ORIGINAL SUBMISSION

BURDOCK GROUP
Consultants

859 Outer Road
Orlando, Florida 32814
p•407.802.1400
f•407.802-1405
888.6.BURDOCK
e•rmatulka@burdockgroup.com

April 18, 2013

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OFFICE OF
FOOD ADDITIVE SAFETY

Paulette M. Gaynor, Ph.D.
Sylvester L. Mosley, Ph.D.
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835
Phone: 240-402-1333

RE: Request to Cease Evaluation of GRN 000450

Dear Drs. Gaynor and Mosley:

As the agent of the notifier, Solazyme Roquette Nutritionals, 225 Gateway Blvd. South San Francisco, CA 94080, I formally request FDA to cease evaluation of the GRAS notification for the use of high lipid *Chlorella protothecoides* S106 flour as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in the diet (GRN 000450) and, further, request your office to replace GRN000450 with the attached submission of notification of GRAS status for the use of high lipid *Chlorella protothecoides* S106 flour as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in the diet. These requests are submitted on the basis of administrative errors, not on the basis of incomplete safety data, as recommended by FDA during a conference call on March 13, 2013.

Please contact me if there are any questions concerning this request.

Sincerely,

(b) (6)

Ray A. Matulka, Ph.D.
Director of Toxicology,
Burdock Group

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Paulette M. Gaynor, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: High Lipid *Chlorella protothecoides* S106 Flour GRAS Notification

Dear Dr. Gaynor:

In accordance with proposed 21 CFR § 170.36 (a notice of a claim for exemption based on a GRAS determination) published in the Federal Register (62 FR 18937-18964), I am submitting, as the agent of the notifier, Solazyme Roquette Nutritionals, 225 Gateway Blvd. South San Francisco, CA 94080 a GRAS notification for the use of high lipid *Chlorella protothecoides* S106 flour as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in the diet at a maximum consumption level of 10,522.4 mg/day. A GRAS expert panel dossier, setting forth the basis for the GRAS determination, as well as *curriculum vitae* of the members of the GRAS panel, are enclosed.

Best regards,

(b) (6)

Ray A. Matulka, Ph.D.
Director of Toxicology

1. GRAS Exemption Claim

A. Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1)

Solazyme Roquette Nutritionals has determined high lipid *Chlorella protothecoides* S106 flour, derived from a non-toxigenic strain of *Chlorella protothecoides*, to be generally recognized as safe (GRAS) as a food ingredient and therefore, exempt from the requirement of premarket approval and from environmental impact, under the conditions of its intended use as described below. The common name for this ingredient is High Lipid Algalin Flour (HLAF) to be used as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in a variety of conventional foods, none of which have a standard of identity[1] (*i.e.*, baked goods, beverages, dairy and egg products, sauces, gravies margarines, salad dressings, and soups). The basis for this finding is described in the following sections.

Signed,

(b) (6)

Date April 18, 2013

Ray A. Matulka, Ph.D.
Director of Toxicology
Burdock Group
859 Outer Road
Orlando, FL 32814

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[1] All food categories designated by Solazyme have been utilized in the estimated dietary intake calculations as appropriate; however, certain categories designated by Solazyme may contain foods for which a standard of identity exists. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity. Solazyme confirms that HLAF will be added only to foods for which a standard of identity does not exist.

(i) Name and Address of the Notifier

Solazyme Roquette Nutritionals, LLC
225 Gateway Blvd
South San Francisco, CA 94080

Agent of the Notifier:

Ray A. Matulka, Ph.D.
Director of Toxicology
Burdock Group
859 Outer Road
Orlando, FL 32814
Telephone: 407-802-1400
Facsimile: 407-802-1405
Email: rmatulka@burdockgroup.com

(ii) Common Name of the Notified Substance

The common name of high lipid *Chlorella protothecoides* S106 flour for the purposes of this GRAS Notification has been defined as:

High Lipid Algalin Flour (HLAF)*

* Synonyms: Algalin Powder, Whole Algalin Powder, Algalin Flour, Whole Algalin Flour (WAF)

(iii) Conditions of Use

HLAF may be used as an ingredient in the food groups shown in Table 1 in order to provide a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening, for consumption levels up to 10,522.4 mg *per* day, by individuals who desire a reduction of cream, milk, eggs/egg yolks, and/or butter/shortening in their diet.

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Table 1. Food groups selected for HLAF supplementation*

Food Category	Intended use level (ppm)
Baked Goods	
Biscuits	50000
Breakfast flour-based foods	30000
Brownies	50000
Cakes	50000
Cookies	50000
Muffins	50000
Beverages Non-alcoholic	
Grain based drinks	50000
Soy based drinks	10000
Breakfast Cereals	
Cold whole grain based cereals	5000
Cheese	
Imitation cheese	10000
Processed cheese and cheese spreads	10000
Non dairy	
Cream substitute and whipped topping	10000
Whipped topping	10000
Creamer	20000
Egg Products**	
Scrambled egg	20000
Egg substitute	50000
Scrambled egg, cholesterol-free, w/ cheese	10000
Fats and Oils	
Imitation butter spreads	80000
Mayonnaise	120000
Salad dressing	85000
Frozen Dairy	
Frozen Yogurt	10000
Ice-cream	40000
Ice-cream fat free	20000
Ice-cream sugar free	50000
Sherbet	40000
Pudding	30000
Custard	40000
Vegetable Gravy	2000
Sauces	50000
Honey mustard	20000
Soy based sauce	10000
Meal Replacement	
Breakfast bar	20000
Granola bar	20000
Protein bar	20000
Instant breakfast drinks	20000
Milk	
Soy	10000
Milk Product	
Yogurt	10000
Snack Food	
Crackers	30000
Salty snacks from grain products	30000
Vegetable and seafood soups	10000
Sweet sauces	5000

Food Category	Intended use level (ppm)

*The food categories correspond to those listed in 21 CFR §170.3(n). ** Fully inspected egg products under FDA jurisdiction. HLAF = High Lipid Algal Flour; ppm = parts *per* million

(iv) Basis of GRAS Determination

Pursuant to 21 CFR § 170.3, the use of HLAF as an ingredient in food categories shown in Table 1 at an intended maximum 90th percentile consumption of 10.52 g *per* day, has been determined GRAS by scientific procedures for its intended conditions of use. The safety of HLAF for this use is supported by publicly available information including, but not limited to a 13-week dietary toxicity study in the rat and two genotoxicity studies (*in vitro* bacteria reverse mutation assay and *in vivo* chromosome aberration assays) (Szabo *et al.*, 2012), as well as, a 28-day repeated dose study on the similarly composed ground yellow high-lipid *C. protothecoides* biomass (Day *et al.*, 2009; FDA, 2012). This determination is based on the views of experts who are qualified by scientific training and experience to evaluate the safety of substances used as ingredients in food.

(v) Availability of Information

The data and information (*i.e.*, published articles, unpublished reports and referenced communications) that serve as a basis for this GRAS determination are available for FDA review and copying during conventional office hours at:

Burdock Group
859 Outer Road
Orlando, FL 32814
Telephone: 407-802-1400
Facsimile: 407-802-1405
Email: rmatulka@burdockgroup.com

Alternatively, data and information that serve as a basis for this GRAS determination may be sent to FDA upon request.

2. Detailed Information about the Identity of the Notified Substance

A. Identity

HLAF is a golden yellow[2] high lipid powder composed of the dried milled biomass of the green microalgae *Chlorella protothecoides* S106. The general descriptive characteristics of HLAF are presented in Table 2.

[2] HLAF is not added to food with the intention of acting as a color. Although HLAF is golden yellow in bulk amounts, in the small amounts used for additions to food, the color is yellowish to pale beige. Under the conditions of maximum usage in foods (up to 120,000 ppm or 12% in mayonnaise), the color of the food to which HLAF is added is not altered. HLAF is, therefore, exempt from the definition of a color additive [FFDCA §201(t) and Title 21 CFR§70.3(f)] because the ingredient is not effective as a coloring agent.

Table 2. General description of HLAF

Appearance	Golden yellow powder
Packaging	Bag-in-box system (15-25 kg)
Storage	Closed package in a cool, dry place (< 25 °C)
Stability	9 months
Intended use	Partial replacement of cream, milk, eggs/egg yolks, and/or butter/shortening
Functionality in food	Source of macronutrients as partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening

HLAF = High Lipid Algal Flour

As a species, *C. protothecoides* is currently assigned to the genus *Chlorella* in the phylum Chlorophyta. Widespread in fresh and salt water, soil and air (Wu *et al.*, 2001), the green microalgae *Chlorella spp.* are nonmotile, unicellular eukaryotes, spherical in shape and having diameters that typically range from 2 – 10 μm (Kay, 1991; Becker, 2007). The cell walls are hemicellulosic, rigid, and account for approximately 10% of the algal dry weight (Becker, 2007). *Chlorella* spp. also characteristically have membrane-bound organelles (Kay, 1991). Axenic[3] cultures of *Chlorella* spp. are easily established, in part because replication under optimum conditions tends to be rapid, often requiring less than two hours (Kay, 1991). Protein and lipid content and the fatty acid profile are known to vary widely with the species, the stage of cell growth (exponential or stationary), and with environmental conditions (*e.g.*, available nutrients, temperature, and light intensity) (Kay, 1991). As an example, the protein content of *Chlorella* spp. have been reported to range from 15 – 60 % on a dry weight basis (Kay, 1991; Becker, 2007).

Although morphologically similar and all unicellular, the species grouped into the genus *Chlorella* have recently been recognized to be phylogenetically divergent (Ferris *et al.*, 2005) and not necessarily related (Tiberg and Einarsson, 1989; Huss *et al.*, 1999). Nomenclature and taxonomic assignments based on traditional identification methods are being re-examined – and when necessary, reclassified – using data from genetic sequence analysis (Ferris *et al.*, 2005). Because species comprising the *Chlorella* genus are a taxonomically complicated group, chemotaxonomic character, DNA[4] base composition and DNA/DNA hybridization are all used to distinguish the morphologically similar species (Huss *et al.*, 1999; Ferris *et al.*, 2005). For example, based on comparative analyses of 18S ribosomal RNA gene sequences, Huss *et al.* (1999) has recommended that only four species remain in the *Chlorella* genus: *C. vulgaris*, *C. lobophora*, *C. sorokiniana*, and *C. kessleri*.

Solazyme obtained *C. protothecoides* UTEX 250 from the University of Texas (UTEX) Culture Collection of Algae in Austin, Texas and assigned the Solazyme internal strain number "S106". UTEX 250 had, in turn, been obtained by UTEX as *C. protothecoides* CCAP211/7C from the Culture Collection of Algae and Protozoa (CCAP), Scottish Association for Marine Science, Argyll Scotland, United Kingdom, where the strain was originally deposited sometime between 1952 and 1955 by A.J. Kluyver [5] as the third isolate of *C. vulgaris* ("3 *C. vulgaris*") taken from freshwater in Delft, Netherlands. Other deposits of this strain include the Meyer 34

[3] Cultures containing a single strain (*i.e.*, not contaminated by or associated with other microorganisms)
[4] DNA = deoxyribonucleic acid
[5] <http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2779>; site accessed April 7, 2011.

and SAG211-7C accessions in the Sammlung von Algenkulturen Gottingen (SAG, Albrecht-von-Haller-Institute for Plant Science, University of Göttingen, Göttingen, Germany).[6] Additional designations by which this strain is known include *C. protothecoides* var. *communis* (Shihira & Krauss (1965)),[7] *C. protothecoides* Krüger, and *Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová (1987). Based on accepted designations, UTEX 250 (S106) may be appropriately considered a strain of *C. protothecoides* or *Auxenochlorella protothecoides*. All *C. protothecoides* (*A. protothecoides*) strains held at the American Type Culture Collection (ATCC, Rockville, Maryland) are preserved under Biosafety Level (BSL) 1 conditions which indicate the microorganisms are not recognized to cause disease in immunocompetent adult humans.[8]

The ecophysiological and biochemical characteristics of *C. protothicoides* include the inability to utilize nitrate as a substrate, the need for thiamine but not for vitamin B_{12}, the lack of secondary carotenoids (most strains) and a tolerance for high temperatures (28 – 34 °C upper limits), acidic pHs (3.5 – 4.0 pH) and high salt concentrations (3 – 4%) (Huss *et al.*, 2002). Morphology for the species includes the absence of pyrenoids, but the presence of a tri-laminar layer within the cell wall (Huss *et al.*, 2002). Reproduction is by endosporulation, an asexual internal process through which two to twelve sporangiospores are produced and released by each parental sporangium during vegetative growth (Ramírez-Romero *et al.*, 2010). It is worth noting that this growth pattern ceases when cells are deprived of nitrogen but continued to be fed sugar. Under nitrogen depleted conditions, the cell begins to synthesize and store copious amounts of oil (Day *et al.*, 2009).

Although similar in appearance, morphology, and composition to other *Chlorella* species, such as *C. vulgaris* and *C. pyrenoidosa*[9] (Robinson and Guzman-Juarez, 1978; Kay, 1991; Brown and Jeffery, 1992; Tokusoglu and Unal, 2003; Ravishankar *et al.*, 2006), *C. protothecoides* alone has the ability, when exposed to glucose, to "de-green" or "etiolize". Genetically, *C. protothecoides* strains (with the single exception of *C protothecoides* var *Acidicola*[10]) have been shown to be closely related to species in the achlorophyllous genus *Prototheca*. Gene-based phylogenetic trees developed from nuclear 18S rRNA[11], plastid 16S rRNA, SSU[12] and LSU[13] rDNA[14], and 26S rDNA domain sequences have repeatedly demonstrated that *A. protothecoides* UTEX 25 (S485), the type strain for *A. protothecoides*, is closely related to the *Prototheca* species (Huss *et al.*, 1999; Tartar *et al.*, 2003; Ueno *et al.*, 2005; Satoh *et al.*, 2010). At least one protein-based phylogenetic tree also confirms these assessments (von Bergen *et al.*, 2009). The relationship is sufficiently homologous that comparative analysis

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[6] < http://sagdb.uni-goettingen.de/detailedList.php?str_number=211-7c>; site accessed April 7, 2011.
[7] <http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2779>; site accessed April 7, 2011.
[8] <http://www.atcc.org/ATCCAdvancedCatalogSearch/AllCollectionSearch/tabid/454/Default.aspx>; site accessed November 30, 2011.
[9] Many of these stains have been reclassified as *C. sorokiniana* or *C. fusca* var. *vacuolata*.
[10] Analysis of 18S RNA gene sequences has demonstrated only a weak phylogenetic relationship to *C. protothecoides* var. *acidcola* which is no longer considered a variety of *C protothicoides* (Huss *et al.*, 2002; Ferris *et al.*, 2005)
[11] rRNA = ribosomal ribonucleic acid
[12] SSU = small subunit of ribulose bis-phosphate carboxylase-oxygenase
[13] LSU = large subunit of ribulose bis-phosphate carboxylase-oxygenase
[14] rDNA = ribosomaldeoxyribonucleic acid

of the phylogeny of the related *A. protothecoides* UTEX 25 (S485) and twelve strains of *Prototheca* were found to form a monophyletic clade (Ueno *et al.*, 2005).

