophycocyanin and C-phycocyanin (Graziani *et al.,* 2013).

To produce the extract, the biomass undergoes a mechanical lysis and soluble components are extracted with water. The phycocyanin-rich *G. sulphuraria* extract may lysed then be treated enzymatically[1], and is filtered, sterilized, dried, and packaged. The final product is a blue powder with a moisture content of ≤5 g/100 g and phycocyanin content of ≥25%. Typical phycocyanin concentrations are approximately 34%. The extract may also be prepared as a formulated product through the addition of up to 50% food-grade maltodextrin/trehalose or others similar sugars used in food industry and up to 5% sodium citrate.

2.2 Taxonomic Classification

Kingdom: *Plantae*
Phylum: *Rhodophyta*
Class: *Cyanophyceae*
Order: *Cyanidiales*
Family: *Galdieriaceae*
Genus: *Galdieria*

The strain utilized by Fermentalg, FCC3424, is a natural variant of *G. sulphuraria* SAG 107.79 (= **UTEX 2393**) using traditional (non-GMO) selection techniques. In particular, FCC3424 has been selected so that the production of phycocyanin is independent of exposure to light. The phylogeny of *Cyanidiophyceae* is shown in Appendix A.

2.3 Common or Usual Name

Phycocyanin-rich *Galdieria sulphuraria* extract.

[1] Optional digestion of carbohydrate components of the extract is carried out using carbohydrase enzyme from *Aspergillus niger* (GRAS Notice 89) (Enzyme Technical Association, 2001)

2.4 Manufacturing

2.4.1 Raw Materials

Along with the *G. sulphuraria* inoculum, the raw materials used in the manufacturing of the biomass include fermentation medium components that are standard ingredients used in the food industry.

2.4.2 Manufacturing Process

The production of the *Galdieria* biomass from which the extract is obtained is carried out using heterotrophic fermentation similar to processes used for the production of yeasts, bacteria, and other microorganisms commonly used in the food industry. The microalgae culture technology developed by Fermentalg allows production of *G. sulphuraria* on an industrial scale, and by using the correct culture conditions, these organisms produce a biomass that is rich in protein, essential amino acids, and minerals. They can also be induced to produce natural antioxidant molecules such as phycocyanin and carotene. As a food ingredient, both the biomass and the resulting extract will be produced using current Good Manufacturing Practice (cGMP) following a Hazard Analysis and Critical Control Point (HACCP) system and in facilities with ISO 22000 certification.

All productions commence from a standardized cryotube from a validated Working Cell Bank of the production strain. Cryobanks are validated to be axenic (without the presence of other organisms), and to ensure that the production strain has not undergone any unexpected change in performance or composition. These tubes are thawed and used to inoculate a chain of precultures that increase the volume of algal culture. The final preculture is used to inoculate the production fermenter in which the microalgae are grown on pre-sterilized medium consisting of an organic carbon substrate with sources of nitrogen, phosphorus, and other minerals (see Table 2.4.1-1) under highly controlled conditions to give a consistent composition at harvest. Production is performed in pre-sterilized enclosed fermentation vessels and carried out in strictly axenic conditions—the only organism in the culture will be Fermentalg's production organism. The enclosed fermentation vessels avoid the potential contamination by bacteria, cyanobacteria, fungi, or environmental contaminants such as heavy metals and pesticides, some of which can produce potential hepatotoxins (microcystins) and neurotoxins (García *et al.,* 2017) that can be encountered in the open pond or raceway systems utilized in the production of spirulina, the most widely used microalgae in human nutrition (Modeste *et al*., 2019).

The main fermentation process may be carried out using a typical fed-batch process followed by maturation, or by continuous culture. An antifoam agent is added separately as required, or may be included at low levels in the culture medium.

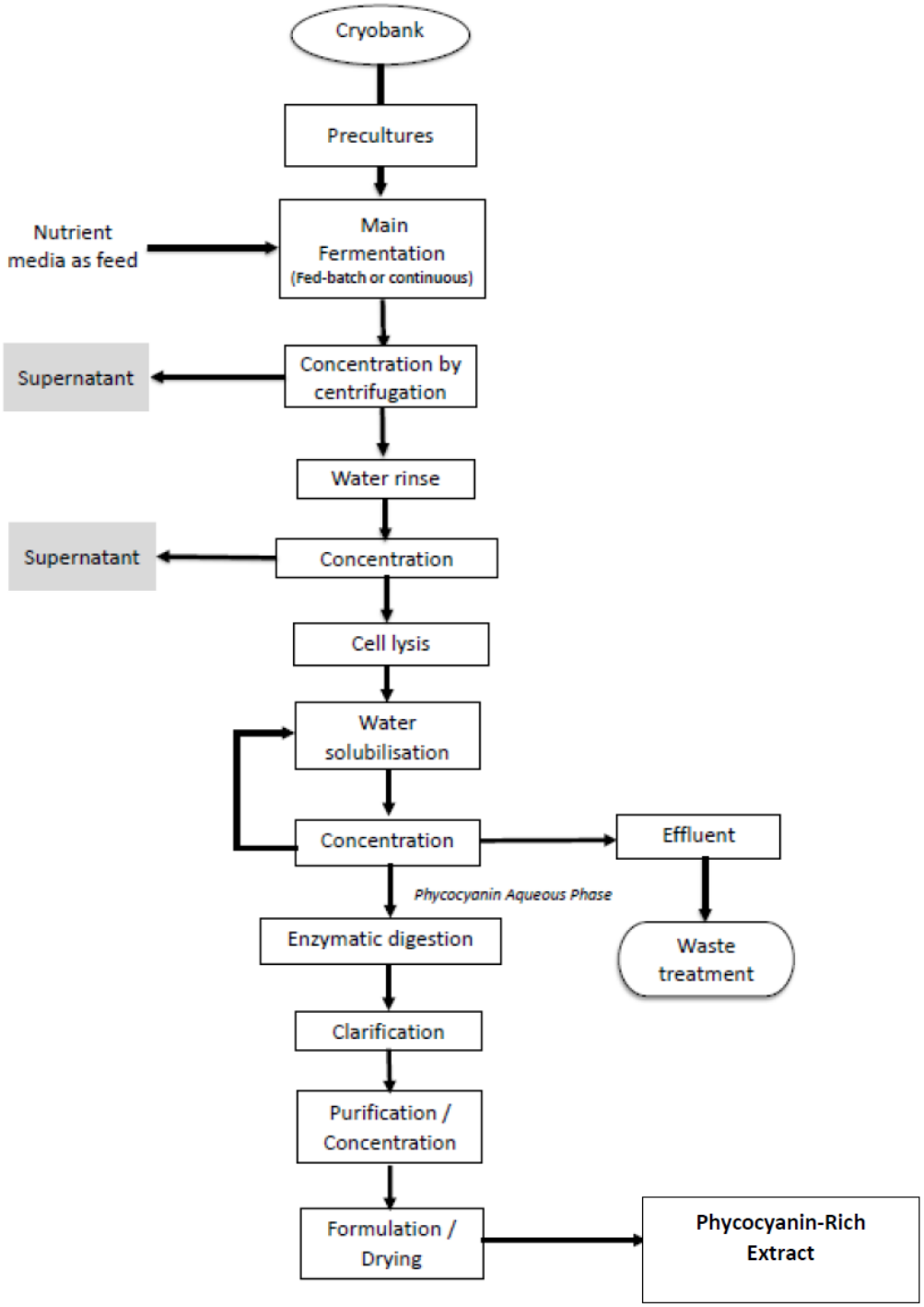
The process is conducted at an established temperature and pH that has been found to support the growth of *Galdieria*, promote the production of phycocyanin and limit the risk of contamination from other organisms. The feed rate for either the fed-batch or continuous process has a major impact on growth rate and pigment production[2]. All steps are run at 42°C and the pH is regulated at 3.0 in reactors. This combination has been found to be effective in supporting growth of *Galdieria* and limiting the risk of contamination from other organisms.