Corroborative Genotypic Information

As previously discussed, delineation of microalgal species based on morphology is problematic due to potentially strong morphological and physiological similarities among diverse species. Molecular indicators such as plastidic 23S rDNA are more reliable indicators of species and strain relatedness. As an example, the plastid 23S rDNA sequences shown in Figure 1, demonstrate that *C. protothecoides* S106 (UTEX 250 (Solazyme source); also known as (aka) SAG 211-7C and CCAP 211/7C; original deposition CCAP(1952-5)) possesses a genotype identical (*i.e.* having 100% homology) to that of Solazyme designated strain *C. protothecoides* S485 (UTEX 25 (Solazyme source); aka, SAG 211-7A, CCAP211/7A, and ATCC 30407). Strain S485 is known formally as *Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová (1987) and is the type strain for *A. protothecoides*. *C. protothecoides* S106 is also shown to share approximately 87.5% homology with another *C. protothecoides* strain, S102 (UTEX B 25 (Solazyme source); aka original deposition CCAP (1952-5)) which is called *C. protothecoides* Krüger (1892) and *A. protothecoides*. In summay, based on plastid 23S rDNA sequencing, *C. protothecoides* S106 is identical (*i.e.* 100% homology) to *A. protothecoides* S485 (type strain of *Auxenochlorella protothecoides*) and only somewhat closely related (approximately 87.5% homology) to *C. protothecoides* S102.

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S102 23S   TGTTGAAGAATGAGCCGGCGACTTAGAAAACGTGGCAAGGTTAAGGAAACGTATCCGGAG
S106 23S   TGTTGAAGAATGAGCCGGCGACTTAGAAAAAGTGGCGTGGTTAAGGAAAAAT-TCCGAAG
S485 23S   TGTTGAAGAATGAGCCGGCGACTTAGAAAAAGTGGCGTGGTTAAGGAAAAAT-TCCGAAG

S102 23S   CCGAAGCGAAAGCAAGTCTGAACAGGGCG----------------------ATTAAGTCA
S106 23S   CCTTAGCGAAAGCGAGTCTGAATAGGGCGATCAAATATTTTAATATTTACAATTTAGTCA
S485 23S   CCTTAGCGAAAGCGAGTCTGAATAGGGCGATCAAATATTTTAATATTTACAATTTAGTCA

S102 23S   TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAGCTTGGGTGACAC
S106 23S   TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAACTTGGGTGATAC
S485 23S   TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAACTTGGGTGATAC

S102 23S   CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT
S106 23S   CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT
S485 23S   CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT

S102 23S   GAAATACCAGTCGAACTCGGAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCGG
S106 23S   GAAATACCAGTCGAACCCGGAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAG
S485 23S   GAAATACCAGTCGAACCCGGAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAG

S102 23S   TTCATA-AGGCTGTCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGCGAAAGCGGTACCA
S106 23S   TACATCTAGTCTATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGTGAAAACGGTACCA
S485 23S   TACATCTAGTCTATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGTGAAAACGGTACCA

S102 23S   AATCGTGGCAAACTCTGAATACTAGATATG-CTATTTATGGGCCAGTGAGACGGTGGGGG
S106 23S   AATCGTGGCAAACTCTGAATACTAGAAATGACGGTGTA-GT---AGTGAGACTGTGGGGG
S485 23S   AATCGTGGCAAACTCTGAATACTAGAAATGACGGTGTA-GT---AGTGAGACTGTGGGGG

S102 23S   ATAAGCTTCATCGTCGAGAGGGAAACAGCCCAGATCACTAGCTAAGGCCCCAAAATGATC
S106 23S   ATAAGCTCCATTGTCAAGAGGGAAACAGCCCAGACCACCAGCTAAGGCCCCAAAATGGTA
S485 23S   ATAAGCTCCATTGTCAAGAGGGAAACAGCCCAGACCACCAGCTAAGGCCCCAAAATGGTA

S102 23S   GTTAAGTGACAAAGGAGGTGAGAATGCAGAAACAACCAGGAGGTTTGCTTAGAAGCAGCC
S106 23S   ATGTAGTGACAAAGGAGGTGAAAATGCAAACACAACCAGGAGGTTGGCTTAGAAGCAGCC
S485 23S   ATGTAGTGACAAAGGAGGTGAAAATGCAAACACAACCAGGAGGTTGGCTTAGAAGCAGCC

S102 23S   ACCCTTTAAAGAGTGCGTAATAGCTCACTG
S106 23S   ATCCTTTAAAGAGTGCGTAATAGCTCACTG
S485 23S   ATCCTTTAAAGAGTGCGTAATAGCTCACTG
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Figure 1. Plastid 23S rDNA sequences for Solazyme designated *C. protothecoides* strains S102, S106 (Solazyme strain of interest) and S485 (*Auxenochlorella protothecoides* type strain)

In similar fashion, Solazyme's *C. protothecoides* S106 strain was compared using partial plastidic 23S rDNA sequencing against 21 *C. vulgaris* strains obtained from five different depository collections. As indicated below, each test strain is identified by its Solazyme designation, common name and strain source; identical strains and other current or previous names are included as parenthetical information for ease of reference:

(1) S183: *C. vulgaris*, UTEX 395 (formerly *C. pyrenoidosa* (Starr and Zeikus 1987));

(2) S184: *C. vulgaris* (type strain), UTEX 259, (CCAP 211/11B; aka *Chlorella candida*; *Chlorella miniata*; *Chlorella vulgaris* var. *vulgaris* f. *vulgaris*; original deposition CCAP 1952-5);
(3) S185: *C. vulgaris*, UTEX 1809 (formerly *C. salina* (Starr and Zeikus 1987));
(4) S187: *C. vulgaris*, UTEX 2714 (*Chlorella vulgaris* Bashan);
(5) S188: *C. vulgaris*, UTEX 30 (CCAP 211/12; SAG 211-12; ATCC 16487; aka *C. vulgaris* Beijerinck f. *viridis* ((Chodat) Fott and Nováková)));
(6) S190: *C. vulgaris*, UTEX 396 (formerly *C. vulgaris* var. *viridis* (Starr and Zeikus 1987));
(7) S191: *C. vulgaris*, UTEX 26 (formerly *Chlorella pyrenoidosa* (Starr & Zeikus 1987); aka *C. photophila* (Shihira & Krauss 1965); *C. emersonii*; *C. fusca* var. *vacuolata*));
(8) S192: *C. vulgaris*, UTEX 265 (CCAP 211/11J; SAG 211-11J; *Chlorella vulgaris* Beijerinck 1890 (Rodhe); aka *C. simplex* (Shihira & Krauss 1965) and *Chlorella vulgaris* var. *vulgaris* f. *vulgaris*; original designation CCAP 1630);
(9) S246: *C. vulgaris*, SAG 30.80 (*Chlorella vulgaris* Beijerinck (Senger 1965); formerly *C. saccharophila*);
(10) S257: *C. vulgaris*, SAG 211.11T (*Chlorella vulgaris* Beijerinck (Ruschmann));
(11) S300: *C. vulgaris,* CCAP 211/19 (*Chlorella vulgaris* Beijerinck 1890 (von Witsch 1946/7));
(12) S301: *C. vulgaris,* CCAP 211/11S (*Chlorella vulgaris* Beijerinck 1890 (Pirson));
(13) S302: *C. vulgaris,* CCAP 211/11Q (*Chlorella vulgaris* Beijerinck (Czurda));
(14) S303: *C. vulgaris,* CCAP 211/11P (SAG 211-11P; *Chlorella vulgaris* Beijerinck (Algéus 1942 Strain B);
(15) S344: *C. vulgaris,* CCAP 211/81 (*Chlorella vulgaris* Beijerinck 1890 (Krienitz (1979); original designation CCAP A36);
(16) S345: *C. vulgaris*, CCAP 211/80 (*Chlorella vulgaris* Beijerinck 1890 (Krienitz (1979); original designation CCAP A35);
(17) S410: *C. vulgaris,* CCALA[15] 263 (*C. vulgaris* Beijerinck (Hindak 1967/61));
(18) S412: *C. vulgaris,* CCALA 266 (*C. vulgaris* Beijerinck (Marsalek 1985/57));
(19) S413: *C. vulgaris,* CCALA 268 (*C. vulgaris* Beijerinck (Gaffron/Bethesda C 1.3.1.));
(20) S497: *C. vulgaris,* CAUP[16] H1993 (*C. vulgaris* Beijerinck (Punčochářová 1981/22)); and

[15] Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň, Czech Republic; <http://www.butbn.cas.cz/ccala/index.php?page=sr&cbl=Algae>; site accessed July 31, 2012.
[16] Culture Collection of Algae of Charles University in Prague (CAUP), Praha, Czech Republic; <http://botany.natur.cuni.cz/algo/caup-list.html>; site accessed July 31, 2012.

(21) S498: *C. vulgaris,* CAUP H1996 (*C. vulgaris* Beijerinck f. *globosa* (Andreeva 1961); *C. vulgaris* var. *vulgaris* f. *globosa*).

Distance values (D values), a numerical metric used to indicate genetic relatedness, were generated from the sequence data; lower D values indicate sequences that are more closely related with "0.000" indicating identical sequences (*i.e.*, 100% homology). Higher values, on the other hand, indicate more divergent sequences. Isolates with minor genetic variation (high homology) are generally accepted as varying by a value of only about 0.005 (Rawat, *et al.*,2005). For perspective, strains having less than 95.5% homology (based on 16S rRNA sequence identity) are routinely regarded as different species (Stackebrandt and Goebel, 1994). Based on an evaluation of the numeric metric used to assess relatedness (Table 3), the partial plastidic 23S rDNA sequence for Solazyme strain S106, the *C. protothecoides* strain of interest, had D values $\geq$ 0.080 relative to the 21 *C. vulgaris* strains examined. When the D values for Solazyme strain S184 (the type strain for *C. vulgaris*) are considered, S184 is seen to be (1) identical to 15 of the other 21 *C. vulgaris* strains tested (D value = 0.000 for all),[17] (2) very closely or reasonably closely matched to three strains (D values from 0.002 to 0.034),[18] and (3) more distantly related to the remaining three strains (D values $\geq$ 0.090),[19] S106 being counted among this last group. An additional comparison of the 23S sequence of *C. protothecoides* S106 using nBLAST analysis to that of GenBank accession No.[20] L43357 *C. vulgaris* Beijerinck (NIES[21]-2170, formerly in the IAM[22] collection as C-27; formerly classified as *C. ellipsoidea* Gerneck) determines homology of 87% (FDA, 2012). In summary, *C. protothecoides* S106 is distantly related to many of the *C. vulgaris* strains tested, including S184 (UTEX 259), but it is also notable that the *C. vulgaris* type strain S184 is less closely related to some *C. vulgaris* isolates (*e.g.*, S412 and S498) than to S106.

[Remainder of this page is blank]

[17] S183, S185, S188, S190, S192, S246, S257, S300, S302, S303, S344, S345, S410, S413, and S497
[18] S187, S197, and S301
[19] S106, S412, and S498
[20] No. = number
[21] National Institute for Environmental Studies, Tsukuba, Japan.
[22] Institute of Applied Microbiology, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences University of Tokyo, Tokyo, Japan.

Table 3. Tabulated Distance values for 23S rDNA sequences of *C. protothecoides* S106 (Solazyme strain of interest) compared to S183, S184 (*C. vulgaris* type strain), S185, S187, S188, S190, S191, S192, S246, S257, S300, S301, S302, S303, S344, S345, S410, S412, S413, S497, and S498. (Lower values indicate sequences that are more closely related, with "0.000"indicating identical sequences. Higher values indicate more divergent sequences.)

	S106 23S	S183 23S	S184 23S	S185 23S	S187 23S	S188 23S	S190 23S	S191 23S	S192 23S	S246 23S	S257 23S	S300 23S	S301 23S	S302 23S	S303 23S	S344 23S	S345 23S	S410 23S	S412 23S	S413 23S	S497 23S	S498 23S
S106 23S		0.090	0.090	0.090	0.080	0.090	0.090	0.095	0.090	0.090	0.090	0.090	0.082	0.090	0.092	0.090	0.090	0.090	0.150	0.090	0.090	0.186
S183 23S	0.090		0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S184 23S	0.090	0.000		0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S185 23S	0.090	0.000	0.000		0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S187 23S	0.080	0.030	0.030	0.030		0.030	0.030	0.033	0.030	0.030	0.030	0.030	0.006	0.030	0.031	0.030	0.030	0.030	0.110	0.030	0.030	0.148
S188 23S	0.090	0.000	0.000	0.000	0.030		0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S190 23S	0.090	0.000	0.000	0.000	0.030	0.000		0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S191 23S	0.095	0.002	0.002	0.002	0.033	0.002	0.002		0.002	0.002	0.002	0.002	0.036	0.002	0.002	0.002	0.002	0.002	0.106	0.002	0.002	0.154
S192 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002		0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S246 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000		0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S257 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000		0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S300 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000		0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S301 23S	0.082	0.034	0.034	0.034	0.006	0.034	0.034	0.036	0.034	0.034	0.034	0.034		0.034	0.034	0.034	0.034	0.034	0.112	0.034	0.034	0.150
S302 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034		0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S303 23S	0.092	0.000	0.000	0.000	0.031	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000		0.000	0.000	0.000	0.109	0.000	0.000	0.156
S344 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000		0.000	0.000	0.106	0.000	0.000	0.152
S345 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000		0.000	0.106	0.000	0.000	0.152
S410 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000		0.106	0.000	0.000	0.152
S412 23S	0.150	0.106	0.106	0.106	0.110	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.112	0.106	0.109	0.106	0.106	0.106		0.106	0.106	0.123
S413 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106		0.000	0.152
S497 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000		0.152
S498 23S	0.186	0.152	0.152	0.152	0.148	0.152	0.152	0.154	0.152	0.152	0.152	0.152	0.150	0.152	0.156	0.152	0.152	0.152	0.123	0.152	0.152	

Solazyme's *C. protothecoides* S106 strain was also compared to eight strains in Solazyme's collection that had previously (prior to 1992) been classified as *C. pyrenoidosa.*

As *C. pyrenoidosa*, these strains had been the subject of several published nutritional studies. With the advent of modern molecular methods of identification, the species in the *Chlorella* genus have undergone significant taxonomic revision. In 1992, many *C. pyrenoidosa* strains were re-evaluated and reclassified as *C. sorokiniana* (Kessler and Huss, 1992) or *C. fusca* var. *vacuolata*. As indicated below, each test strain is identified by Solazyme designation, current classification and strain source; previous names and identical strains and/or other current or former names are also included as parenthetical information:

(1) S129: *C. sorokiniana*, UTEX 1230 as *C. pyrenoidosa* (formerly *C. pyrenoidosa* (Starr & Zeikus 1987); *C. vulgaris* fo. *tertia* and *C. vulgaris* group; ATCC 22521; CCAP 211/8K as *C. vulgaris* f. *tertia;* CCAO 259 as *C. sorokiniana*; SAG 211-8K as *C. sorokiniana*; originally designated Tx 7-11-05);
(2) S134: *C. sorokiniana* UTEX 1666, (formerly *C. pyrenoidosa*);
(3) S135: *C. sorokiniana* UTEX 1670 (formerly *C. pyrenoidosa* (Starr & Zeikus 1987); originally designated B1E-B2P);
(4) S140: *C. sorokiniana* UTEX B1810 (formerly *C. sorokiniana* var. *pacificensis* (Starr & Zeikus 1987));
(5) S167: *C. fusca* var. *vacuolata* UTEX 251 (formerly *C. pyrenoidosa*; SAG 211-8B; CCAP 211/8B; CCAUP H 6401; ATCC 11469);
(6) S168: *C. fusca* var. *vacuolata* UTEX 252 (formerly *C. pyrenoidosa*; aka *Scenedesmus* sp.; *Chlorella emersonii* var. *emersonii*; SAG 211-8C as *C. fusca*; CCAP 211/8C as *Chlorella emersonii* var. *globosa* (Shihira & Kraus (1965));
(7) S545: *C. sorokiniana* UTEX 1664; and
(8) S547: *C. sorokiniana* UTEX 1668 (formally *C. pyrenoidosa* (Starr and Zeikus 1987).

Evaluation of the D values generated from partial plastidic 23S rDNA sequencing (Table 4) indicate that Solazyme strain S106, the *C. protothecoides* strain of interest, has D values $\geq$ 0.076 ($\leq$ 88% homology), which shows slightly greater homology than was seen between S106 and the 21 previously examined *C. vulgaris* isolates. In addition, Solazyme strains S129, S134 (UTEX 1666), S135 (UTEX 1670), S545 (UTEX 1664), and S547 (UTEX 1668) are seen to be identical (D value = 0.000 for all), which is not surprising, considering that UTEX strains 1664 through 1671 are mutants of UTEX 1663, a sub-isolate of Tx-7-11-05 (UTEX 1230 or S129). As with the *C. vulgaris* isolates examined above, strain S106 is less closely related to most of the strains previously classified as *C. pyrenoidosa* than they are to one another. The exception is strain S168, whose 23S sequence shows less homology to the other strains in this set than S106 shows to the other strains.

In summary, partial plastidic 23S rDNA sequences were used to assess the genetic relatedness between S106, *C. protothecoides* and two genera of *Chlorella* previously studied and used as foods or dietary supplements, namely *C. vulgaris* and *C. pyrenoidosa*. Algal strain S106 is less closely related to these isolates than to S485 (type strain for *A. protothecoides*), against which S106 demonstrated 100% homology. In addition, two of the *C. vulgaris* isolates, S412 and S498 are less closely related to S184 (UTEX 259), the *C. vulgaris* type strain, than is S106 and

S168, previously classified as *C. pyrenoidosa*, is more distantly related to the other *C. pyrenoidosa* isolates than is S106.

Table 4. Tabulated Distance values for 23S rDNA sequences of *C. protothecoides* S106 (Solazyme strain of interest) compared to other Chlorella strains S129, S134, S135, S140, S167, S168, S545, and S547. (Lower values indicate sequences that are more closely related, with "0.000"indicating identical sequences. Higher values indicate more divergent sequences.)

	S106 23S	S129 23S	S134 23S	S135 23S	S140 23S	S167 23S	S168 23S	S545 23S	S547 23S
S106 23S		0.076	0.076	0.076	0.086	0.090	0.182	0.076	0.076
S129 23S	0.076		0.000	0.000	0.022	0.049	0.158	0.000	0.000
S134 23S	0.076	0.000		0.000	0.022	0.049	0.158	0.000	0.000
S135 23S	0.076	0.000	0.000		0.022	0.049	0.158	0.000	0.000
S140 23S	0.086	0.022	0.022	0.022		0.041	0.164	0.022	0.022
S167 23S	0.090	0.049	0.049	0.049	0.041		0.142	0.049	0.049
S168 23S	0.182	0.158	0.158	0.158	0.164	0.142		0.158	0.158
S545 23S	0.076	0.000	0.000	0.000	0.022	0.049	0.158		0.000
S547 23S	0.076	0.000	0.000	0.000	0.022	0.049	0.158	0.000	

Common or Usual Name:

The common name of HLAF has been defined as "High Lipid Algalin Flour". Synonyms include Algalin Powder, Whole Algalin Powder, Algalin Flour, Whole Algalin Flour (WAF).

B. Regulatory status

HLAF from the dried milled biomass of *C. protothecoides* S106 has not been approved for use in food by FDA,[23] FEMA,[24] USDA,[25] or EU. Algalin oil from *C. protothecoides* to be used as a food oil in a variety of foods excluding meat and poultry products, however, holds GRAS status as GRAS Notification 000384.[26]

C. Composition

The chemical composition of HLAF is summarized in Table 5. HLAF is a mixture of fiber, ash, protein, fat, and sucrose.

[23] FDA = United States Food and Drug Administration
[24] FEMA = Flavor Extract Manufactures Associations
[25] USDA = United States Department of Agriculture
[26] <http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=384>; site accessed January 25, 2013.

Table 5. Typical analysis of the major components of HLAF

Analysis	Batch Analysis Results (n = 5)	
	Range	Average
Moisture (%)	2.0 – 2.5	2.3
Fiber (%)	29.6 – 33.6	31.5
Ash (%)	1.5 – 3	2.0
Protein (%)	4.4 – 7.1	6.0
Fat (%)	49.9 – 53.8	52.0
Oleic Acid (%)	32.2 – 33.7	33.1
Sucrose	5.9 – 6.5	6.2

HLAF = High Lipid Algal Flour

D. Method of Manufacture of HLAF

HLAF is manufactured by fermenting and harvesting cultures of *C. protothecoides* S106. A pure, clonally isolated culture is initially used to prepare a master seed bank from which working seed vials are prepared. Three samples from the master and each working seed bank are characterized by molecular genotyping to demonstrate that they are genetically identical (*i.e.*, 100% homology between the six chromosomal footprints and 100% homology between their 23S ribosomal deoxyribonucleic acid (DNA) sequences).[27] For a production lot, a cryo-preserved working seed vial is thawed and the contents used to inoculate a flask culture, which is transferred into larger flasks at mid-log phase, and then to standard, industrial seed fermenters. Throughout the aseptic fermentation process, pH, temperature, agitation and aeration rates are controlled, and glucose or sucrose and nutrients are added. Lipid production is induced by limiting inorganic nitrogen during the fermentation process. Following completion of fermentation, the cells are inactivated by pasteurization, and separated from the culture broth by centrifugation. The separated cells are washed with water to remove the medium and other non-biomass related material, concentrated, and milled (>30% cells disrupted). After milling, the pH is adjusted to neutral and food-grade antioxidants are added. The biomass, including undisrupted cells and cell walls, is then pre-heated (to facilitate homogenization and drying), homogenized, dried, and packaged. If needed, food grade flow agents may be added to assist processing through drying and packaging; at present the use of such agents is not part of the standard process. All ingredients used during manufacture are safe and suitable.[28] A graphical depiction of the manufacturing process is presented in Figure 2.

The final product, HLAF, is available in quantities of 15 – 25 kg packed in a bag-in-box arrangement and stored at temperatures <25 °C.

[27] When tested, the six 23S ribosomal DNA sequences also demonstrated 100% identity to the 23S reference sequence for the original *C. protothecoides* S106 isolate.

[28] Regarding labeling concerns related to the eight major food allergens, one component of the fermentation media contains wheat. Four manufacturing ingredients presented in the attached Dossier in Support of the Generally Recognized as Safe (GRAS) Status of HLAF as a Food Ingredient contained soy and/or soybean oil; these ingredients are no longer used.

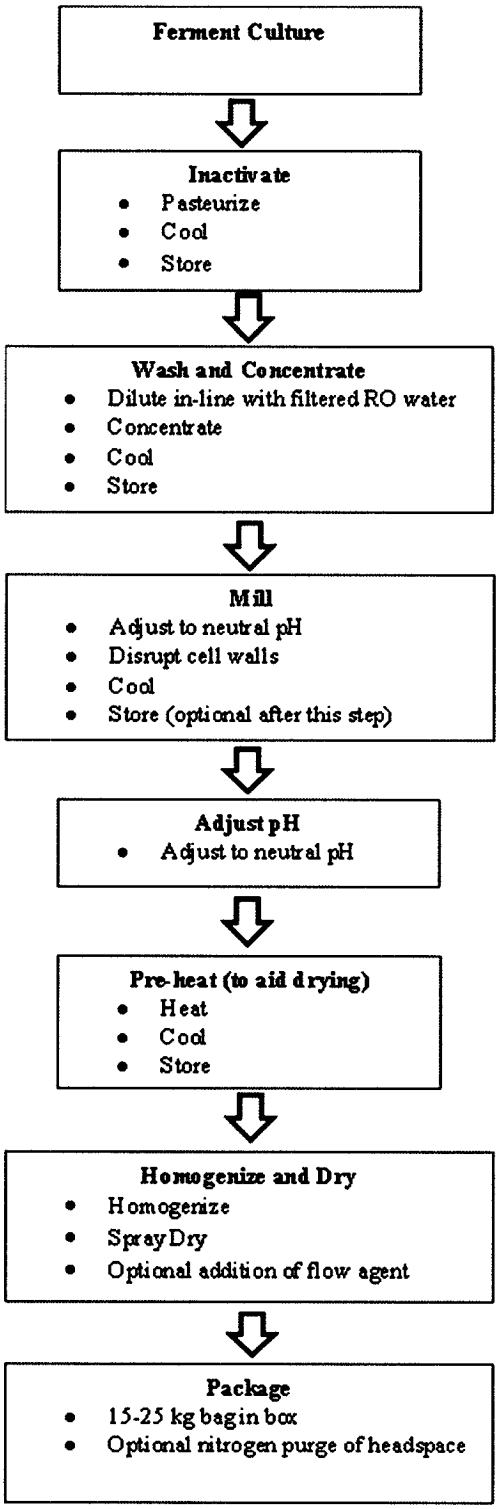

Figure 2. HLAF production schematic

E. Specifications for Food Grade HLAF

Specifications provided in Table 6 for bulk HLAF include appearance, lead, arsenic, mercury, cadmium, chromium,[29] bacteria, yeast and mold, and the absence of *Escherichia coli*, *Salmonella* and *Pseudomonas aeruginosa*.

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[29] Cobalt is not included in the product specifications because it is not present in HLAF. The presence of cobalt in the algal flour of GRN 330 was due to the inclusion of cobalt as a trace mineral in the media formulation used to produce the algal flour. In response to FDA concern, the formulation was modified to eliminate cobalt as a trace mineral in the media used in the production of HLAF.

Table 6. Specifications for HLAF

Analysis	Method	Specification	Batch Analysis Results (n = 3) Range	Average
Appearance	C-M-00023-000 Appearance [a]	Golden yellow powder	Conforms	Conforms
Moisture (%)	AOAC 930.15	≤ 10	2.0 – 2.5	2.3
Fiber (%)	AOAC 991.43	10 – 50	29.6 – 33.6	31.5
Ash (%)	AOAC 942.05	< 10	1.5 – 3	2.0
Protein (%)	AOAC 990.03	2 – 15	4.4 – 7.1	6.0
Fat (%)	AOAC 954.02	40 – 70	49.9 – 53.8	52.0
Oleic Acid (%) [b]	Q-019-00 Rev 00 Calculations for Various Analytical Data [c]	≥ 25	32.2 – 33.7	33.1
Sucrose (%)	AOAC 980.13	0 – 10	5.9 – 6.5	6.2
Heavy metals				
Lead (ppm)	EPA 3050/6020 USP 730	< 0.5	0.067 – 0.193	0.136
Arsenic (ppm)	EPA 3050/6020 USP 730	< 0.2	0.033 – 0.05	0.0423
Mercury (ppm)	EPA 3050/6020 USP 730	< 0.1	< 0.005	< 0.005
Cadmium (ppm)	EPA 3050/6020 USP 730	< 0.1	< 0.001 – 0.0038	0.0027
Chromium (ppm)	EPA 3050/6020 USP 730	< 2	0.073 – 0.597	0.409
Microbiological Limits				
Aerobic Plate Count (cfu/g)	AOAC 990.12	< 10,000	100 – 420	240
Coliform (cfu/g)	AOAC 966.24	≤ 5	< 3	< 3
E. coli (in 10 g)	USP 32, NF 27, 2009	Negative	Negative	Negative
Staphylococci (in 10 g)	USP 32, NF 27, 2009	Negative	Negative	Negative
Salmonella (in 25 g)	AOAC 2004.03	Negative	Negative	Negative
Pseudomonas aeruginosa (in 10 g)	USP 32, NF 27, 2009	Negative	Negative	Negative
Yeast (cfu/g)	Chapter 18, FDA-BAM, 7th ed.	< 100	< 10	<10
Mold (cfu/g)	Chapter 18, FDA-BAM, 7th ed.	< 100	< 10 to 10	< 10

[a] C-M-00023-000 Appearance (Solazyme, internal method, Communicated by e-mail March 30, 2011)
[b] Main fatty acid present; for minor constituents see Day *et al.* (2009).
[c] Q-019-00 Rev 00 Calculations for Various Analytical Data (Solazyme, internal method, Communicated by e-mail March 30, 2011)
AOAC = Association of Analytical Chemists; cfu = colony-forming units; EPA = Environmental Protection Agency; FDA-BAM = Food and Drug Administration Bacteriological Analytical Manual; g = gram; HLAF= High Lipid Algalin Flour; n = number; ppm = parts *per* million; USP = United States Pharmacopeia; methods available upon request

3. Self-Limiting Levels of Use

The quantity of HLAF used as a replacement ingredient for cream, milk, eggs/egg yolks, and/or butter/shortening would be self-limiting due to potential unpalatability.

4. Estimated Daily Intake

The intake profile (amount and frequency) by individuals in USDA's What We Eat in America (WWEIA) Continuing Survey of Food Intakes by Individuals 2003-2004 (Dwyer *et al.*, 2003)[30] was used to calculate the estimated daily intake (EDI) of HLAF for individuals consuming the food groups selected for the addition of HLAF as described below and in the attached Dossier in Support of the Generally Recognized as Safe (GRAS) Status of HLAF as a Food Ingredient. The individual foods selected for addition of HLAF are provided in Appendix 2 of the attached Dossier. HLAF will be added only to foods for which a standard of identity does not exist.

The means and 90th percentile EDIs were calculated only for HLAF intake following addition of HLAF to the selected food groups. The means and 90th percentile EDIs were not calculated for current HLAF intake from natural sources as no information regarding current intakes of HLAF from natural sources was discovered during a comprehensive search of the published literature. HLAF added to the selected foods at the levels specified in Table 1 would provide a mean and 90th percentile HLAF consumption of 4.70 and 10.52 g/day, respectively (Table 7).

Table 7. Predicted intake of HLAF following supplementation of selected foods at the indicated levels (Table 1) for individuals consuming selected supplemented foods

	Per User (mg/day)	
HLAF intake from:	**Mean**	**90th Percentile**
Possible maximum consumption with HLAF as an added ingredient to food	4,699	10,522

HLAF = High Lipid Algal Flour

5. Absorption, Distribution, Metabolism and Elimination (ADME)

The lipids, proteins, and carbohydrates found in HLAF (a high lipid *Chlorella protothecoides* S106 flour) are expected to be digested, absorbed, metabolized and excreted through the same normal physiological processes by which other plant materials common to the human diet are digested and utilized for macronutrients.

6. Basis of GRAS Determination

The determination that HLAF is GRAS is on the basis of scientific procedures, as described below and in the attached Dossier in Support of the Generally Recognized as Safe (GRAS) Status of HLAF as a Food Ingredient. On the basis of the data and information

[30] USDA (2006) What We Eat In America, NHANES 2003-2004; Documentation and Data Files. U.S. Department of Agriculture, Agriculture Research Service; http://www.ars.usda.gov/Services/docs.htm?docid=15044; site visited September 28, 2011.

described below and in the attached dossier and other publicly available information, there is consensus among experts qualified by scientific training and experience to evaluate the safety of substances added to food, that HLAF is GRAS under the intended conditions of use.

6.1. Acute studies

No acute toxicity studies related to *C. protothecoides* or *A. protothecoides* were discovered in the scientific literature.