[2] *G. sulphuraria* biomass contains naturally occurring phycocyanin and other phytopigments but neither the biomass nor the extract thereof is intended for use as a color additive when used as an ingredient in foods as described herein.

The biomass undergoes a mechanical lysis before soluble components, including phycocyanin, are extracted with water. The phycocyanin-rich *G. sulphuraria* extract may be treated enzymatically[3], before it is filtered, sterilized, dried, and packaged. An overview of the manufacturing process is shown in Figure 2.4.2-1.

[3] An optional treatment to reduce the size of carbohydrate molecules in the extract can be carried out using carbohydrase enzyme from *Aspergillus niger* (GRAS Notice 89) (Enzyme Technical Association, 2001)

Figure 2.4.2-1 Schematic Overview of the Manufacturing Process for Phycocyanin-Rich *Galdieria sulphuraria* Extract



2.5 Product Specifications and Batch Analyses

2.5.1 Specifications

The physical parameters (appearance, odor, and taste), compositional (phycocyanin and moisture content), and heavy metal (arsenic, cadmium, lead, and mercury) specifications for phycocyanin-rich *G. sulphuraria* extract are presented in Table 2.5.1-1.

Table 2.5.1-1 Physical and Chemical Specifications for Phycocyanin-Rich *Galdieria sulphuraria* Extract

Specification Parameter	Specification	Method
Physical Properties		
Appearance	Blue powder	Visual
Odor/taste	Neutral	Organoleptic
Composition		
Phycocyanin (% w/w on a dry basis)	≥25	Internal, Spectrophotometric
Moisture	≤5 g/100 g	Internal, Thermo-gravimetry
Heavy Metals		
Arsenic (mg/kg on a dry basis)	≤0.5	Internal, ICP-MS
Cadmium (mg/kg on a dry basis)	≤1	Internal, ICP-MS
Lead (mg/kg on a dry basis)	≤0.5	Internal, ICP-MS
Mercury (mg/kg on a dry basis)	≤0.05	Internal, ICP-MS

ICP-MS = inductively coupled plasma mass spectrometry.

2.5.2 Microbiological Specifications

The microbiological specifications for phycocyanin-rich *G. sulphuraria* extract are presented in Table 2.5.2-1.

Table 2.5.2-1 Microbiological Specifications for Phycocyanin-Rich *Galdieria sulphuraria* Extract

Specification Parameter	Specification	Method
Aerobic microorganisms 30°C (CFU/g)	$\leq 10^5$	XP V08-034
Thermotolerant coliforms (CFU/g)	≤10	3M-01/02-09/89-C equivalent to NF V08-060
Sulfite-reducing anaerobic bacteria (CFU/g)	≤100	NF V08-061
Clostridium perfringens (CFU/g)	<1	NF EN ISO 7937
Coagulase positive Staphylococci (CFU/g)	≤100	BKR 23/10-12/15 equivalent to NF EN ISO 6888-2
Listeria monocytogenes (Negative/25 g)	Negative/25 g	AES 10/03-09/00
Salmonella spp (Negative/25 g)	Negative/25 g	BKR 23/07-10/11 BRD 07/11-12/05

CFU = colony forming units.

2.5.3 Batch Analysis

Analysis of 5 batches of phycocyanin-rich *G. sulphuraria* extract demonstrates that the manufacturing process as described in Section 2.4 produces a consistent product that meets specifications. A summary of the chemical analysis for the 5 batches of phycocyanin-rich *G. sulphuraria extract* is presented in Table 2.5.3-1.

Table 2.5.3-1 Summary of the Chemical Product Analysis for 5 Batches of Phycocyanin-Rich *Galdieria sulphuraria* Extract

Specification Parameter	Manufacturing Batch				
	EP_A946	EP_A947	EP_A948	EP_A949	EP_A950
Composition					
Protein (g/100 g)	43.6 (± 1.4)	46.9 (± 1.5)	43.8 (± 1.4)	50.9 (± 1.6)	47.5 (± 1.5)
Total carbohydrates (g/100 g)	50.1	45.9	49.1	43.3	47.1
Available carbohydrates (g/100 g)	15.7	3.8	21.1	11.6	7.2
Lipids (g/100 g)	<0.6	<0.6	<0.6	<0.6	<0.6
Moisture (g/100 g)	1.6 (± 0.5)	2.3 (± 0.5)	1.9 (± 0.5)	1.0 (± 0.5)	0.9 (± 0.5)
Ash (g/100 g)	4.72 (± 0.47)	4.83 (± 0.48)	5.22 (± 0.51)	4.72 (± 0.47)	4.55 (± 0.46)
Phycocyanin Concentration					
Phycocyanin concentration (% w/w on a dry basis)	33.01 (± 0.523)	35.33 (± 0.86)	36.29 (± 4.10)	37.13 (± 0.50)	32.90 (± 0.15)
Heavy Metals					
Arsenic (mg/kg on a dry basis)	<0.05	<0.05	<0.05	<0.05	<0.05
Cadmium (mg/kg on a dry basis)	0.138 (± 0.041)	0.128 (± 0.038)	0.176 (± 0.053)	0.158 (± 0.047)	0.128 (± 0.038)
Lead (mg/kg on a dry basis)	<0.08 (± 0.02)	<0.02 (± 0.01)	<0.02	<0.02	0.04 (± 0.01)
Mercury (mg/kg on a dry basis)	<0.005	<0.005	<0.005	<0.005	<0.005

A summary of the microbiological analysis for the 5 batches of phycocyanin-rich *G. sulphuraria extract* is presented in Table 2.5.3-2.

Table 2.5.3-2 Summary of the Microbiological Product Analysis for 5 Batches of Phycocyanin-Rich *Galdieria sulphuraria* Extract

Specification Parameter	Manufacturing Batch				
	EP_A946	EP_A946	EP_A946	EP_A946	EP_A946
Aerobic microorganisms 30°C (CFU/g)	<10	<10	<10	<10	<10
Sulfite-reducing anaerobic bacteria (CFU/g)	<10	<10	<10	<10	<10
Clostridium perfringens (CFU/g)	<1	<1	<1	<1	<1
Thermotolerant coliforms (CFU/g)	<10	<10	<10	<10	<10
Coagulase positive Staphylococci (CFU/g)	<10	<10	<10	<10	<10
Listeria monocytogenes (Negative/25 g)	Negative	Negative	Negative	Negative	Negative
Salmonella spp (Negative/25 g)	Negative	Negative	Negative	Negative	Negative

CFU = colony forming units.

2.6 Additional Characterization

Although phytopigments, such as carotenoids and chlorophyll are present in *G. sulphuraria*, these materials are not water-soluble, and thus they are not present in the water-extracted phycocyanin-rich *G. sulphuraria* extract in detectable amounts. Chlorophyll was shown to be below the level of detection (0.002 mg/g) in 5 batches of phycocyanin-rich *G. sulphuraria* extract.

The use of pre-sterilized enclosed fermentation vessels during production minimizes the risk of environmental contamination. Fermentalg has demonstrated the absence of other potential environmental contaminants (polyaromatic hydrocarbons, pesticides, polychlorinated compounds) in phycocyanin-rich

G. sulphuraria extract. Microcystins are algal toxins that are produced by some strains of cyanobacteria and which can contaminate products produced from spirulina. Although these toxins are not known to occur in *Galdieria* and the method of production in closed systems mean that the presence of microcystins is not expected, Fermentalg has nonetheless demonstrated their absence in the 5 batches of phycocyanin-rich *G. sulphuraria* extract. Impurity analysis is presented in Table 2.6-2. These data demonstrate the absence of potentially toxic impurities in phycocyanin-rich *G. sulphuraria* extract.

Table 2.6-2 Summary of Impurity Analysis for 5 Batches of Phycocyanin-Rich *Galdieria sulphuraria* Extract

Parameter (µg/kg)	Batch Number				
	EP_A946	EP_A947	EP_A948	EP_A949	EP_A950
Benzo(a)pyrene	<0.5	<0.5	<0.5	<0.5	<0.5
Benz(a)anthracene	<0.5	<0.5	<0.5	<0.5	<0.5
Benzo(b)fluoranthene	<0.5	<0.5	<0.5	<0.5	<0.5
Chrysene	<0.5	<0.5	<0.5	<0.5	<0.5
Sum of 4 PAHs[a]	<0.5	<0.5	<0.5	<0.5	<0.5
Multi-residue pesticide[b]	No	No	No	No	No
Multi-residue pesticide[c]	No	No	No	No	No
Sum of non-dioxin PCB	0.12 (± 0.02)	0.077 (± 0.015)	0.052 (± 0.010)	0.043 (± 0.009)	0.013 (± 0.003)
Sum of PCB type dioxins (ng/kg)	0.012 (± 0.002)	0.007 (± 0.0011)	0.021 (± 0.003)	0.0034 (± 0.0005)	0.011 (± 0.002)
Sum of PCDD/PCDF (ng/kg)	0.049 (± 0.007)	0.022 (± 0.003)	0.024 (± 0.004)	0.017 (± 0.003)	0.027 (± 0.004)
Sum of PCDD/PCDF and sum of PCB type dioxins (ng/kg)	0.061 (± 0.009)	0.029 (± 0.004)	0.045 (± 0.007)	0.02 (± 0.004)	0.038 (± 0.006)
Microcystin LA	<0.1	<0.1	<0.1	<0.1	<0.1
Microcystin LF	<0.1	<0.1	<0.1	<0.1	<0.1
Microcystin LR	<0.11	<0.11	<0.11	<0.11	<0.11
Microcystin LW	<0.06	<0.06	<0.06	<0.06	<0.06
Microcystin RR	<0.02	<0.02	<0.02	<0.02	<0.02
Microcystin YR	<0.15	<0.15	<0.15	<0.15	<0.15

PAH = polycyclic aromatic hydrocarbons; PCB = polychlorinated biphenyls; PCDD = polychlorinated dibenzodioxins; PCDF = polychlorinated dibenzofurans.

[a] Benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene, Chrysene.

[b] GC250.

[c] LC350.

2.7 Stability

The compositional and microbiological stability of 5 batches of phycocyanin-rich *G. sulphuraria* extract, including the phycocyanin concentration and purity, was evaluated by Fermentalg under accelerated storage conditions (40°C, 75% relative humidity) for 6 months. As shown in Tables 2.7-1 through 2.7-3, the phycocyanin from *G. sulphuraria* remains in compliance with the specifications for at least 6 months. Extrapolating these results to real-time conditions, these data demonstrate that the ingredient is likely to remain stable for a period of 2 years at room temperature.

Table 2.7-1 Stability for Phycocyanin-Rich *Galdieria sulphuraria* Extract: T0 Months

	Batch Number				
Parameter	**EP_A946**	**EP_A947**	**EP_A948**	**EP_A949**	**EP_A950**
Composition					
Protein (g/100 g)	43.6 (± 1.4)	46.9 (± 1.5)	43.8 (± 1.4)	50.9 (± 1.6)	47.5 (± 1.5)
Total carbohydrates (g/100 g)	50.1	45.9	49.1	43.3	47.1
Available carbohydrates (g/100 g)	15.7	3.8	21.1	11.6	7.2
Lipids (g/100 g)	<0.6	<0.6	<0.6	<0.6	<0.6
Moisture (g/100 g)	1.6 (± 0.5)	2.3 (± 0.5)	1.9 (± 0.5)	1.0 (± 0.5)	0.9 (± 0.5)
Ash (g/100 g)	4.72 (± 0.47)	4.83 (± 0.48)	5.22 (± 0.51)	4.72 (± 0.47)	4.55 (± 0.46)
Phycocyanin Concentration					
Phycocyanin concentration (%)	33.01 (± 0.523)	35.33 (± 0.86)	36.29 (± 4.10)	37.13 (± 0.50)	32.90 (± 0.15)
Purity (-)	2.63 (± 0.02)	2.57 (± 0.03)	2.71 (± 0.69)	2.65 (± 0.03)	2.43 (± 0.02)
Microbial Stability					
Aerobic microorganisms 30°C (CFU/g)	<10	<10	<10	<10	<10
Sulfite-reducing anaerobic bacteria (CFU/g)	<10	<10	<10	<10	<10
Clostridium perfringens (CFU/g)	<1	<1	<1	<1	<1
Thermotolerant coliforms (CFU/g)	<10	<10	<10	<10	<10
Coagulase positive Staphylococci (CFU/g)	<10	<10	<10	<10	<10
Listeria monocytogenes (Negative/25 g)	Negative	Negative	Negative	Negative	Negative
Salmonella spp (Negative/25 g)	Negative	Negative	Negative	Negative	Negative

CFU = colony forming units; T = time.

Table 2.7-2 Stability for Phycocyanin-Rich *Galdieria sulphuraria* Extract: T3 Months

	Batch Number				
Parameter	**EP_A946**	**EP_A947**	**EP_A948**	**EP_A949**	**EP_A950**
Composition					
Protein (g/100 g)	45.3 (± 1.4)	46.3 (± 1.4)	43.8 (± 1.4)	50.7 (± 1.6)	48.4 (± 1.5)
Total Carbohydrates (g/100 g)	48.7	46.7	49.8	44.5	47.3
Available Carbohydrates (g/100 g)	17.9	14.5	25.4	10.8	14.1
Lipids (g/100 g)	<0.6	<0.6	<0.6	<0.6	<0.6
Moisture (g/100 g)	1.0 (± 0.5)	2.1 (± 0.5)	1.1 (± 0.5)	<0.5	<0.5
Ash (g/100 g)	4.94 (± 0.49)	4.91 (± 0.48)	5.25 (± 0.51)	4.81 (± 0.48)	4.33 (± 0.44)
Phycocyanin Concentration					
Phycocyanin concentration (%)	28.67 (± 0.13)	29.96 (± 0.16)	30.02 (± 0.77)	32.52 (± 0.32)	29.80 (± 2.15)
Purity	2.32 (± 0.00)	2.21 (± 0.01)	2.38 (± 0.12)	2.35 (± 0.01)	2.32 (± 0.12)
Microbial Stability					
Aerobic microorganisms 30°C (CFU/g)	<100	<100	<100	<100	<100
Sulfite-reducing anaerobic bacteria (CFU/g)	<10	<10	<10	<10	<10
Clostridium perfringens (CFU/g)	<1	<1	<1	<1	<1

Table 2.7-2 Stability for Phycocyanin-Rich *Galdieria sulphuraria* Extract: T3 Months

	Batch Number				
Parameter	**EP_A946**	**EP_A947**	**EP_A948**	**EP_A949**	**EP_A950**
Thermotolerant coliforms (CFU/g)	<10	<10	<10	<10	<10
Coagulase positive Staphylococci (CFU/g)	<100	<100	<100	<100	<100
Listeria monocytogenes (Negative/25 g)	Negative	Negative	Negative	Negative	Negative
Salmonella spp (Negative/25 g)	Negative	Negative	Negative	Negative	Negative

CFU = colony forming units; T = time.