6.2. Short term repeated-dose studies

In a 28-day repeated-dose toxicity study (Day *et al.*, 2009) HSD:SD® rats (*n* = 10/sex/group) were each provided diets *ad libitum* containing 0 ppm (placebo control), 25,000 ppm (low-dose), 50,000 ppm (mid-dose) and 100,000 ppm (high-dose) ground yellow high-lipid[31] *C. protothecoides* S106 biomass. The test diets for the Day *et al.* (2009) study were formulated using the AIN-93G Rodent Diet (Research Diets, Inc., New Brunswick, NJ) as the basal diet to which sufficient test substance was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose groups. The low-, mid-, and high dose diets were equivalent to 1794, 3667, and 7557 mg/kg bw[32]/day, respectively in males and 1867, 3918, and 8068 mg/kg bw/day, respectively, in females. Following the treatment period, the rats from each group were terminated on Day 31 (males) or Day 32 (females). This study was performed in compliance with OECD[33] Guidelines for the Testing of Chemicals, Section 4 (Part 407): Health Effects, *Repeated Dose 28-day Oral Toxicity Study in Rodents* (1995) and with Good Laboratory Practice (GLP) in accordance with OECD Principles of Good Laboratory Practice (as revised in 1997)[34] and US FDA GLP: 21 CFR 58, 1987.

The daily administration of the ground yellow high-lipid *C. protothecoides* biomass material at dietary concentrations up to 100,000 ppm in the feed was well-tolerated by the rats in the 28-day study. Consumption of this high-lipid algal biomass was not found to affect health or growth as measured by viability, appearance, behavior, body weight, body weight gain, food consumption, or food efficiency. No treatment-related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, or histopathology of animals in any group. Although statistical significance was shown for several parameters, none were attributable to ingestion of the test substance because the changes were noted only sporadically, did not demonstrate a dose-response relationship, were within the ranges historically observed in the age and strain of rats used in this study, and/or were also observed in the control group. These observations are summarized below:

(a) One control female died from suspected accidental overdose of anesthesia on Day 29 during orbital sinus bleeding. This occurrence was an isolated incident and not related to the test substance. (b) Incidental findings during clinical observation included a scab on the head of one mid-dose male (Days 2-6) and an abrasion on the nose of one low-dose female (Day 3). (c) A minor unilateral observation was also made in one control female during ophthalmologic examination on Day 25. This finding was sporadic and not associated with the test substance. (d)

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[31] 48% lipid
[32] bw = body weight
[33] OECD = Organisation for Economic Co-operation and Development
[34] OECD Environmental Health and Safety Publication, Series on Principles of Good Laboratory Practice and Compliance Monitoring – Number 1. Environment Directorate, Paris 1998.

A minimally significant increase in food consumption was observed in high-dose males during Week 2 ($P < 0.05$) compared to the control group. Although food consumption by high-dose males did not differ significantly from the control group during the other three weeks, overall food consumption (Days 0-28) was also statistically higher for high-dose males compared to the controls ($P < 0.05$). Because increased consumption was slight and not accompanied by corresponding changes in body weight or food efficiency, the finding was not toxicologically significant and not necessarily related to the test substance. (e) A statistically significant decrease in mean food efficiency was reported in mid-dose males during Week 3 ($P < 0.05$) compared to control males. This decrease was short term and, therefore, judged to be incidental and not related to the test substance. (f) Statistically significant decreases in mean corpuscular hemoglobin concentration (MCHC) and absolute basophil concentrations were noted for the males of the high-dose and low-dose groups, respectively, compared to the control group ($P < 0.05$ for both). Because these Day 29 hematology findings were not accompanied by any other clinical or histopathologic change and no dose-dependent relationship was demonstrated, the findings were judged to be not attributable to treatment. (g) A significant increase in the absolute large unstained cell concentration in mid-dose group females compared to the control group ($P < 0.05$) was not found to be toxicologically relevant because the change was sporadic (not present at Day 29) and did not demonstrate a dose-dependent relationship. (h) Clinical chemistry results revealed statistically significant increases in creatinine in mid-dose males and triglycerides in high-dose males on Day 15 and blood urea nitrogen in mid-dose males on Day 29 compared to the control group ($P < 0.05$ for all). Cholesterol in high-dose females was significantly elevated compared to the control group ($P < 0.05$) on Day 15. Because these findings did not present with consistency, dose-response relationship, or corresponding clinical or histopathological changes, they were not related to the test substance. (i) Clinical chemistry results from Days 15 and 29 revealed statistically significant increases in alkaline phosphatase in high-dose group males ($P < 0.05$ for both). These findings did not demonstrate a dose-response relationship and were not present in females, nor were they accompanied by corresponding clinical or histopathologic change, nor were there corresponding changes in liver or kidney weight; they were, therefore, judged not to be toxicologically relevant. (j) Findings for Day 29 urinalysis included a statistically significant increase in specific gravity and a decrease in urine volume in mid-dose females compared to controls ($P < 0.05$ for both). These changes were not dose-related nor were they accompanied by clinical or histopathologic change, nor were liver or kidney weights affected. The changes were judged not to be treatment related. (k) Macroscopic observation revealed a statistically significant increase in absolute adrenals ($P < 0.05$), adrenals-to-body ($P < 0.01$) and adrenals-to-brain ($P < 0.05$) weights in the mid-dose males compared to the control group values. Due to the absence of clinical or histopathological changes, these observations were incidental.

Under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for ground yellow high-lipid *C. protothecoides* biomass in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponded to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats.

The findings of an unpublished 28-day trial of dietary *C. protothecoides* in the Syrian golden hamster (sex not specified; $n = 15$/group) (Harding and Jones, 2008) corroborated the findings of the published Day *et al.* (2009) study. Control animals received a hyperglycemic-hypercholesterolemic diet *ad libitum*; the treatment groups received the same diet supplemented

with 2.5% or 5.0% (*w/w*)[35] *C. protothecoides* (equivalent to ~3000 or ~6000 mg/kg bw/day, respectively). No statistically significant effect was observed in body weight, food intake, body composition (*e.g.*, *percentage* body fat), or plasma triglycerides, cholesterol, protein, or albumin for the treatment groups compared to the control group. Compared to the control group, plasma glucose was significantly reduced in both low- and high-dose groups ($P < 0.05$ for both), but without affecting triglycerides or total cholesterol. Although plasma insulin concentrations for the treatment group did not differ significantly compared to the control group, plasma insulin in the 2.5% group was significantly elevated compared to plasma insulin in the 5.0% group ($P < 0.05$). Oxygen consumption was also found to be significantly greater in the 2.5% group than in the control group ($P < 0.05$), but without affecting production of carbon dioxide. The authors concluded that *C. protothecoides* in a hyperglycemic-hypercholesterolemic diet decreased plasma glucose independent of plasma insulin and further determined that "consumption of *C. protothecoides* at 2.5% and 5% of total diet (w/w) [~3000 and ~6000 mg/kg bw/day] appeared to be safe as there was no difference between groups in the liver production total plasma protein or albumin" (Harding and Jones, 2008).

In summary, although statistically significant effects were noted for several endpoints in a 28-day repeated-dose study in rats (Day *et al.*, 2009), these were not found to be related to administration of the test substance, a ground yellow high-lipid[36] *C. protothecoides* S106 biomass material. Under the conditions of the study, the NOAEL for high-lipid *C. protothecoides* biomass in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponded to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats. In a corroborative, unpublished 28-day repeated-dose study in hamsters, consumption of *C. protothecoides* up to ~6000 mg/kg bw/day was determined by the authors to be safe (Harding and Jones, 2008). The short-term repeated-dose toxicity studies in rodents are summarized in Table 8.

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[35] w/w = weight/weight
[36] 48% lipid

Table 8. 28-Day short term repeated-dose toxicity studies in rodents

Duration	Species (#/dose group)	Dose/Route	Results/Notes	Reference
28 days	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm in diet	Dietary ground yellow high-lipid *C. protothecoides* biomass was well-tolerated; NOAEL was 7557 mg/kg bw/day in males and 8068 mg/kg bw/day in females. No reported adverse effects.	Day *et al.* (2009)
28 days	Syrian golden hamster (15/group; sex not specified)	0, 2.5% and 5.0% *C. protothecoides* in diet (equivalent to 0, ~3000, and ~6000 mg/kg bw/d)	Plasma glucose significantly reduced in low- and high-dose groups compared to the control ($P < 0.05$ for both). Plasma insulin for treatment groups did not differ significantly compared to control, but plasma insulin in low-dose group was significantly elevated compared to high-dose group ($P < 0.05$). Oxygen consumption significantly greater in low-dose group than in control group ($P < 0.05$). Consumption of *C, protothecoides* at up to ~6000 mg/kg bw/day was well tolerated.	Harding and Jones (2008); unpublished report; Corroborative

C. Protothecoides = *Chlorella protothecoides*; F = Female; HLAF= High Lipid Algal Flour; M = Male; NOAEL = No Adverse Effect Level; ppm = parts *per* million; # = number

6.3. Subchronic studies

In a 13-week repeated-dose subchronic toxicity study (Szabo *et al*, 2012), HSD:SD® rats (n = 10/sex/group) were provided *ad libitum* diets containing 0 ppm (Group 1 placebo control), 25,000 ppm (Group 2), 50,000 ppm (Group 3) and 100,000 ppm (Group 4) HLAF (equivalent to 0, 1249, 2478, and 4807 mg/kg bw/day, respectively in males and 0, 1413, 2739, and 5366 mg/kg bw/day, respectively, in females). The test diets were formulated using the DIO Rodent® basal diet to which sufficient HLAF was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose groups. Following the treatment period, the rats from each group were terminated on Day 93 (males) or Day 94 (females). The subchronic repeated-dose toxicity study in rodents is summarized in Table 9.

This study was performed in compliance with OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Part 408): Health Effects, *Repeated Dose 90-Day Oral Toxicity Study in Rodents* (1998) and with GLP in accordance with OECD Principles of Good Laboratory Practice (as revised in 1997)[34] and US FDA GLP: 21 CFR 58, 1987. The study protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC) of PSL[37], Dayton, NJ, which is fully accredited by Accreditation of Laboratory Animal Care International (AAALAC International) (Accredited Unit No. 000939).

The daily administration of HLAF at dietary concentrations up to 100,000 ppm was well-tolerated by the rats in the 13-week study. Consumption of HLAF was not found to affect health or growth as measured by viability, condition, behavior, body weight, body weight gain, food consumption, or food efficiency. No treatment-related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, or histopathology of animals in any group. Although statistical significance was shown for several

[37] PSL = Product Safety Labs

parameters, none were attributable to ingestion of HLAF because the changes were within the ranges historically observed in the age and strain of rats used in this study, did not demonstrate a dose-dependent relationship, were noted only sporadically, and/or were also observed in the control group. These observations are summarized below:

(a) A firm, round, subcutaneous lesion on the left side of the neck (cranial) area in one Group 2 female was first noted and palpated on Day 70 during cage-side observation. Monitored daily from Day 70 to Day 94 when the animal was terminated, the mass was collected for pathological evaluation and later identified as an adenocarcinoma of mammary gland origin. The lesion was an isolated finding limited to this one female. (b) A statistically significant decrease in food consumption was observed in Group 4 males on Days 14-21, 35-56, and 84-91 ($P < 0.05$ for all) and overall, Days 0-91 ($P < 0.05$) as compared to the control group. Because decreased consumption occurred during three discrete weeks out of 13 weeks, was minimal on an individual basis regarding the Day 0-91 assessment and was not accompanied by decreases in body weight or food efficiency, the finding was not adverse. (c) A statistically significant increase in mean food efficiency in Group 3 females on Days 21-28 ($P < 0.05$) and overall 0-91 Days ($P < 0.01$), compared to control females was not dose-related and was therefore considered not to be treatment-related. (d) A statistically significant decrease in mean daily food consumption was reported for Group 4 females compared to the control group ($P < 0.01$) on Days 63-70. This decrease was short term and, therefore, judged to be incidental and not related to the test substance. (e) A vitreal hemorrhage obscuring the optic nerve was reported in the right eye of a Group 1 (control) female on Day 90. The finding was interpreted to result from a sporadically occurring retinal vascular abnormality or hyaloid artery remnant. (f) Statistically significant increases in platelet concentration were noted for the females of Group 2 and Group 4 compared to the control group ($P < 0.05$ for all). Because these Day 91 hematology findings were not accompanied by any other clinical or histopathologic change and no dose-response relationship was demonstrated, the findings were judged to not be attributable to HLAF treatment. (g) A significant increase in the absolute reticulocytes in Group 3 females compared to the control group ($P < 0.05$) was not found to be toxicologically relevant because the change was sporadic, was not accompanied by any other clinical or histopathologic change and were not dose-dependent. (i) Clinical chemistry results from Day 91 revealed statistically significant increases in alkaline phosphatase in Group 2 and Group 3 males, decreased aspartate aminotransferase in Group 2 females, and an increase in total protein in Group 2 females compared to their respective control groups ($P < 0.05$ for all). These findings did not demonstrate a dose-response relationship, nor were they accompanied by corresponding clinical or histopathologic change; they were, therefore, judged not to be toxicologically relevant. (j) A firm, white lesion in the right kidney of a Group 4 male corresponded to a neoplasm having morphologic features consistent with a renal mesenchymal tumor. The tumor was an isolated finding, limited to this one male. (k) The enlarged, pale, mottled thymus noted in a Group 2 male lacked any histologic correlate and was spontaneous in origin. (l) In comparison to controls, Group 3 male relative adrenal-, brain-, and testis-to-body weight ratios were significantly decreased, while the kidney-to-brain weight ratio was significantly increased ($P < 0.05$ for all). In Group 2 and 3 females, absolute liver weights, liver-to-body weight and liver-to-brain weight ratios were significantly increased when compared to the control group ($P < 0.01$ for all Group 2 parameters; $P < 0.05$ for Group 3). All changes were incidental and not toxicologically relevant as no change demonstrated a dose-dependent relationship or was found in the high-dose group.

In summary, although statistically significant effects were noted for several endpoints, none were attributable to ingestion of HLAF because the changes were within the ranges historically observed in the age and strain of rats used in this study, did not demonstrate a dose-dependent relationship, were noted only sporadically, and/or were also observed in the control group. Under the conditions of this study, the NOAEL for HLAF in the diet is 100,000 ppm, the highest dietary concentration provided in the study, which corresponds to a dietary NOAEL of 4807 mg/kg bw/day in male rats and 5366 mg/kg bw/day in female rats.

Table 9. 90-Day subchronic repeated-dose toxicity study in rodents

Duration	Species (#/dose group)	Dose/Route	Results/Notes	Reference
13 weeks	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm HLAF in diet (equivalent to 0, 1249, 2478, and 4807 mg/kg bw/day, respectively in males and 0, 1413, 2739, and 5366 mg/kg bw/day, respectively, in females)	Dietary HLAF was well-tolerated; NOAEL was 4807 mg/kg bw/day in males and 5366 mg/kg bw/day in females; No adverse effects	Szabo *et al.* (2012).