Table 2.7-3 Stability for Phycocyanin-Rich *Galdieria sulphuraria* Extract: T6 Months

	Batch Number				
Parameter	**EP_A946**	**EP_A947**	**EP_A948**	**EP_A949**	**EP_A950**
Composition					
Protein (g/100 g)	45.4 (± 1.4)	41.4 (± 1.3)	44.3 (± 1.4)	50.3 (± 1.6)	48.4 (± 1.5)
Total Carbohydrates	47.3	49.6	49.3	44.0	44.8
Available Carbohydrates (g/100 g)	13.6	16.7	24.1	12.0	10.5
Lipids (g/100 g)	0.7 (± 0.6)	<0.6	<0.6	<0.6	0.9 (± 0.6)
Moisture (g/100 g)	1.6 (± 0.5)	4.0 (± 0.5)	1.0 (± 0.5)	0.7 (± 0.5)	1.3 (± 0.5)
Ash (g/100 g)	4.97 (± 0.29)	4.99 (± 0.29)	5.44 (± 0.30)	4.91 (± 0.29)	4.68 (± 0.28)
Phycocyanin Concentration					
Phycocyanin concentration (%)	27.72 ± 0.48	27.99 ± 0.47	28.82 ± 0.17	29.59 ± 0.12	26.80 ± 0.16
Purity	2.04 ± 0.00	2.00 ± 0.04	2.06 ± 0.02	2.01 ± 0.01	2.01 ± 0.05
Microbial Stability					
Aerobic microorganisms 30°C (CFU/g)	<10	<10	<10	<10	<10
Sulfite-reducing anaerobic bacteria (CFU/g)	<10	<10	<10	<10	<10
Clostridium perfringens (CFU/g)	<1	<1	<1	<1	<1
Thermotolerant coliforms (CFU/g)	<10	<10	<10	<10	<10
Coagulase positive Staphylococci (CFU/g)	<10	<10	<10	<10	<10
Listeria monocytogenes (Negative/25 g)	Negative	Negative	Negative	Negative	Negative
Salmonella spp (Negative/25 g)	Negative	Negative	Negative	Negative	Negative

CFU = colony forming units; T = time.

Part 3. §170.235 Dietary Exposure

3.1 Estimated Intake of Phycocyanin-Rich *Galdieria sulphuraria* Extract

3.1.1 Background

Given the intended use of phycocyanin-rich *G. sulphuraria* extract is as an ingredient in all foods (excluding infant formulas and USDA-regulated products), conducting a full National Health and Nutrition Examination Survey (NHANES) assessment would result in an overestimation of intakes. Therefore, to estimate the anticipated intake of phycocyanin-rich *G. sulphuraria* extract, a similar approach as presented in the GRAS Notice for the C-phycocyanin-enriched water extract of the cyanobacterium *Arthrospira maxima* or *Arthrospira platensis* (also known as *Spirulina maxima* or *Spirulina platensis*) (GRN 424) was used given the similarity between the extracts of spirulina and *G. sulphuraria—i.e.,* both ingredients are phycocyanin-rich water extracts, with similar proposed conditions of use, *i.e*., up to 250 mg/serving (Desert Lake Technologies LLC, 2012).

3.1.2 Methods

In GRN 424, the estimated daily intake of spirulina was determined based on the maximum intended use level of the ingredient per serving (250 mg), as well as the number of servings containing spirulina assumed to be consumed daily (up to 18.2 servings) (Desert Lake Technologies LLC, 2012). The reference for the number of servings in this GRAS Notice was considered outdated (Basiotis *et al*., 2000); as such, data from the Food Patterns Equivalents Database (FPED) was investigated (USDA ARS, 2019a). This database converts the foods and beverages in the Food and Nutrient Database for Dietary Studies component of the NHANES survey to the 37 USDA Food Patterns components (USDA ARS, 2019b). The Food Patterns are measured as 'cup equivalents' of Fruit, Vegetables, and Dairy; 'ounce equivalents' of Grains and Protein Foods; 'teaspoon equivalents' of Added Sugars; 'gram equivalents' of Solid Fats and Oils; and the 'number' of Alcoholic Drinks. For the purposes of the current assessment, each of these unit equivalents was considered to be equal to a 'serving', and the data was utilized to determine the overall number of daily servings for each age group of the U.S. population.

3.1.3 Intake Estimates for Phycocyanin-Rich *Galdieria sulphuraria* Extract

The most recent release of the FPED provides information for the 2015-2016 release of the NHANES (USDA, 2019; USDA ARS, 2019a,b; CDC, 2020). Males aged 20 to 29 years were determined to consume the greatest number of servings of all food groups combined, at 21 servings of food and beverages per day. In contrast, females aged 60 to 69 years of age and 70 years and older consumed the smallest number of total daily servings of food at 13 servings of food per day.

Phycocyanin-rich *G. sulphuraria* extract is intended for use at 250 mg per serving in all foods excluding infant formulas and USDA regulated products. Similar to the approach used for GRN 424, assuming that 10% of a person's daily food intake contains phycocyanin-rich *G. sulphuraria* extract, this is equivalent to 2.1 servings by the highest food consumers (Desert Lake Technologies LLC, 2012). Using the maximum intended level of use (*i.e.*, 250 mg per serving), the highest intake of phycocyanin-rich *G. sulphuraria* extract is 525 mg per day. Product specifications indicate the phycocyanin concentration of the extract is ≥25%, while values of approximately 35% are typically observed. As a result, consumption of 525 mg per day of the extract would result in intakes of 184 mg of phycocyanin per day, equivalent to 3.1 mg/kg body weight/day for a 60-kg individual.

Using the same methodology, a low-end consumer (as observed among females aged 60 to 69 years or 70 years and older) is anticipated to consume 1.3 servings per day of phycocyanin-rich *G. sulphuraria* extract, which results in a total intake of 325 mg per day of phycocyanin-rich *G. sulphuraria* extract, based on the maximum intended use level of up to 250 mg phycocyanin-rich *G. sulphuraria* extract per serving. Based on a phycocyanin concentration of 35%, consumption of 325 mg of the extract would result in intakes of 114 mg/ of phycocyanin per day, equivalent to 1.9 mg/day for a 60 kg individual.

Therefore, the intake of phycocyanin-rich *G. sulphuraria* extract is estimated to range between 325 and 525 mg/day.