F = Female; HLAF = High Lipid Algal Flour; M = Male; NOAEL = No Adverse Effect Level; ppm = parts *per* million; # = number

6.4. Other studies

The roles that three different algal strains (*C. protothecoides* 902[38], *P. zopfii* 822[39], and *C. vulgaris* 1206[40]) could play in the detoxification of chlordecone-poisoned rats were explored and compared by Pore *et al.* (1984) in a series of inter-related studies. In previous work (Conte and Pore, 1973; Pore, 1984), both the living cells and cell walls of *C. protothecoides* 902 had been shown to have a high binding affinity for chlordecone, similar to that of cholestyramine.[41] In the current study all animals were female SD rats (175-199 g; 43-51 days of age) and all received 2.8-5.2 μCi of ^{14}C chlordecone[42] plus ~0.05 mg unlabeled chlordecone in corn oil *via* intraperitoneal injection on Day 0. For the initial ingestion study, a subset of the rats (n = 6) received a diet supplemented with 4 g/day (20-23 g/kg bw/day) of freeze-dried *C. protothecoides* 902 starting on Day 4 and continuing through Day 17; the corresponding control group (n = 6) received the control diet without algal supplementation. As determined by scintillation counting

[38] 902 *Chlorella protothecoides* BTR (Biotech Research, Inc.) 902; reported depository entries includes ATCC 75667

[39] *Prototheca zopfii* Krüger (Cooke 1962/62-344); aka *P. stagnora* 62-344, and *P. moriformis*; depository designations include ATCC 16527 and UTEX 1442; ATCC classifies the microorganism as BSL 1.

[40] No additional strain information is available.

[41] Cholestyramine is an ion exchange resin and the primary material used to treat cases of chlordecone intoxication. Cholestyramine binds to chlordecone in the intestine (during primary poisoning or later when absorbed chlordecone is secreted back into the intestine) which prevents reabsorption and allows for elimination with the feces. Without assistance, chlordecone is neither detoxified by the body nor is it effectively eliminated.

[42] 10 mCi/mmol ^{14}C chlordecone

of feces (the primary means of chlordecone excretion) collected on Days 4-13, the half-life ($t_{½}$) of chlordecone in the rat was 19.0 days for the *C. protothecoides*-treated group and 35.5 days for the control animals. All rats in the initial study were sacrificed on Day 17. In confirmation of the feces-based findings, the residual ^{14}C-chlordecone radioactivity of abdominal adipose samples[43] from *C. protothecoides*-treated rats was significantly lower than radioactivity in the control group ($P < 0.01$).

In a series of shorter four-day studies, the $t_{½}$ of chlordecone in the rat (as determined by radioactivity in the feces) was 18.7 days for the *C. protothecoides*-treated group ($n = 6$), significantly less than the 40.3 days for the control group ($n = 6$) ($P < 0.01$). When *C. protothecoides* cell walls (isolated by hydrolysis in sulfuric acid from 4/g/day equivalent of *C. protothecoides* 902) were fed to chlordecone-treated rats ($n = 6$), the $t_{½}$ was 19.4 days, comparable to the freeze-dried intact cells. In comparison, neither administration (4 g/day) of intact *Prototheca zopfii* nor of intact *C. vulgaris* exhibited a similar effect on the chlordecone $t_{½}$ in the rat; measured half-lives (based on radioactivity of feces) were 32.7 days for *C. vulgaris* and 29.5 days for *P. zopfii*. The authors proposed the difference in chlordecone $t_{½}$ was due to the presence of relatively large amounts of sporopollenin[44] (2% of cell dry weight) in the outer trilaminar layer of *C. protothecoides* cell walls. No sporopollenin is present in the cell walls of *C. vulgaris*; intermediate amounts are present in *P. zopfii* cell walls. The authors did not report any adverse effects in the rats that were associated with consumption of living *C. protothecoides* 902 cells or isolated cell walls.

In summary, exposure of chlordecone-treated rats to either living cells or the cell walls of *C. protothecoides* 902 (20-23 g/kg bw/day) resulted in a significantly decreased chlordecone $t_{½}$ compared to the control group. An intermediate effect was observed when intact *P. zopfii* was consumed, but no effect occurred when rats ate intact *C. vulgaris*. The authors believed the reduction of ^{14}C-chlordecone was due to the high amounts of sporopollenin (~2% dry cell weight) in the *C. protothecoides* cell walls. *C. vulgaris* cell walls contained no sporopollenin and *P. zopfii* contained an intermediate amount. The authors did not report any adverse effects associated with consumption of the living cells or the cell walls of the algae. This study is summarized in Table 10.

[Remainder of this page is blank]

[43] Retro-kidney and mesentary

[44] Rare but natural oxidative carotenoid polymers that occur in a few microorganisms and plants

Table 10. Other studies in the rodent model

Method	Concentration	Subjects (#/group)	Results	Reference
Detoxification study, 17-d	0 or 4 g/d (0 or 20-23 g/kg bw/d) live *C. protothecoides* 902 in feed after rats received 2.8-5.2 μCi of ^{14}C chlordecone plus ~0.05 mg unlabeled chlordecone in corn oil *via* I.P. injection (Day 0)	Female SD rats (6/group)	Chlordecone $t_{½}$ in rats fed live 902 was significantly reduced (18.7-19.0 d), compared to the control group (35.5 d) ($P < 0.01$)	Pore *et al.* (1984)
Detoxification studies, 4-d	Live *P. zopfii* 822, live *C. vulgaris* 1206 or cell walls of *C. protothecoides* 902 in feed after rats received 2.8-5.2 μCi of ^{14}C chlordecone plus ~0.05 mg unlabeled chlordecone in corn oil *via* i.p. injection (Day 0)	Female SD rats (6/group)	Chlordecone $t_{½}$ in rats fed 902 cell walls (19.4 d) was comparable to $t_{½}$ in live-902 group; Chlordecone $t_{½}$ in rats fed live 822 or live 1206 were 29.5 and 32.7 d, respectively, and did not differ from the control. The authors did not report any adverse effects to consumption of live cells or cell walls.	Pore *et al.* (1984)

i.p.= Intraperitoneal; $t_{½}$ = half life

6.5. Genotoxicity

The mutagenic potential of HLAF was evaluated by bacterial reverse mutation assay (*i.e.* Ames test) using standard plate incorporation (Experiment I) and pre-incubation (Experiment II) methods with and without S9 metabolic activation[45] (Szabo *et al.*, 2012). Tester strains included *Salmonella typhimurium* TA100 and TA1535 and *Escherichia coli* WP2uvrA for detection of base-pair substitutions and *S. typhimurium* TA98 and TA1537 for detection of frame-shift mutations. The assays were performed in compliance with the international guidelines of (1) the Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted July 21, 1997 and (2) Commission Regulation (EC) No. 440/2008 B.13/14, "Mutagenicity – Reverse Mutation Test using Bacteria", dated May 30, 2008. The assays were also conducted under GLP conditions.[55] Based on pre-experiments to evaluate cytotoxicity, 5000 μg/plate HLAF was selected as the highest dose for all test strains and conditions, except for TA 1537 in Experiment II without activation, where 1000 μg/plate was selected as the highest dose due to cytotoxic effects of the test substance. For the 5000 μg/plate series, five lower dose levels (2500, 1000, 316, 100 and 31.6 μg/plate) were prepared and tested; for the 1000 μg/plate series, six lower dose levels (316, 100, 31.6, 10.0, 3.16 and 1.00 μg/plate) were prepared. In the absence of activation, sodium azide served as positive control for *S. typhimurium* TA100 and TA1535, 4-nitro-*o*-phenylene-diamine for *S. typhimurium* TA98 and TA1537 and methylmethanesulfonate for *E. coli* WP2uvrA. In the presence of activation, the positive control for all bacterial strains was 2-aminoanthracene. The negative control for all tests was distilled water and the solvent control was dimethyl sulfoxide.

[45] S9 metabolic activation = The supernatant fraction of rat liver homogenate (derived from male Wistar rats pretreated with the inducers phenobarbital and β-naphthoflavone) mixed with Cofactor-1.

Precipitation of HLAF was visually observed in all test strains at concentrations of 2500 μg/plate and higher in Experiment I and II in the absence of S9 mix and in all test strains at a concentration of 5000 μg/plate in Experiment I and II in the presence of S9. In Experiment I cytotoxic effects of the test substance were observed in test strain TA 1537 at concentrations of 316 μg/plate and higher without metabolic activation and at concentrations of 2500 μg/plate with metabolic activation. In Experiment II these same effects were observed in test strains TA 98 and TA 100 at concentrations of 316 μg/plate and higher without metabolic activation and in test strain TA 1537 at concentrations of 10.0 μg/plate and higher without metabolic activation and concentrations of 316 μg/plate and higher with metabolic activation. Regardless of the presence or absence of metabolic activation, dose-dependent increases in the number of revertant colonies of greater than twice the negative control values were not observed in any strain treated with HLAF in either experiment. Under the conditions of this study, HLAF was not a mutagen.

The clastogenic potential of HLAF was evaluated in an *in vivo* bone marrow chromosome aberration test in the mouse (Szabo *et al.*, 2012). The assays were performed under GLP conditions[55] and in compliance with international guidelines.[46] Prior to the main experiment, a range-finding study (OECD guideline 475) was used to determine the maximum tolerated dose (MTD) of HLAF. Due to a lack of observed toxicity, the MTD defaulted to 2000 mg/kg bw, the highest dose evaluated for the assay (in accordance with OECD guideline 475). This dose was then selected for use in the main study. Prepared in cottonseed oil one hour before treatment, HLAF at a dose of 2000 mg/kg bw was administered *via* gavage to ten male and ten female NMRI mice (7-13 weeks old) in a single application at study initiation. Negative control mice (n = 10/sex) were given the vehicle in similar volumes (10 ml/kg bw). Positive control animals (n = 5/sex) received a single dose of 40 mg/kg bw of cyclophosphamide in physiological saline *via* i.p. injection. Bone marrow cells were harvested 24 hours and 48 hours post-administration for the treatment and negative control groups (n = 5/sex/group/time) and 24 hours post-administration for the positive control group. After bone marrow cells were fixed and stained, 100 metaphases[47] were scored for cytogenic damage to determine the incidence of structural chromosomal aberrations (*i.e.*, breaks, fragments, deletion exchanges, chromosomal disintegrations and gaps). In addition, approximately 1000 cells were evaluated for cytotoxicity to determine the mitotic index (% of cells in mitosis). Although the positive controls (male and female groups) exhibited biologically and statistically significant increases in aberrant cell values ($P < 0.01$ for both *vs.* corresponding negative control), no dose dependent, biologically relevant or statistically significant increase in the incidence of aberrant cells or in the mitotic index was observed in the 24 hour- or 48 hour-dose groups compared to their corresponding negative control groups. The findings (aberrant cell values and mitotic indices) for all groups except for those of the positive control were in the range of the historic negative controls. Under the conditions of this study, HLAF was not clastogenic.

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[46] The Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 475, "Mammalian Bone Marrow Chromosome Aberration Test'', adopted July 21, 1997 and Commission Regulation (EC) No. 440/2008 B.11, "Mutagenicity –Mammalian Bone Marrow Chromosome Aberration Test", dated May 30, 2008.

[47] For animals that showed a distinct positive result in fewer than 100 metaphases, 50 metaphases were examined (in accordance with OECD Guideline 475).

In summary, HLAF did not exhibit mutagenicity or clastogenicity under the conditions of, respectively, the bacterial reverse mutation and *in vivo* bone marrow chromosome aberration assays. The genotoxicity studies are summarized in Table 11.

Table 11. Genotoxicity studies

Assay	Test system	Concentrations	Results	Reference
Ames assay	*S. typhimurium* TA98, TA100, TA1535, and TA1537; and *E. coli* WP2uvrA	Experiment I (standard plate incorporation): Up to 5000 µg/plate in DMSO for all test strains and conditions (with and without S9 activation) Experiment II (pre-incubation): Up to 5000 µg/plate in DMSO for all test strains and conditions except for TA 1537 without S9 activation where 1000 µg/plate was highest dose due to cytotoxic effects.	Not mutagenic	Szabo *et al.* (2012)
In vivo bone marrow chromosome aberration assay	NMRI Mouse (10M/10F/group)	0 and 2000 mg/kg bw in cottonseed oil *via* gavage; single dose	Not clastogenic	Szabo *et al.* (2012)

bw = body weight; DMSO = Dimethyl sulfoxide; F = Female; M = Male

6.6. Carcinogenesis

No studies addressing carcinogenicity were discovered in the scientific literature for *C. protothecoides* or *A. protothecoides* .

7. Human-Related Observations and Concerns

7.1. Organoleptic observations

Although HLAF is not intended to be consumed as a standalone food, the ingredient has a slightly sweet, fatty taste with a creamy mouth-feel texture. When used at its intended levels in food, taste of the final food is not adversely affected. In creamy products such as ice cream, HLAF provides a more indulgent mouth-feel.

7.2. Allergenic potential

The evaluation of the allergenicity of the food ingredient rests on three pillars: (1) digestibility of the protein, (2) allergenic status of the protein source in the scientific literature, and (3) comparison of the amino acid sequences in the ingredient protein(s) with sequences in known allergenic proteins.

7.2.1. Digestibility

As *per* the ADME statement (Section 5.), the proteins found in HLAF are expected to be digested, absorbed, metabolized and excreted through the same normal physiological processes by which other plant materials common to the human diet are digested and utilized for macronutrients.

7.2.2. Allergenic status in the scientific literature

A search of the scientific literature did not indicate any association of allergy or allergic response to *C. protothecoides* or to any *Auxenochlorella* or *Prototheca* species. Published studies of allergy instead have primarily been limited to *Chlorella* species[48] *C. vulgaris*, *C. pyrenoidosa*, *C. saccharophila* and *C. homosphaera* (Tiberg *et al.*, 1990a; Tiberg *et al.*, 1990b). Although allergy to *Chlorella* has generally presented as hypersensitization resulting from exposure to algae-infested waters or respiratory allergy to airborne[49] algae (Bernstein and Safferman, 1966; Bernstein and Safferman, 1973; Tiberg *et al.*, 1995; Chrisostomou *et al.*, 2009), there is a published case study of oral allergy in an 11-year-old boy (Yim *et al.*, 2007). Three months into a daily regimen of Chlorella food supplements (200 mg/tablet, 10 tablets/day), acute tubulointerstitial nephritis[50] was diagnosed in the child. Renal function improved with cessation of the *Chlorella*[51] supplements and a six-month regimen of corticosteroids. A follow-up skin prick test (100 mg and 200 mg *Chlorella* in 5 ml distilled water) six months after steroid therapy failed to produce positive wheal reactions for the 100 mg or 200 mg test amounts. In a previous study by Tiberg and Einarsson (1989), the allergenic potencies of eight strains within four species of *Chlorella* (*C. vulgaris*, C. *homosphaera*, C. *saccharophila*, and *C.fusca*) were compared in an attempt to define a representative extract preparation for the genus. Due to the highly variable allergenic activity of the eight strains, the authors concluded that development of a representative extract preparation for the genus was unlikely and that "selection of a strain is a crucial step for the production of an allergen extract from *Chlorella*." An evaluation of the scientific literature did not indicate any association of allergy or allergic response to any *C. protothecoides* or *A. protothecoides* species.

7.2.3. Comparison of potential allergens using translated genomic sequences

The Structural Database of Allergenic Proteins[52] (SDAP) administered by the University of Texas Medical Branch, Galveston, TX, is a worldwide database that contains all allergens from the IUIS (International Union of Immunological Societies) website,[53] supplemented with information from the scientific literature and from major sequence (SwissProt,[54] PIR,[55] and NCBI[56]) and structure (PDB[57]) databases. To assess the allergenic potential of the S106 proteome *in silico*, the basic local alignment search tool (BLAST) was used to query the entire amino acid sequence of the SDAP against the S106 genome, obtained *via* Next-Generation Sequencing with assembly of fragmented sequences. For comparison, the SDAP was also BLASTed against translations of the genomes of *Zea mays* (corn), *Lycopersicon esculentum*

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[48] When considering species identified in articles published prior to 2000, the possibility of reclassification is a concern especially for *C. vulgaris* and *C. pyrenoidosa* species. Verification against the culture collections of the depositories is recommended.

[49] Aerosolized or associated with house dust

[50] Tubulointerstitial nephritis is a common cause of acute renal failure from immune-mediated tubulointerstitial injury; hypersensitivity reactions can induce allergic response in the renal interstitium (Yim *et al.*, 2007).

[51] Composition of the tablets was not defined beyond the genus.

[52] http://fermi.utmb.edu/SDAP/

[53] http://www.allergen.org

[54] SwissPROT = a protein database that contains sequences translated from the EMBL Nucleotide Sequence Database, prepared by the European Bioinformatics Institute

[55] PIR = Protein Information Resource

[56] NCBI = National Center for Biotechnology Information

[57] PDB = Brookhaven Protein Data Bank, a database of three-dimensional structures

(tomato), and *Saccharomyces cerevisiae* (yeast). In addition, a translated, expressed sequence tag (EST) database from *Triticum aestivum*, (wheat) was also BLASTed. BLAST results are presented in a graphical format in Figure 3. As can be seen, similar to *S. cerevisiae* with 843 BLAST hits, the translated genome of S106 has relatively few BLAST hits (1635 hits) against the SDAP allergens. Corn (12,879 hits), tomato (15, 870 hits) and wheat (130,116 hits), on the other hand, have significantly greater numbers of hits. The well-known foods included in the evaluation (*i.e.*, corn, wheat, and tomato) have greater numbers of hits and with higher e-values (*i.e.*, more proteins that are more closely related to known allergenic epitopes), than S106 does.

In silico analysis of the potential allergens in S106 was provided by BLAST of the SDAP against five different translated genomes (corn, tomato, S106 and yeast) and one translated EST database (wheat). Genomes from three different food crops (*i.e.*, corn, tomato and wheat), had dramatically greater numbers of BLAST hits than did S106 indicating that from an allergenicity standpoint, the protein component of whole S106 biomass poses little risk as a potential human allergen.

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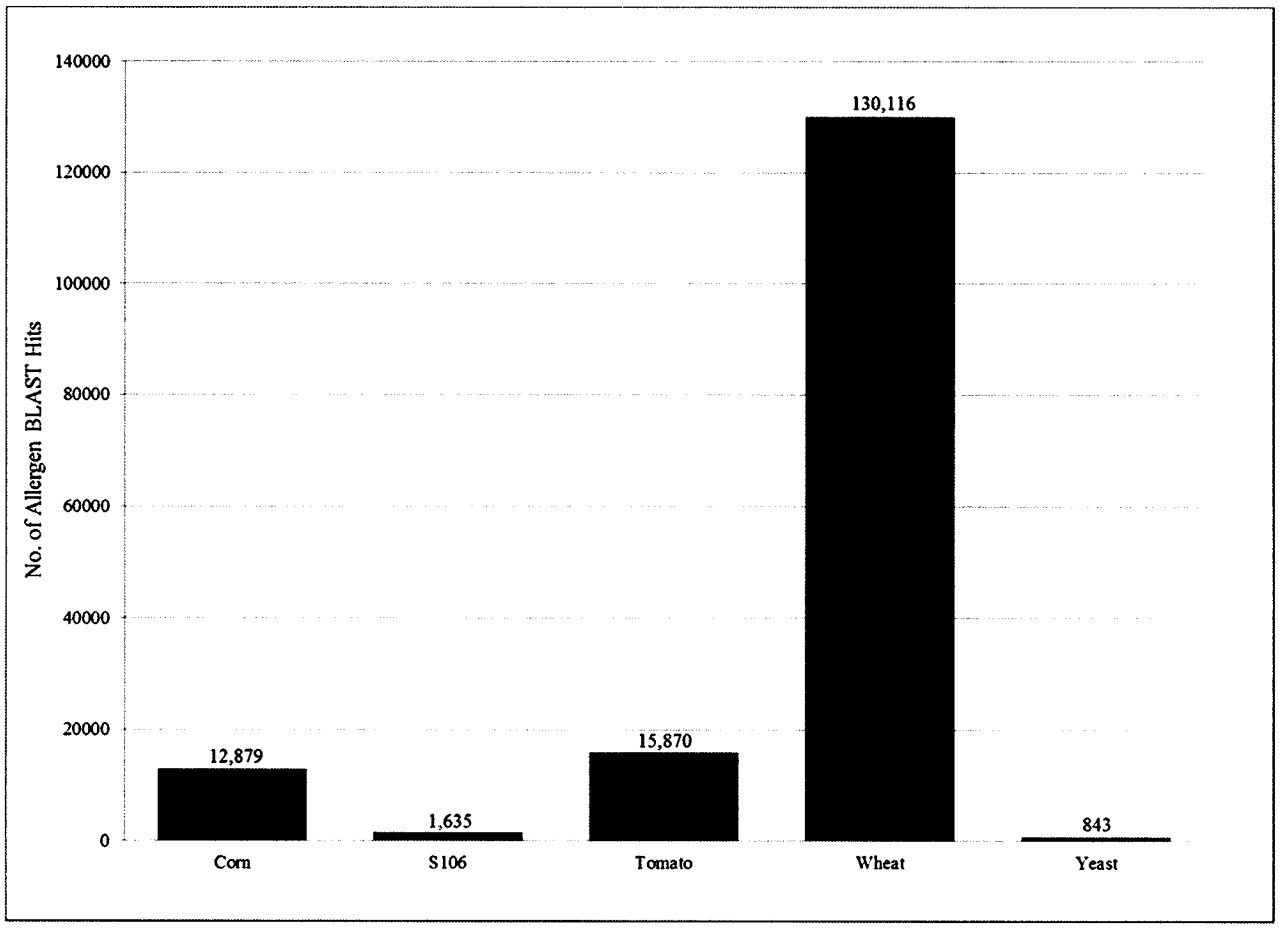

Figure 3. Number of putative allergen hits in various translated genomes or transcriptomes by BLAST with the SDAP.

7.2.4. Supportive observations in humans

The potential for induced allergenic response to HLAF, when consumed as a food, was investigated *via* dermal sensitization in human subjects (Szabo *et al.*, 2012). The study design was in accordance with the applicable guidelines for the protection of human subjects for research as outlined in 21 CFR 50 and in accordance with the accepted standards for Good Clinical Practices (GCPs), International Conference on Harmonization (ICH Expert Working Group, 1996) and the standard practices of Thomas J. Stephens and Associates, Inc. (Carrollton, TX). Performance of a dermal patch test in humans as a means of predicting food allergy by the occurrence of contact dermatitis is a noninvasive approach that holds low risk for the safety of the participants. Written informed consent complying with 21 CFR §50.25 was obtained from each subject prior to study enrollment. A total of 130 healthy subjects enrolled in the Human Repeat Insult Patch test (HRIPT) for HLAF and 110 healthy human subjects[58] (28 men, 82 women; average subject age of 45.01 ± 13.01 years) completed the study.

[58] The 110 subjects completing the study identified their ethnicity/race as follows: 11 African Americans (10.0%), 1 Asian (0.9%), 76 Caucasians (69.1%), 13 Hispanics (11.8%), 2 Native Americans (1.8%), 1 Pacific Islander (0.9%), and 6 subjects of mixed ethnicity (5.5%).

The HRIPT developed by Rizer and Nozawa (Trookman *et al.*, 2011) consisted of two phases, an induction phase and a challenge phase. The three-week induction phase included nine patch periods. During each period, sufficient HLAF test material to cover the test site (approximately 2 cm^2 in area) was applied under a patch to healthy skin on the upper back of each subject. The patch was removed 48 hours after application or about two hours before a study visit. Induction sites were graded 48 to 72 hours after each application using a standardized skin irritation scale (Berger and Bowman, 1982). A minimum of twelve days after application of the last induction patch, challenge patches were applied to the original test site and to a naïve site, also on the upper back. These were removed 48 hours after application or about two hours before the study visit. Challenge sites were graded 48 hours and 96 hours post-application. Occlusive patches (non-woven cotton pads covered by Blenderm tape and held securely to skin on all sides with a porous, hypoallergenic tape) were used for the first two patch periods in the induction phase; semi-occlusive patches (non-woven cotton pads covered and held securely to skin on all sides with a porous, hypoallergenic tape) were used for all later patch periods in the induction phase and for the entire challenge phase.

Of the 130 subjects who enrolled, one was discontinued for noncompliance, 16 for missing more than one study visit, and one withdrew for a cardiac condition unrelated to the test material. Two additional subjects were withdrawn after grading of the first and second occlusive patches determined moderate site reactions to the test material. One subject was scored during the first grading *(i.e.*, evaluation of the first patching) as having severe erythema (very intense redness) with edema (swelling), bullae[59] and spreading at the application site; this subject withdrew from the study and the reaction was followed to resolution. The second subject was scored during the first grading as having severe edema with bullae at the application site. For this subject, an alternate site was used for the second patching and both the original and alternate sites were evaluated during the second grading. At the second grading, the original site scored "severe" for erythema with edema and papules[60] and the alternate site scored as "severe" edema with bullae; the subject was then withdrawn and the reactions were followed to resolution.

Because increasing instances of site irritation were broadly observed among the study participants at the second grading (37%; Table 12), semi-occlusive patches were substituted for occlusive patches for the remainder of the study. At the ninth (final) grading in the induction phase, the test sites of four participants (3.6%) exhibited a mild erythema[61] (faint to definite pink) while the test sites of 106 participants (96.4%) presented with no visible erythema. After the change to semi-occlusive patching, no cumulative irritation was observed in the induction phase. The results of the challenge phase demonstrated that the test material did not induce an allergic response in the study group. At the 96-hour grading in the challenge phase, the original application sites of three subjects (2.7%) and naïve site of one subject (0.9%) exhibited mild erythema. No other subjects demonstrated irritation.

Regarding the two subjects withdrawn during the occlusive portion of the test (one after the first and one after the second patch period), the total evidence suggests that these individuals

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[59] Vesicles (small, circumscribed elevations having translucent surfaces so that fluid is visible (*i.e.*, blister-like)) with a diameter > 0.5 cm; vesicles may coalesce to form one or a few large blisters that fill the patch site.
[60] Small red solid elevations; when touched the affected surface has a granular feeling.
[61] Because redness can result from local irritation, mild erythema alone is not treated as potentially indicative of allergenic response.

are more likely to have experienced irritation, rather than allergic response. Although pre-existing skin sensitization is strongly suggested by the rapidity (first of nine 48-h patch periods), severity (edema and papules/bullae), and persistence of the responses (3-4 weeks to resolve) in both subjects, neither of the responses was confirmed (*via* oral food challenge) to result from a true allergic response. Because erythema is known to result from local irritation (Cudowska and Kaczmarski, 2005), gradings of mild erythema (faint to definite pink) without infiltration are usually regarded as negative and a positive reaction is assessed only if erythema with infiltration or papules is present. Distinguishing irritation from allergy can, however, be challenging for test materials with inherent irritant potential (McNamee *et al.*, 2008).[62] In addition, it is widely known that repeated exposure to a dermal irritant can result in a cumulative irritation reaction (Trookman *et al.*, 2011) and that the use of occlusive patching (an exaggerated exposure condition frequently used for this very reason), can exacerbate irritant effects (McNamee *et al.*, 2008).

In this study, HLAF was applied as a dry solid to subjects' backs under occlusive patching without dilution or extraction.[63] At the end of the second patch period (occlusive) more than one-third (37%) of study volunteers exhibited some degree of patch site irritation (*i.e.*, erythema) with three individuals also presenting with papules and a fourth with edema. The use of semi-occlusive patching during the remainder of the study was found to resolve nearly all irritation concerns in the 110 remaining subjects for the remaining weeks of the study (Table 12). At the end of the induction phase, only four subjects presented with mild erythema at the application site. At the end of the challenge phase, three presented with mild erythema at the original site and only one at the alternative (naïve) site. As an additional consideration, while atopic patch tests (APTs) have been shown to generate negative predictive values of about 90%[64] and have been found particularly useful in predicting late phase (T-cell-mediated) reactions[65] (Cudowska and Kaczmarski, 2005; Chung *et al.*, 2010) positive predictive values for this APT have been substantially lower (50% and 57% compared to DBPCFC[66] for milk and egg allergy, respectively, (Chung *et al.*, 2010); 29% for milk, casein, egg white and yolk (Peron *et al.*, 2011). A tendency for false positives (20% compared to DBPCFC for milk allergy in older children; (Cudowska and Kaczmarski, 2005) is a recognized limiting factor of APT.

In summary, although mild-moderate irritation was identified in several subjects, HLAF did not, under the conditions of this test, induce contact sensitization (allergic contact dermatitis) in any subject completing the study which suggests a low likelihood of food allergy.

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[62] Wheat gluten is an example of a test material suspected of inducing false positive (irritant) reactions (Turjanmaa *et al.*, 2006; McNamee *et al.*, 2008).

[63] When a test material is a potential irritant, dilution or extraction is recommended as a means of decreasing the incidence of false positive responses (Turjanmaa *et al.*, 2006; McNamee *et al.*, 2008).

[64] 95.9% and 88.9% compared to double-blind placebo-controlled food challenge (DBPCFC) for milk and egg allergy, respectively;(Chung *et al.*, 2010); and 90% for milk, casein, egg white and yolk (Peron *et al.*, 2011).

[65] In comparison, skin prick tests (SPT) and food-specific IgE antibodies *via* serological assay are indicative of early (IgE-mediated) food allergy reaction (Cudowska and Kaczmarski, 2005).

[66] DBPCFC = Double-Blind Placebo-Controlled Food Challenge

Table 12. The score frequencies for the test material at each grading time point during the induction and challenge phases.

Score	Induction Phase									Challenge Phase			
	G1	G2	G3	G4	G5	G6	G7	G8	G9	48 O	96 O	48 A	96 A
0	81	68	99	106	98	106	106	105	106	106	107	103	109
0P	0	0	1	0	3	0	0	0	0	0	0	0	0
0R	0	1	0	0	0	0	0	0	0	0	0	0	0
1	27	36	9	3	9	4	4	3	4	4	3	7	1
1P	2	3	1	1	0	0	0	2	0	0	0	0	0
2	0	1	0	0	0	0	0	0	0	0	0	0	0
2E	0	1	0	0	0	0	0	0	0	0	0	0	0
Total	110	110	110	110	110	110	110	110	110	110	110	110	110

0 = No visible erythema; **1** = Mild erythema (faint pink to definite pink); **2** = Moderate erythema (definite redness); 48 = 48-hour observation; 96 = 96-hour observation; A = Alternate (naïve) site; E = Edema; G = Grading; O = Original site; P = papules, R = Removal of the patch at other than the assigned time

7.3. Pathogenic Potential

7.3.1. Pathogenic status in the scientific literature

Even though microalgae are ubiquitous organisms, algal infections are rare in humans and other mammals. Of those that do occur, the most common are protothecosis and chlorellosis, pseudofungal diseases caused, respectively, by opportunistic members of the achlorphyllous *Prototheca* spp. and green *Chlorella* spp. (Ramírez-Romero *et al.*, 2010). The two *Prototheca* species known to be disease agents are *P. wickerhamii*, the cause of most human protothecosis and *P. zopfii,* the usual causative agent in animals (Chandler *et al.*, 1978; Jagielski and Lagneau, 2007). Thus far, the *Chlorella* species responsible for incidents of chlorellosis have not yet been identified. Chlorellosis is usually differentiated from protothecosis by the color of the lesions and/or affected organs. Protothecosis lesions tend to be ivory, white or cream-colored like the achlorophyllous causative organism; chlorellosis lesions, on the other hand, are generally bright green or emerald in color (Chandler *et al.*, 1978).