Fermentalg also calculated a conservative intake estimate assuming 50% of a person's daily food servings (a maximum of 10.5 servings/day) contained phycocyanin-rich *G. sulphuraria* extract. Under this assumption, a person would consume approximately 2.6 g of phycocyanin-rich *G. sulphuraria* extract/day, resulting in consumption of about 910 mg phycocyanin/day (equivalent to approximately 15 mg phycocyanin/kg body weight/day for a 60-kg individual.

3.1.4 Summary and Conclusion

To estimate the anticipated intake of phycocyanin-rich *G. sulphuraria* extract, a similar approach as presented in the GRAS Notice for the C-phycocyanin-enriched water extract of the cyanobacterium *Arthrospira maxima* or *Arthrospira platensis* (also known as *Spirulina maxima* or *Spirulina platensis*) (GRN 424) was used. Using the same approach to estimate consumer exposure as that used in GRN 424, Fermentalg assumed that up to 10% of a person's daily food intake contains the extract at the maximum intended level of use (*i.e.*, 250 mg/serving). Using data from the FPED, which converts the foods and beverages in the Food and Nutrient Database for Dietary Studies component of the NHANES (USDA ARS, 2019b) to the 37 USDA Food Patterns components, males aged 20 to 29 years were determined to consume the greatest number of servings of all food groups combined, at 21 servings of food and beverages per day. In contrast, females aged 60 to 69 years of age and 70 years and older consumed the smallest number of total daily servings at 13 servings of food and beverages per day.

Using the maximum intended level of use (*i.e.*, 250 mg per serving), the highest intake of phycocyanin-rich *G. sulphuraria* extract is 525 mg per day. Product specifications indicate the phycocyanin concentration of the extract is ≥25%, while values of approximately 35% are typically observed. As a result, consumption of 525 mg per day of the extract would result in intakes of 184 mg of phycocyanin per day, equivalent to 3.1 mg/kg body weight/day for a 60-kg individual.

Fermentalg expects that the addition of phycocyanin-rich *G. sulphuraria* to foods sold in the U.S. will be substitutional for other phycocyanin-containing extracts (*i.e.*, C-phycocyanin-enriched water extract of the cyanobacterium *Arthrospira maxima* or *Arthrospira platensis*) rather than additive.

Part 4. §170.240 Self-Limiting Levels of Use

No known self-limiting levels of use are associated with the phycocyanin-rich *G. sulphuraria* extract.

Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Not applicable.

Part 6. §170.250 Narrative and Safety Information

6.1 Introduction

The safety of phycocyanin-rich *G. sulphuraria* is supported by a series of published studies conducted on the ingredient (Athané *et al.*, 2020). The subchronic toxicity of phycocyanin-rich *G. sulphuraria* extract was compared to that of *S. platensis* phycocyanin in a published 90-day repeat dose study. In addition, the potential mutagenicity and genotoxicity of *G. sulphuraria* extract was evaluated in the bacterial reverse mutation test and an *in vitro* micronucleus test. Additional details are provided in Section 6.3.1 to 6.3.4. In addition, the safety of the source organism, *G. sulphuraria*, is supported by a series of toxicity studies conducted on the biomass. These studies, which include a 14-day dose-range finding study, subchronic toxicity study and genotoxicity studies, are presented in Section 6.4-1 to 6.4-2.

All studies were performed in compliance with the following principles of Good Laboratory Practice (GLP): Organisation for Economic Co-operation and Development (OECD) Principles of GLP (as revised in 1997), ENV/MC/CHEM (98) 17 (OECD, 1998a), Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 (EC, 2004), and Annexe 2 à l'article D523-8 du code de l'environnement (Legifrance, 2007).

Fermentalg also conducted comprehensive searches of the published scientific literature through of March of 2021 for *G. sulphuraria*. The search was conducted on databases including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, and ToxFile®. No additional studies or clinical trials of *G. sulphuraria* were identified.

As the extract is rich in protein, a discussion of potential allergenicity is provided in Section 6.5.

6.2 Absorption, Distribution, Metabolism and Excretion

No studies investigating pharmacokinetic parameters of the phycocyanin-rich *G. sulphuraria* extract were identified. However, as the ingredient is considered primarily as a nutritive macro-ingredient in foods, the body is expected to act upon it through similar physiological processes of digestion common to other edible plant and algal derived foodstuffs commonly consumed in the human diet.

6.3 Toxicological Studies

6.3.1 Subchronic and Chronic Studies

A 90-day repeat dose study of phycocyanin-rich *G. sulphuraria* extract was conducted on male and female Wistar rats according to the OECD Guideline for Testing on Chemicals (Test Guideline 408) (OECD, 1998b). Ten rats/sex were administered 250, 2,500, or 4,000 mg/kg body weight/day phycocyanin-rich *G. sulphuraria* extract (33.3% phycocyanin) *via* oral gavage divided into 2 equal doses due to viscosity and dispensed in the morning and afternoon. The doses were selected based on a previous 14-day dose range finding study. A group of 10 male and 10 female Wistar rats was administered a daily 2,012 mg/kg body weight/day dose of a reference extract, *S. platensis* phycocyanin extract (66.2% spirulina), *via* oral gavage for 90 days. High-dose and reference control animals received 1,332 mg/kg phycocyanin. An equivalent dose of rich C-phycocyanin extract from spirulina was used as a comparator in the 90-day repeated dose study. Additional groups of 5 rats/sex from the vehicle control and high-dose groups were further observed

for 28 days post-treatment to evaluate persistence, reversibility, or delayed occurrence of toxic effects. A vehicle control group received analytical-grade water only.

Detailed clinical examinations were performed on the rats before initiation and weekly during the study. During the test administration period, daily examinations for signs of toxicity, morbidity and mortality were conducted as well as weekly recordings of body weight and food consumption. Eyes were assessed by means of ophthalmoscope in the control and high-dose groups. In Week 13 of the study, sensory reactivity, assessment of grip strength and motor activity were recorded. Blood and urine analyses were performed at the end of the treatment and recovery period.

Following treatment (and recovery period), sacrificed rats had detailed necropsy, the weight of organs (kidneys, liver, adrenal, testes, epididymides, uterus, thymus, spleen, brain, ovaries, heart, thyroid gland, pituitary gland, prostate, seminal vesicles) measured and histopathological evaluation on all tissues in all rats from control and high-dose groups.

No mortality was recorded in treated group of rats in the 90-day or 28-day recovery groups following exposure to the phycocyanin-rich *G. sulphuraria* extract. No clinical abnormalities were observed in rats treated with test and reference extracts and vehicle control. No ocular abnormalities were recorded following ophthalmological examinations. The neurotoxic potential of test and reference extracts was found to be negative following assessment of sensory reactivity, grip strength and motor activity. Body weight gain and average food consumption were not affected up to and including the maximum dose of 4,000 mg/kg body weight/day in the treatment and recovery period.

Hematological parameters [hemoglobin, packed cell volume, total red blood cell (RBC) count, total and differential white blood cell counts, RBC indices, platelet count, activated partial thromboplastin and prothrombin time and reticulocyte count] of male and female rats treated with test and reference extract were found to be comparable to control group rats following treatment and recovery time. There was no change in plasma levels of total protein, albumin, globulin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphate, glucose, creatinine, calcium, total cholesterol, phosphorus, total bilirubin, urea nitrogen, urea, sodium, potassium, and triglycerides (low-density lipoprotein, high-density lipoprotein, triiodothyronine, thyroxine, thyroid stimulating hormone) in male and female rats. No adverse effects were detected in urinalysis data.