Protothecosis typically manifests in animals and humans as localized (*i.e.*, cutaneous or articular) or disseminated systemic infection.[67] In the cow, protothecosis is commonly diagnosed as the cause of bovine mastitis. Although infection in cats and goats is also usually localized and cutaneous, infection in dogs can be severe, involving the eyes, brain, and internal organs (Jagielski and Lagneau, 2007; Satoh *et al.*, 2010). Infections in humans usually occur from contamination of wounds or other breaks in the skin with dirty water. On a yearly basis, 2-5 cases of infection are typically reported, with 108 total cases of human protothecosis reported within a 25 year period (Krcméry Jr., 2000). Protothecosis in humans most commonly presents as skin infection (Torres *et al.*, 2003). Individuals who become ill with systemic infection are nearly always already debilitated or immunocompromised (Torres *et al.*, 2003; Jagielski and Lagneau, 2007; Ramírez-Romero *et al.*, 2010; Satoh *et al.*, 2010).

In comparison, chlorellosis has been reported in a variety of domestic and wild animals, including dogs, cattle, sheep, gazelle, dromedaries and beavers, but in only one human. The single reported incident of human infection presented as localized cutaneous lesions in surgical

[67] The infection of internal organs that often results in altered immune response.

wounds exposed to river water (Ramírez-Romero *et al.*, 2010); the causative species was not identified. Although infection in the human patient was mild, chlorellosis can cause systemic disease in livestock (Chandler *et al.*, 1978; Ramírez-Romero *et al.*, 2010). Most bovine infections are mild (*i.e.*, infected lymph nodes found during slaughterhouse inspection); chlorellosis in sheep, however, is often enteric and frequently the cause of morbidity and mortality (Chandler *et al.*, 1978; Ramírez-Romero *et al.*, 2010). Although infection usually occurs through environmental contamination of open wounds, infection in animals has also been associated with drinking sewage water or stagnant water, or grazing pasture irrigated with untreated sewage water (Ramírez-Romero *et al.*, 2010).

7.3.2. Biosafety level designation

As previously defined in Section 2.A. 'Identity', the source microorganism for HLAF, *C. protothecoides* S106 (UTEX 250, aka SAG 211-7C and CCAP 211/7C; original deposition CCAP(1952-5)), is appropriately considered a strain of *Auxenochlorella protothecoides* in addition to being a strain of *C. protothecoides.* Further, S106 has been demonstrated to be identical to the *Auxenochlorella protothecoides* type strain (*C. protothecoides* S485, aka UTEX 25; SAG 211-7A, CCAP211/7A, and ATCC 30407). All *C. protothecoides* (*A. protothecoides*) strains held at the ATCC in Rockville, Maryland, are preserved under BSL 1 conditions which indicate the microorganisms are not recognized to cause disease in immunocompetent adult humans.[8]

7.3.3. Corroborative pathogenicity study

As corroborative evidence supporting a BSL 1 designation for the *C. protothecoides* S106 source organism utilized in the production of HLAF, a pathogenicity study designed to monitor the possible survival or propagation of Microbial Pest Control Agents (MPCA) was conducted using the rat model (Solazyme Roquette Nutritionals, 2012a). The in-life dosing and tissue collection portion of the study was based on US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 3a: *Short-Term Toxicity Studies with Rodents* (2003) and US EPA Health Effects Test Guidelines, OPPTS[68] 885.3050, Acute Oral Toxicity/Pathogenicity (1996); and conducted under GLP.[69] The tissue culture and analysis portion of the MPCA study was also conducted under GLP[70] in accordance with the EPA Microbial Pesticide Test Guidelines, OPPTS 885.3050, "Acute Oral Toxicity/ Pathogenicity", US Environmental Protection Agency, Prevention, Pesticides and Toxic Substances (7101). EPA 712-C-96-315, February 1996.

In order to evaluate whether or not *C. protothecoides* S106 microalgae could survive or propagate in the rat, 32 HSD:SD® rats (12 weeks old; mean weight ±20% for each sex) were randomly assigned to either the treatment (n = 12/sex) or vehicle control (n = 4/sex) group. Either live microalgal cells (~2.5 x 10^8 CFU[71]/rat) in 2 ml vehicle (Defined EBO2[72] minus Co^{2+}

[68] OPPTS = Office of Prevention, Pesticides, and Toxic Substances

[69] US EPA GLP: Toxic Substances Control Act (TSCA): 40 CFR 792, 1989 and US FDA GLP: 21 CFR 58, 1987

[70] Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on June 20, 2002 (BGBl. I Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407); the OECD Principles of Good Laboratory Practice (as revised in 1997); and OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998

[71] CFU = Colony Forming Units

and nitrogen) or 2 ml vehicle alone was administered *via* oral gavage to each rat as an acute dose. All treatment animals (24 rats) and half of the vehicle control animals (2 males, 2 females) were housed in one room. The remaining vehicle control animals (2 males, 2 females) were housed in a separate room. All rats were provided 2016CM Harlan Teklad Global Rodent Diet® (Harlan Teklad, Inc., Indianapolis, IN) and filtered tap water *ad libitum*. All rats were monitored for viability twice *per* day with cage-side observations twice the first day and once *per* day thereafter. Body weights for control rats were recorded during acclimation and on Days 1, 3, 7, 14 and 21 with terminal weights recorded on Day 22. Body weights for all treatment rats were recorded during acclimation and on Day 1, with terminal weights recorded for the first subgroup (n = 3/sex) on Day 4. Body weights for the second subgroup (n = 3/sex) were recorded on Day 7 with terminal weights recorded on Day 8. Third subgroup body weights (n = 3/sex) were recorded on Days 7 and 14 with terminal weights recorded on Day 15. Fourth subgroup body weights (n = 3/sex) were recorded on Days 7, 14 and 21 with terminal weights recorded on Day 22. Tissue[73], blood (*i.e.*, EDTA[74]/citrate buffered blood and plasma), and fecal samples were collected over a period of three weeks post-dosing (Days 3 (feces only), 4, 8, 15, and 22), transferred (under controlled conditions of 2-8 °C) from PSL (Dayton, NJ) to BSL Bioservice (Planegg, Germany) for analysis.

No mortalities occurred during the in-life portion of the study. No effect in clinical signs, mean body weight or mean body weight gain were found in the treatment groups, compared to the control groups. In addition, no differences were identified in any monitored parameter between the control groups (housed with or isolated from the test group). Although two incidental findings (a small-appearing kidney in one control male and one treatment female) were reported at necropsy, no macroscopic differences were observed among the groups.

Tissue, blood, plasma, and fecal samples were analyzed for the presence of *C. protothecoides* cells using a surface spread method with plate counting.[75] All sample and control plates were prepared in triplicate with each plate being analyzed in duplicate. After incubation (3-8 days at 30±2 °C), all plates prepared from tissue, blood, and feces samples in addition to negative control (basal medium) and positive control plates (basal medium inoculated with 1:100, 1:1000 and 10,000 dilutions of 2-4 day old *C. protothecoides* S106 cultures) were analyzed by plate counting for CFU/plate of the microalgae. Plate counting determined that algal colony counts were below the limits of detection[76] for all treatment and control tissue, blood

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[72] Defined EB02 = A proprietary media formulation developed by Solazyme containing: 3 g/L K_2HPO_4, 5.66 g/L $Na_2HPO_4 \cdot 7H_2O$, 1.2 g/L citric acid monohydrate, 1 g/L $(NH_4)_2SO_4$, 0.23 g/L $MgSO_4 \cdot 7H_2O$, 0.03 g/L $CaCl_2 \cdot 2H_2O$, 1 mL trace elements minus Co^{2+}, 0.225 ml/L antifoam (Sigma 204), 1.5 ml DAS vitamins, glucose to 40 g/L. Defined EB02 minus Co^{2+} and nitrogen was prepared as above except ammonium sulfate was omitted.

[73] Brain, lung, liver, spleen, kidney, and lymph nodes

[74] EDTA = Ethylenediaminetetraacetic acid

[75] Prior to initiation of the pathogenicity study in the rats, the surface spread method was validated for quantification of *C. protothecoides* S106 in the spiked tissues (brain, lung, liver, spleen, kidney and lymph nodes), blood and feces of control animals (Solazyme Roquette Nutritionals, 2012b). The surface spread method is the method of choice for quantification of colony forming units of aerobic microorganisms (USP <61>).

[76] All limits of detection (LODs) were normalized to the volume (blood) or weights (tissues and feces) of the samples: <5 CFU/ml plasma and heparinized blood; <14 - < 20 CFU/g brain; <15 - <19 CFU/g lung; <14 - <19 CFU/g liver; <14 - <20 CFU/g spleen; <14 - <19 CFU/g kidney; <14 - <18 CFU/g lymph node; <14 - <31 CFU/g feces for 29 of 32 feces samples with the LOD for one sample at < 54 CFU/g, for a second at < 67 CFU/g, and for a third at < 115 CFU/g (all extremely small, highly diluted samples from Day 15).

(except for unreadable plates from three control animal samples), plasma, and feces samples for all collection days. Of all plates prepared for analysis, one sample from each of three control animals (two female Day 22 heparinized bloods and one male Day 22 brain) could not be read due to overgrowth by molds and red-colony bacteria. Although the analyses of nearly all fecal samples[77] from all days were also affected by rapid overgrowth of molds, each of the fecal plates could still be analyzed and each reported an algal count below the limit of detection. No presence or growth of *C. protothecoides* S106 was observed on the negative control plates (< 1 CFU/ml negative control inoculum, equivalent to < 1 CFU/plate). The results of the positive controls, expressed as CFU/ml positive control inoculum, (equivalent to CFU/plate), were directly comparable to the CFU/mL of the 2-4 day *C. protothecoides* S106 cultures.

In summary, one incident of chlorellosis has been documented in a human. The infection arose from the exposure of surgical wounds to contaminated water and was limited to cutaneous lesions. Because lesional organisms are rarely cultured for genotyping, there is no supportive evidence that *C. protothecoides* was or was not the opportunistic *Chlorella* species in the one human patient or in the animals that are occasionally infected. Because only one case of chlorellosis in humans was reported compared to 108 reported cases of human protothecosis, it is highly unlikely that *C. protothecoides* has or will be the cause of human disease. In addition, *C. protothecoides* and *A. protothecoides* spp. cultures have a BSL 1 rating. Further, as corroborative evidence supporting the limited findings of one incident of opportunistic infection in one human in the scientific literature (from an unknown *Chlorella* spp.) and the BSL 1 designation for *C. protothecoides* and *A. protothecoides* spp., no adverse or toxic effect related to treatment was observed in any rat in the pathogenicity study. No viable count of *C. protothecoides* S106 was detected in any examined tissues or fluids collected during a three-week post-treatment period after the administration of the live algal culture. Under the conditions of the pathogenicity study in rodents, *C. protothecoides* S106 was not acutely toxic, pathogenic or toxigenic. Taken together with the record in the scientific literature and the nonpathogenic BSL rating, it is highly unlikely that consumption of *C. protothecoides* S106 or HLAF derived from *C. protothecoides* S106 would result in a pathogenic incident.

7.4. Photosensitive dermatitis

In 1977, at least 23 residents of Tokyo were diagnosed with photosensitive dermatitis[78] (Tamura *et al.*, 1979; Jassby, 1988). Investigation revealed that each patient had consumed the same brand of *Chlorella* tablet ("Kenbi Chlorella") and, further, that the tablets contained PheideA and its ester in high quantities (up to 8.2 mg/g total PheideA; (Jassby, 1988). A subsequent feeding trial in mice confirmed the association when administration of suspect tablets induced the phototoxic response and administration of *Chlorella* tablets having lower concentrations of Pheides (< 10% of suspect concentrations) did not. The researchers were also able to establish a linear dose-response relationship between inflammation severity and the Pheide content of the administered tablets (Jitsukawa *et al.*, 1984; Jassby, 1988).

Pheophorbide-associated phototoxicity occurs after ingested Pheides enter the circulatory system and distribute throughout the body. When Pheides that have deposited in the skin or other

[77] All treatment fecal samples from Days 4, 8, 15, and 22; seven out of eight control fecal samples from Day 22.
[78] Skin inflammations characterized by rash and itchiness that develop on exposure to light. Usual presentation is cutaneous lesions on the face and dorsa (backs) of the hands. In severe cases, lesions necrotize and scar.

near-surface tissues are exposed to light, oxygen is generated and the fatty acids of nearby cell membranes are subsequently oxidized which leads to cell rupture, damage to dermal capillaries and escalating inflammation (Jitsukawa *et al.*, 1984; Jassby, 1988). A change in the manufacturing process (the use of ethanol during granule formation of the dried *Chlorella* prior to pelleting) was determined to have been the cause of the high PheideA concentrations (Jitsukawa *et al.*, 1984) in the Tokyo incident. The concentrations of existing and potential total PheideA permitted in algae preparations were limited, respectively, to 0.8 mg/g and < 1.2 mg/g by the Japanese Public Health Ministry in 1981 (Jassby, 1988).

Because Pheides are natural degradation products of chlorophylls (Chls), they are present in all photosynthetic plants. In nature, Chl catabolites, including the phototoxic Pheides, typically increase in concentration during leaf senescence, fruit ripening or other incidents of "de-greening" (Jassby, 1988; Hörtensteiner *et al.*, 2000). During "de-greening" events, Pheides form from Chl through the step-wise loss of the phytol residue *via* chlorophyllase followed by the loss of the central magnesium atom *via* magnesium dechelatase (Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000). As mentioned previously, certain manufacturing steps can affect Pheide formation in Chl-containing microalgae products including the use of ethanol, acetone or methanol during processing which can enhance chlorophyllase activity and production of Pheides. On the other hand, heating Chl-containing materials to 100 °C for three minutes is sufficient to inactivate the chlorophyllase enzyme (Jassby, 1988). To ensure against Pheide formation in preparations of microalgae that contain Chl, enzyme inactivation is often combined with an avoidance of processing steps using ethanol, acetone or methanol (Jassby, 1988).

The mechanism of Chl breakdown has been extensively studied in *C. protothecoides* (Hörtensteiner *et al.*, 1998; Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000), the bleaching or de-greening of which under high glucose/nitrogen deficient conditions has been compared to the Chl catabolism of senescing plants (Hörtensteiner *et al.*, 2000), albeit with some substantial differences:

(1) Higher plants contain exclusively Chl-a. Although Chl-a is the major Chl in all algae, species within Division Chlorophyta, such as *C. protothecoides* also contain Chl-b (Becker, 1994).
(2) Because *C. protothecoides* contains both Chl-a and Chl-b, colored catabolites of Chl degradation form for both Chls (Hörtensteiner *et al.*, 1998).
(3) In higher plant species the majority of intermediate and final Chl catabolites are colorless, but in *C. protothecoides*, the main intermediate catabolites are the green-colored Pheides (PheideA and PheideB) with the final catabolites being the red-colored Red Chl catabolites (RCC-a and RCC-b) (Hörtensteiner *et al.*, 1998; Hörtensteiner, 1999).
(4) After rapid formation from the green-colored Pheides in a two-step ring-opening of the porphyrin macrocycle (Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000), algal RCCs are excreted into the medium. In higher plants, RCC-a (there is no RCC-b) is further degraded into a variety of Fluorescent and Nonfluorescent Chlorophyll Catabolites (FCCs and NCCs) (Hörtensteiner, 1999) which are then deposited into the vaucoles of senescent mesophyll cells (Hörtensteiner *et al.*, 1998).