The values of absolute and relative weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries and heart, thyroid gland (fixed), pituitary gland and prostate + seminal vesicles with coagulating glands as a whole complex of male and female rats treated with test item, were found to be comparable with those of the control rats at the end of treatment period and also at the end of the recovery period. No treatment-related gross pathological or histopathological changes were observed in the rats.

The no-observed-adverse-effect-level (NOAEL) of phycocyanin-rich *G. sulphuraria* extract was determined to be ≥4,000 mg/kg body weight/day equivalent to a dose of 1,332 mg/kg phycocyanin. A similar lack of toxicity was observed with spirulina extract.

6.3.2 Short-Term Tests for Genotoxicity

6.3.2.1 In Vitro Micronucleus Test in L5178Y TK$^{+/-}$ Mouse Lymphoma Cells

The potential of phycocyanin-rich *G. sulphuraria* extract to induce an increase in the frequency of micronucleated cells was evaluated in a mouse cell line L5178Y TK$^{+/-}$. A preliminary test showed phycocyanin-rich *G. sulphuraria* extract was non-cytotoxic and freely soluble. The highest dose level selected for the main experiment was 2,000 µg/mL. For the main experiment phycocyanin-rich *G. sulphuraria* extract was dissolved in water and added to the cell medium culture at 125, 250, 500, 1,000, and 2,000 µg/mL, with and without a rat liver metabolic activation system (+/-S9). The following methods were used; 3-hour treatment and 24-hour recovery or 24-hour treatment and no recovery (-S9), 3-hour treatment and 24-hour recovery (+S9). The vehicle control was water. Positive controls for 3-hour treatments are 1 µg/mL clastogen mitomycin C and 0.5 µg/mL aneugen colchicine (-S9) or 6 µg/mL clastogen cyclophosphamide (+S9). Positive controls for 24-hour treatments are 1 µg/mL clastogen mitomycin C and 0.5 µg/mL aneugen colchicine (-S9). A cytotoxicity assessment was also performed under the same treatment and dose levels.

Cytotoxicity was determined by the population doubling (PD) of cells. Micronuclei were analyzed for 3 dose levels of the test item (+/- S9), for the vehicle and positive controls, in 1,000 mononucleated cells per culture, totaling 2,000 mononucleated cells per dose. For each treated and control culture, the number of cells with micronuclei and number of micronuclei per cell were recorded separately.

The mean population doubling and the mean frequencies of micronucleated cells for the vehicle controls were within the acceptance criteria. Positive control cultures showed clear statistically significant increases in the frequency of mononucleated cells.

No notable cytotoxicity was observed at any of the tested dose levels or tested conditions. Following the 3-hour treatments (+/- S9 mix) or the 24-hour treatment without S9 mix, no statistically significant or dose-related increase in the frequency of micronucleated cells was noted in comparison to the corresponding vehicle control.

It can be concluded that phycocyanin-rich *G. sulphuraria extract* did not induce any chromosome damage or damage to the cell division apparatus in cultured L5178Y TK$^{+/-}$ mouse lymphoma cells (+/-S9) under the experimental conditions of the test study.

6.3.2.2 Bacterial Reverse Mutation Test

This study evaluated the potential of test item, phycocyanin-rich *G. sulphuraria* extract, to induce reverse mutations in *Salmonella typhimurium*. Five strains of *S. typhimurium* were used: TA 1535, TA 1537, TA 98, TA 100, and TA 102. Following a preliminary toxicity test, phycocyanin-rich *G. sulphuraria* extract was tested in 2 independent experiments, both with and without a metabolic activation system (S9) at 312.5, 625, 1250, 2,500, and 5,000 µg/plate. The vehicle control was water. Positive controls without S9 mix included sodium azide (TA 1535, TA 100), 9-aminoacridine (TA 1537), 2-nitrofluorene (TA 98) and mitomycin C (TA 102). Positive controls with S9 mix were 2-anthramine (TA 1535, TA 1537, TA 98) and benozo(a)pyrene (TA 100).

The direct plate incorporation method was used to treat the *S. typhimurium* strains, except for the second experiment (+S9) which followed the pre-incubation method (60 minutes, 37°C). Each strain was exposed to 5 dose levels of Cyan-PC (3 plates/dose level). Following a 48 to 72-hour incubation at 37°C, toxicity was evaluated based on an observation in the decrease in number of revertant colonies and/or thinning of the bacterial lawn.

No notable increases in the number of revertants or signs of toxicity, including thinning of the bacterial lawn or a decrease in the number of revertants, were noted in any of the *S. typhimurium* strains (+/- S9 mix). Hence, the test item, phycocyanin-rich *G. sulphuraria* extract did not show any mutagenic activity in the bacterial reverse mutation test in the presence or absence of a rat liver metabolizing system.

6.4 Toxicological Studies of *Galdieria sulphuraria* Biomass

Because the phycocyanin-rich *G. sulphuraria* extract is a water extract of *G. sulphuraria* and is not chemically modified, toxicological studies on the *G. sulphuraria* biomass are pertinent and pivotal in supporting the safety of this ingredient.

6.4.1 Subacute and Subchronic Toxicity Studies

The toxicity of *G. sulphuraria* biomass has been investigated by Fermentalg in a 14-day dose range finding study and in a 13-week toxicity study conducted in rats. In the 14-day dose range finding study, dried *G. sulphuraria* biomass was administered daily by gavage to Sprague-Dawley rats (3 animals/sex/group) for 2 weeks at dose levels of 0, 500, 2,000, and 5,000 mg/kg/day, under a constant dosage volume of 10 mL/kg. Although higher mean adrenal weights were observed in males at the 2,000 and 5,000 mg/kg/day dose levels, values remained within the range of historical control data and was deemed to be incidental. Based on these results, 5,000 mg/kg body weight/day was selected as the highest dose for the 13-week toxicity study.

In the 13-week toxicity study followed by a 4-week recovery period, rats were administered (15 animals/sex/group) dried *G. sulphuraria* biomass twice daily at dose levels of 0, 500, 2,000, and 5,000 mg/kg/day (0, 250, 1,000, and 2,500 mg/kg body weight twice daily). A significant reduction in food consumption in high-dose males were reported; however, this finding was not considered to be adverse due to its low magnitude and non-adverse effect on body weight and may have been caused by the viscosity or caloric density of the test item, which may have contributed to satiety. Changes in hematology, blood biochemistry and urine parameters were reported to be reversible and were also considered to be non-adverse in view of their low amplitude. In males administered with 2,000 and 5,000 mg/kg body weight/day and all females, a slight increase in TSH concentration was reported; however, these findings were considered to be non-adverse in view of their low amplitude and as there were no effects reported in histopathology or on T3 and T4 concentrations. Based on these results, Fermentalg concludes the NOAEL of dried *G. sulphuraria* biomass was 5,000 mg/kg/day, the highest dose tested.

6.4.2 Short-Term Tests for Genotoxicity

Genotoxicity studies of *G. sulphuraria* biomass were evaluated in bacterial and mammalian test systems. *G. sulphuraria* biomass showed no mutagenic activity in the bacterial reverse mutation test with *Salmonella typhimurium* strains and did not induce any chromosome damage or damage to the cell division apparatus in cultured L5178Y $TK^{+/-}$ mouse lymphoma cells, either in the presence or in the absence of a rat liver metabolizing system.

6.5 Allergenicity

Searches of the scientific literature and public health-related databases did not find any indication of potential allergenicity of any member of the *Galdieriaceae* family, to which *G. sulphuraria* belongs, and specifically, as expected because the ingredient has not yet been marketed for use in food, no reports of allergic reactions to the dried biomass of *G. sulphuraria* dried biomass powder were identified.

To evaluate potential allergenicity, Fermentalg evaluated the digestibility of its phycocyanin, extracted from *G. sulphuraria*, under experimental gastric phase conditions. In addition, bioinformatic analysis was performed to compare proteins encoded in the genome of *G. sulphuraria* to those of known allergens. Details are provided in Sections 6.5.1 and 6.5.2.

6.5.1 Pepsin Digestion

Major food allergens have been shown to resist digestion by pepsin *in vitro*, in contrast to non-allergenic proteins (mainly enzymes) that were rapidly digested (Astwood *et al*., 1996). On this basis, the digestibility of Fermentalg's phycocyanin ingredient, extracted from *G. sulphuraria*, was evaluated under experimental gastric phase conditions. Pepsin digestive enzyme was added to Fermentalg's phycocyanin from *G. sulphuraria* or to phycocyanin from spirulina (*Arthrospira platensis*), and the protein components were evaluated by gel electrophoresis at 0, 5, 60, 120, 240, and 360 minutes post-enzyme addition. No intact phycocyanins were present in the pepsin treatment samples from either source after 5 minutes of digestion, with samples containing only small peptides <10 kDa. Following 60 minutes of incubation, only trace levels of small peptides remained. Protein from both sources remained relatively stable in control samples that received no pepsin digestive enzyme. These results indicate that phycocyanin apoprotein is likely to undergo rapid degradation under normal digestive conditions, suggesting it may be non-allergenic. The authors also concluded that, when consumed as a food component, the phycocyanin from *G. sulphuraria* or spirulina are expected to be digested similarly.

6.5.2 Bioinformatics

A bioinformatics search of the potential proteins encoded in the genome DNA of *G. sulphuraria* against the AllergenOnline database, version 19, was performed in April 2019 by Fermentalg (FARRP, 2019). The search used a genomic DNA sequence file prepared of their cultured algal species (identified as strain FCC3424). Evaluation of the sequence reads identified a genome of 26.4 Mbp. The reads were quality checked using FastQC and cleaned using PRINSEQ by trimming of low-quality bases. Two assemblers were used, SPAdes and Trinity, to identify probably proteins using 25 as kmer length for Trinity and 21, 33, 55, and 77 for SPAdes. Post assembly polishing was accomplished using Pilon. For the FCC3424 strain, the assembly metrics for SPAdes were 1,998 contigs, largest contig 294001B, N50 54420B, N75 16958B, L50 66, L75 164, and GC% 40.27, while the assembly metrics for Trinity were 2,890 contigs, largest contig 154130B, N50 24717B, N75 11797B, L50 292, L75 677, and GC% 40.30. The reads mapped at 99.67% for SPAdes and 99.59% for Trinity. Genes with functional annotation were predicted using the *Galdieria* model from AUGUSTUS. tRNA were predicted using tRNAscan-SE and rRNA were predicted using barrnap. The number of predicted proteins for strain FCC3424 was 5,701 from SPAdes and 11,976 from Trinity.

In a preliminary review of allergenicity, the predicted proteins were compared to the AllergenOnline.org version 19 database using the Holland Computing Center server at the University of Nebraska, running FASTA version 35 in batch mode (FARRP, 2019). Sequences of >35% identity to any allergen in this database, over segments of at least 80 amino acids were compiled in Excel spreadsheets. Those were compiled into lists of matches to the AllergenOnline allergens. The genomic predictions using SPAdes and Trinity identified potential matches to 64 allergens using predicted sequences from FCC3424. The matched proteins were then compared to the National Center for Biotechnology Information (NCBI) Protein database using BLASTP to understand the significance of unique matches that might represent potential risks of allergy. The output files are included within the report provided in Appendix B. Although there were a few potential matches to clinically important food allergens, such as tropomyosin of shrimp, aldolase, and enolase sequences, the comparison to the general NCBI Protein database using BLASTP indicated that these proteins are not likely to represent plausible risks of allergy as the general population of other organisms currently used in the food chain have many higher identity matches than those found for *G. sulphuraria*. The full report is provided in Appendix B.

A secondary review was conducted to identify protein regions from the predicted protein dataset of the entire *G. sulphuraria* genome (5,921 peptides and proteins) that could facilitate immunoglobulin E (IgE) binding. This review compared the predicted peptide and proteins of *G. sulphuraria* against the AllergenOnline.org version 19 database for 8-amino acid (8 mer) exact matches in accordance with suggested CODEX guidelines (Codex Alimentarius, 2003, 2009; FARRP, 2020). 61 predicted proteins from the *G. sulphuraria* genome met the requirements for further evaluation as potential allergen risks, of which 41 had been previously evaluated in the preliminary sliding 80-mer review described above. The remaining 20 sequences highlighted as potential allergen risks were evaluated and assessed individually. Sequences were first assessed *via* sequence alignment using NCBI BLASTP tools to determine if there is a closer match between the full allergen identified by the 8-mer match and the predicted *G. sulphuraria* protein, or if there were additional epitopes present within the predicted protein. A second check for allergenic risk was conducted by searching the NCBI database for the identified allergen to determine if other common foodstuffs shared greater similarity with the identified allergens than the predicted *G. sulphuraria* protein. Finally, the NCBI database was searched with the predicted protein using BLASTP to identify if the predicted *G. sulphuraria* proteins are found in common foodstuffs. Using these 3 checks it was concluded that none of the 20 additional potential allergens identified *via* the 8-mer CODEX identity search criterion posed allergenic risks due to the lack of similarity with known allergens either within the 8 mer or any wider homology (Codex Alimentarius, 2003, 2009). The output files are provided along with the full report in Appendix B.

6.6 GRAS Panel Evaluation

Fermentalg has concluded that the phycocyanin-rich *G. sulphuraria* extract is GRAS for use in conventional food products, as described in Section 1.3, on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of phycocyanin-rich *G. sulphuraria* extract, as discussed herein, and on consensus among a panel of experts (the GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Michael W. Pariza, Ph. D. (Director Emeritus, Food Research Institute, University of Wisconsin-Madison and Member, Michael W. Pariza Consulting LLC); John A. Thomas, Ph.D., F.A.T.S., (Adjunct Professor, Department of Pharmacology & Toxicology, Indiana University School of Medicine); and David Bechtel, Ph.D., D.A.B.T. (Bechtel Consulting).

The GRAS Panel, convened by Fermentalg, independently and critically evaluated all data and information presented herein, and also concluded that the phycocyanin-rich *G. sulphuraria* extract is GRAS for use in conventional food products as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel, and evaluation of such data as it pertains to the proposed GRAS uses of the phycocyanin-rich *G. sulphuraria* extract is presented in Appendix C.

6.7 Conclusion

Based on the above data and information presented herein, Fermentalg has concluded that the phycocyanin-rich *Galdieria sulphuraria* extract is GRAS, on the basis of scientific procedures, for use in food and beverage products as described in Section 1.0. General recognition of Fermentalg's GRAS conclusion is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of the phycocyanin-rich *Galdieria sulphuraria* extract in food, who similarly concluded that the proposed uses of the phycocyanin-rich *Galdieria sulphuraria* extract are GRAS on the basis of scientific procedures.

Fermentalg has reviewed the available data and information and are not aware of any data and information that are, or may appear to be, inconsistent with our conclusion of GRAS status.

The phycocyanin-rich *Galdieria sulphuraria* extract therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the *Code of Federal Regulations*.

Part 7. §170.255 List of Supporting Data and Information

Astwood JD, Leach JN, Fuchs RL (1996). Stability of food allergens to digestion in vitro. Nat Biotechnol 14(10):1269-1273. DOI:10.1038/nbt1096-1269.

Athané A, Demol J, Brosset-Vincent S, Aguenou C, Krisa S, Courtois, A, et al. (2020). The safety evaluation of phycocyanin-enriched *Galdieria sulphuraria* extract using 90-day toxicity study in rats and in vitro genotoxicity studies. Toxicol Res Appl 4:1-15. DOI:10.1177/2397847320929991.

Basiotis PP, Lino M, Dinkins JM (2000). *Consumption of Food Group Servings: People's Perceptions vs. Reality*. (Nutrition Insights 20). Hyattsville (MD): U.S. Department of Agriculture (USDA), Center for Nutrition Policy and Promotion (CNPP). Available at: https://fns-prod.azureedge.net/sites/default/files/nutrition_insights_uploads/Insight20.pdf.

CDC (2020). *National Health and Nutrition Examination Survey (NHANES): 2015-2016*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2015 [Page last reviewed: 2/21/2020].

Codex Alimentarius (2003). *Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganisms*. (CAC/GL 46-2003; Adopted in 2003, Annexes II and III adopted in 2008). Rome Italy: Codex Alimentarius. Available at: http://www.fao.org/fao-who-codexalimentarius/standards/list-of-standards/en/ [Select required standard].

Codex Alimentarius (2009). *Foods Derived from Modern Biotechnology, 2nd edition*. (Codex Alimentarius). Geneva, Switz.: World Health Organization (WHO) / Rome, Italy: Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/docrep/011/a1554e/a1554e00.htm.

Desert Lake Technologies LLC (2012). *Notice to US Food and Drug Administration that the use of CyaninPlus™ is Generally Recognized as Safe*. (GRN No. 424). Prepared by Klamath Falls (OR): Desert Lake Technologies, LLC. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=424 [Dec. 6, 2012 - FDA response - no questions].

EC (2004). Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version). Off J Eur Union 47(L50):44-59. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1563890415256&uri=CELEX:32004L0010 [Consolidated Version: 20/04/2009].

Enzyme Technical Association (2001). *GRAS Notification - Exemption Claim for Ten Microbially Derived Enzyme Preparations that are the Subject of a GRAS Affirmation Petition, GRASP 3G0016*. (GRN No. 89). Prepared by Washington (DC): Enzyme Technical Association. Submitted to Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=89 [Apr. 3, 2002 - FDA response - no questions].

FARRP (2019). *AllergenOnline Version 19: Home of the FARRP Allergen Protein Database*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org [Released: February 10, 2019].

FARRP (2020). *AllergenOnline Version 20: Home of the FARRP Allergen Protein Database*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org [Released: February 10, 2020].

García JL, de Vicente M, Galán B (2017). Microalgae, old sustainable food and fashion nutraceuticals. Microb Biotechnol 10(5):1017-1024. DOI:10.1111/1751-7915.12800. Cited In: Modeste et al., 2019.

Graziani G, Schiavo S, Nicolai MA, Buono S, Fogliano V, Pinto G, et al. (2013). Microalgae as human food: chemical and nutritional characteristics of the thermo-acidophilic microalga *Galdieria sulphuraria*. Food Funct 4(1):144-152. DOI:10.1039/c2fo30198a.

Gross W, Schnarrenberger C (1995). Heterotrophic growth of two strains of the acido-thermophilic red alga *Galdieria sulphuraria.* Plant Cell Physiol 36(4):633-638. DOI:10.1093/oxfordjournals.pcp.a078803. Cited In: Modeste et al., 2019.

Legifrance (2007). Article Annexe II à l'article D523-8: Principes de L'OCDE de Bonnes Pratiques de Laboratoire (BPL) [Annex II to Article D523-8: OECD principles of good laboratory practices (GLP)]. Dans: *Code de l'environnement [Code of the Environment]*. Paris, France: Legifrance, Secrétariat général du Gouvernement**.** Available at: https://www.legifrance.gouv.fr/affichCodeArticle.do?idArticle=LEGIARTI000006840001&cidTexte=LEGITEXT000006074220&dateTexte=20071016.

Modeste V, Brient A, Thirion-Delalande C, Forster R, Aguenou C, Griffiths H, et al. (2019). Safety evaluation of *Galdieria* high-protein microalgal biomass. Toxicol Res Appl 3:2397847319879277 [13pp]. DOI:10.1177/2397847319879277.

Moon M, Mishra SK, Kim CW, Suh WI, Park MS, Yang J-W (2014) Isolation and characterization of thermostable phycocyanin from *Galdieria sulphuraria*. Korean J Chem Eng 31(3):490-495.

OECD (1998a). *OECD Principles of Good Laboratory Practice*. (Series on Principles of Good Laboratory Practice and Compliance Monitoring, no. 1 [ENV/MC/CHEM(98)17]). Paris, France: Organisation for Economic Co-Operation & Development (OECD), Environment Directorate, Chemicals Group and Management Committee, OECD Environmental Health and Safety Publications. Available at: http://www.oecd-ilibrary.org/environment/oecd-principles-on-good-laboratory-practice_9789264078536-en [As revised in 1997].

OECD (1998b). Repeated dose 90-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline no 408) [Updated & Adopted: 21 September 1998]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en.

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006). Commercial applications of microalgae. J Biosci Bioeng 101(2):87-96. DOI:10.1263/jbb.101.87. Cited In: Graziani *et al.*, 2013.

U.S. FDA (2019). *U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration)*. Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/.

Part	Section §	Section Title
170—Food additives	170.30	Eligibility for classification as generally recognized as safe (GRAS)
172—Food additives permitted for direct addition to food for human consumption	172.135	Disodium EDTA
176 – Indirect Food Additives: Paper and Paperboard Components	176.180	Boric acid
182—Substances generally recognized as safe	182.1320	Glycerin
	182.6285	Dipotassium phosphate
	182.8997	Zinc sulfate
184—Direct food substances affirmed as generally recognized as safe	184.1095	Sulfuric acid
	184.1143	Ammonium sulfate
	184.1193	Calcium chloride
	184.1261	Copper sulfate
	184.1298	Ferric citrate
	184.1443	Magnesium sulfate
	184.1446	Manganese chloride
	184.1857	Corn sugar
	184.1979	Whey

USDA (2019). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2015-2016.* Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release [Last Modified: 10/17/2019].

USDA ARS (2019a). *Food Patterns Equivalents Database [FPED]*. Beltsville (MD): U.S. Department of Agriculture (USDA), Agricultural Research Service (ARS), Food Surveys Research Group. Available at: https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/food-surveys-research-group/docs/fped-overview/ [Last Modified: 10/17/2019].

USDA ARS (2019b). *USDA Food and Nutrient Database for Dietary Studies 2015-2016 [FNDDS]*. Beltsville (MD): U.S. Department of Agriculture (USDA), Agricultural Research Service (ARS), Food Surveys Research Group. Available at: https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/food-surveys-research-group/docs/fndds/ [Last Modified: 05/21/2019].