Ordinary commercial *Chlorella* products generally contain 2-3% dry weight Chl content (Jassby, 1988). *C. protothecoides* Krüger ACC[79] 25, however, has been shown to have no detectable Chl and diminished chloroplast structures[80] when "glucose-bleached" in high glucose/low nitrogen medium (Grant and Hommersand, 1974). HLAF is generated from *C. protothecoides* S106, a strain that has been shown by Solazyme to be identical to *C. protothecoides* UTEX 25 which is also known as *C. protothecoides* Krüger ACC NO. 25.[81] After growth in (and isolation from) the nitrogen-limited medium of the Solazyme manufacturing process, Chl is not present and chloroplasts are minimized in the algae and subsequent HLAF ingredient.

8. Evaluation

HLAF is a high lipid flour composed of the dried milled biomass of the microalgae *Chlorella protothecoides* S106. HLAF can be used as a substitute for cream, milk, eggs/egg yolks, and/or butter/shortening in baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups.

Although statistically significant effects were noted for several endpoints in a published 28-day short-term repeated-dose study in rats, these were not found to be related to administration of a ground yellow high-lipid *C. protothecoides* biomass material. Under the conditions of the study, the NOAEL for the biomass material in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats. In corroborative support of these findings, an unpublished 28-day short-term repeated-dose study in hamsters demonstrated that consumption of *C. protothecoides* at up to ~6000 mg/kg bw/day was safe.

In a published 13-week subchronic toxicity study in rats, statistically significant effects were noted for several endpoints, but none were attributable to ingestion of HLAF because the changes were within the ranges historically observed in the age and strain of rats used in this study, did not demonstrate a dose-dependent relationship, were noted only sporadically, and/or were also observed in the control group. Under the conditions of this study, the NOAEL for HLAF in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 4807 mg/kg bw/day in male rats and 5366 mg/kg bw/day in female rats.

The results of the bacterial reverse mutation and *in vivo* bone marrow chromosome aberration assays indicate that HLAF is neither mutagenic nor clastogenic.

In a study exploring the effect of consuming live cells or the isolated cell walls of *C. protothecoides* 902 (20-23 g/kg bw/day) on the $t_{½}$ of chlordecone in C^{14}-chlordecone-treated rats, no adverse effects associated with consumption of the living cells or the cell walls of the algae were reported by the authors.

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[79] Algal Culture Collection at Indiana University, Bloomington, IN.

[80] As measured by absorbance, Chl returns to normal levels within 48 hr of providing a "greening" medium (little/no glucose, high nitrogen) (Grant and Hommersand, 1974).

[81] UTEX 25 was initially designated as ACC No. 25 when the collection was held by Indiana University. In 1976 the ACC at Indiana University was transferred to the University of Texas and became the core of the UTEX collection. <http://www.sbs.utexas.edu/utex/insideUtex.aspx>; site accessed December 12, 2011.

HLAF has a low likelihood of being a food allergen. HLAF proteins are expected to be digested, absorbed, metabolized and excreted through the same normal physiological processes by which other plant materials common to the human diet are digested and utilized for macronutrients. In addition, a search of the scientific literature did not indicate any association of allergy or allergic response to *C. protothecoides* or to any *Auxenochlorella* or *Prototheca* species. Also, a BLAST of the translated genome for S106 garnered few hits, indicating little risk of S106 being a human allergen. Finally, under the conditions of the HRIPT conducted in healthy male and female human subjects, HLAF did not induce contact sensitization (allergic contact dermatitis) in any subject completing the study, although mild-moderate irritation (but not allergic response) was identified in several subjects. The results of the study therefore indicate a low likelihood of food allergy. Taken together, HLAF is highly unlikely to be a food allergen.

Human infection by *C. protothecoides* is also extremely unlikely. Only one case of human chlorellosis has been documented in the literature and this infection was localized and cutaneous, the result of surgical wounds exposed to contaminated water. In addition, *C. protothecoides* and *A. protothecoides* strains are categorized as nonpathogenic BSL 1 microorganisms in respected culture collections such as ATCC. Corroboratively, no viable counts of *C. protothecoides* S106 were detected in any of the examined tissues from a three-week pathogenicity study in which rats were acutely dosed with live *C. protothecoides* S106 (> 2.5×10^8 CFU/rat). Under the conditions of this study, *C. protothecoides* S106 was not pathogenic.

Due to the use of nitrogen-limited medium in the Solazyme manufacturing process, *C. protothecoides* S106 cells are effectively de-greened. Assay of Solazyme's ground yellow high-lipid *C. protothecoides* biomass reported PheideA concentrations below the detection limit of 0.5 ppm, concentrations that are well below the permitted limit of 0.8 mg/g PheideA in algal preparations imposed by the Japanese Public Health Ministry. The results of the conducted assay indicate that insufficient PheideA is present in HLAF for photosensitivity to occur.

The NOAEL for HLAF in a 13-week subchronic toxicity study in male and female rats was 100,000 ppm, the highest dietary concentration provided. The dietary NOAEL for HLAF in male and female rats was 4807 and 5366 mg/kg bw/day, respectively (equivalent to 288,408 mg/day in a 60 kg human).

The estimated daily intake of HLAF is based on the 90th percentile HLAF consumption levels from foods supplemented with HLAF; the estimated daily intake of HLAF when added to foods was determined to be 10.52 g/day. Thus, the potential theoretical maximum HLAF consumption at the 90th percentile may reach 10.52 mg/day. This theoretical intake level represents a conservative estimate because it is unlikely that an individual would consume HLAF from conventional foods at the 90th percentile level.

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DOSSIER IN SUPPORT OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF HIGH LIPID ALGALIN FLOUR FROM CHLORELLA PROTOTHECOIDES STRAIN S106 AS A FOOD INGREDIENT

July 13, 2012

FINAL

Panel Members

Joseph F. Borzelleca, Ph.D.

Eric A. Johnson, Ph.D.

Gary M. Williams, M.D.

801 N. Orange Avenue, Suite 710 • Orlando, FL 32801 • Phone: 407.802.1400 • Fax: 407.802.1405

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DOSSIER IN SUPPORT OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF HIGH LIPID ALGALIN FLOUR FROM CHLORELLA PROTOTHECOIDES STRAIN S106 AS A FOOD INGREDIENT

TABLE OF CONTENTS

1. EXECUTIVE SUMMARY 4
2. INTRODUCTION 4
2.1. Description of Organism 4
2.2. Genotype 6
2.2.1. History of use 13
2.2.2. Current uses 14
2.3. Proposed use or uses 15
2.4. Mechanism of action 15
2.5. Regulatory status 15
3. DESCRIPTION, SPECIFICATIONS AND MANUFACTURING PROCESS 16
3.1. Description and Specifications 16
3.2. Manufacturing Process 18
3.3. Stability 21
4. ESTIMATED DAILY INTAKE 23
5. ABSORPTION, DISTRIBUTION, METABOLISM AND ELIMINATION (ADME) 25
6. SAFETY EVALUATION 25
6.1. Acute Studies 25
6.2. Short Term Repeated-Dose Studies 25
6.3. Subchronic Studies 28
6.4. Other Studies 30
6.4.1. Pathogenicity in rodents 30
6.4.2. Chlordecone detoxification in rodents 33
6.5. Genotoxicity 35
6.6. Carcinogenesis 37
6.7. Observations in Humans 37
6.7.1. Allergenicity Potential 38
6.7.2. Infection 41

6.7.3. Photosensitive dermatitis 42
7. EVALUATION 44
8. CERTIFICATION 46
10. SIGNATURES 48
11. REFERENCES 49
12. APPENDIX 1 55
13. APPENDIX 2 60

DOSSIER IN SUPPORT OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF HIGH LIPID ALGALIN FLOUR[1] FROM CHLORELLA PROTOTHECOIDES S106 AS A FOOD INGREDIENT

1. EXECUTIVE SUMMARY

Solazyme Roquette Nutritionals, LLC (hereinafter referred to as Solazyme) convened an independent panel of recognized experts (hereinafter referred to as the Expert Panel)[2], qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, to determine the Generally Recognized As Safe (GRAS) status of the proposed uses of a high lipid algalin flour (hereinafter referred to as HLAF) from *Chlorella protothecoides* Strain S106 as a food ingredient based on scientific procedures. HLAF is to be added to foods identified herein, such that the 90th percentile consumption from all categories may be up to 10.52 g/day. Solazyme assures that all relevant, unpublished information in its possession related to the safety of HLAF has been supplied to and summarized by the Burdock Group in this dossier. A comprehensive search of the scientific literature was conducted through December 14, 2011 for safety and toxicity information on *Chlorella protothecoides*, HLAF and related substances and, has been summarized in this dossier by the Burdock Group. This information, along with supporting documentation, was made available to the Expert Panel. In addition, the Expert Panel independently evaluated materials deemed appropriate and necessary. Following an independent, critical evaluation, the Expert Panel conferred and unanimously agreed that the proposed uses of HLAF are safe when consumed up to 10.52 g/day.

2. INTRODUCTION

HLAF is a high lipid (40-70% fat) algal flour composed of the milled dried biomass of the microalgae *Chlorella protothecoides* Strain S106. HLAF will be used as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups. HLAF will only be added to foods for which a standard of identity does not exist. This dossier is a summary of the scientific evidence that supports the Generally Recognized As Safe status of HLAF as a human food ingredient.

2.1. Description of Organism

HLAF is a golden yellow high lipid powder composed of the milled dried biomass of *C. protothecoides* S106. As a species, *C. protothecoides* currently belongs to the genus *Chlorella* in the phylum Chlorophyta. Widespread in fresh and salt water, soil and air (Wu *et al.*, 2001), the green microalgae *Chlorella spp.* are nonmotile, unicellular eukaryotes, spherical in shape and

---

[1] The term "flour" as used herein refers to "algal flour," a dried milled biomass of the microalgae *Chlorella protothecoides* and should not be confused with the standardized name of cereal based flour described in 21 CFR 137.105 *et seq.*).

[2] Modeled after that described in Section 201(s) of the Federal Food, Drug, and Cosmetic Act, as amended. See also attachments (*curriculum vitae*) documenting the expertise of the Panel members.

have diameters that typically range from 2 – 10 μm (Kay, 1991; Becker, 2007). The cell walls are hemicellulosic, rigid, and account for approximately 10% of the algal dry weight (Becker, 2007). *Chlorella* spp. also characteristically have membrane-bound organelles (Kay, 1991). Axenic[3] cultures of *Chlorella* spp. are easily established, in part because replication under optimum conditions tends to be rapid, often requiring less than two hours (Kay, 1991). Protein and lipid content and the fatty acid profile are known to vary widely with the species, the stage of cell growth (exponential or stationary), and with environmental conditions (*e.g.*, available nutrients, temperature, and light intensity) (Kay, 1991). As an example, the protein contents of *Chlorella* spp. have been reported to range from 15 – 60 % on a dry weight basis (Kay, 1991; Becker, 1994).

Although morphologically similar and all unicellular, the species grouped into the genus *Chlorella* have recently been recognized to be phylogenetically divergent (Ferris *et al.*, 2005) and not necessarily related (Tiberg and Einarsson, 1989; Huss *et al.*, 1999). Nomenclature and taxonomic assignments based on traditional identification methods are being re-examined – and when necessary, reclassified – using data from genetic sequence analysis (Ferris *et al.*, 2005). Because species comprising the *Chlorella* genus are a taxonomically complicated group, chemotaxonomic character, DNA[4] base composition and DNA/DNA hybridization are all used to distinguish the morphologically similar species (Huss *et al.*, 1999; Ferris *et al.*, 2005). For example, based on comparative analyses of 18S ribosomal RNA gene sequences, Huss *et al.* (1999) have recommended that only four species remain in the *Chlorella* genus: *C. vulgaris*, *C. lobophora*, *C. sorokiniana*, and *C. kessleri*.

Solazyme obtained *C. protothecoides* UTEX 250 from the University of Texas (UTEX) Culture Collection of Algae in Austin, Texas and assigned the Solazyme internal strain number "S106". UTEX 250 had, in turn, been obtained by UTEX as *C. protothecoides* CCAP211/7C from the Culture Collection of Algae and Protozoa (CCAP), Scottish Association for Marine Science, Argyll Scotland, United Kingdom, where the strain was originally deposited sometime between 1952 and 1955 by A.J. Kluyver [5] as the third isolate of *C. vulgaris* ("3 *C. vulgaris*") taken from freshwater in Delft, Netherlands. Other deposits of this strain include the Meyer 34 and SAG211-7C accessions in the Sammlung von Algenkulturen Göttingen (SAG, Albrecht-von-Haller-Institute for Plant Science, University of Göttingen, Göttingen, Germany).[6] Additional designations by which this strain is known include *C. protothecoides* var. *communis* Shihira and Krauss (1965),[7] *C. protothecoides* Krüger and *Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová (1987). Based on accepted designations, UTEX 250 (S106) may be appropriately considered a strain of *C. protothecoides* or *Auxenochlorella protothecoides*. All *C. protothecoides* (*A. protothecoides*) strains held at the American Type Culture Collection (ATCC, Rockville, Maryland) are preserved under Biosafety Level 1

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[3] Cultures containing a single strain (*i.e.*, not contaminated)
[4] DNA = deoxyribonucleic acid
[5] <http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2779>; site accessed April 7, 2011.
[6] < http://sagdb.uni-goettingen.de/detailedList.php?str_number=211-7c>; site accessed April 7, 2011.
[7] <http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2779>; site accessed April 7, 2011.

conditions which indicate the microorganisms are not recognized to cause disease in adult humans.[8]

The ecophysiological and biochemical characteristics of *C. protothecoides* include the inability to utilize nitrate as a substrate, the need for thiamine but not for vitamin B_{12}, the lack of secondary carotenoids (most strains), and a tolerance for high temperatures (28 – 34 °C upper limits), acidic pH (3.5 – 4.0 pH) and high salt concentrations (3 – 4%) (Huss *et al.*, 2002). Morphology for the species includes the absence of pyrenoids, but the presence of a tri-laminar layer within the cell wall (Huss *et al.*, 2002). Reproduction is by endosporulation, an asexual process through which two to twelve sporangiospores are produced and released by each parental sporangium during vegetative growth (Ramírez-Romero *et al.*, 2010). This growth pattern ceases when cells are deprived of nitrogen but continued to be fed sugar. Under nitrogen depleted conditions, the cell begins to synthesize and store copious amounts of oil (Day *et al.*, 2009).

Although similar in appearance, morphology, and composition to other *Chlorella* species, such as *C. vulgaris* and *C. pyrenoidosa*[9] (Robinson and Guzman-Juarez, 1978; Kay, 1991; Brown and Jeffery, 1992; Tokusoglu and Unal, 2003; Ravishankar *et al.*, 2006), *C. protothecoides* alone has the ability, when exposed to glucose, to "de-green" or "etiolize". Genetically, *C. protothecoides* strains (with the single exception of *C protothecoides* var *Acidicola*[10]) have been shown to be closely related to species in the achlorophyllous genus *Prototheca.* Gene-based phylogenetic trees developed from nuclear 18S rRNA[11], plastid 16S rRNA, SSU[12] and LSU[13] rDNA[14], and 26S rDNA domain sequences have repeatedly demonstrated that *A. protothecoides* UTEX 25 (S485), the type strain for *A. protothecoides*, is closely related to the *Prototheca* species (Huss *et al.*, 1999; Tartar *et al.*, 2003; Ueno *et al.*, 2005; Satoh *et al.*, 2010). At least one protein-based phylogenetic tree also confirms these assessments (von Bergen *et al.*, 2009). The relationship is sufficiently homologous that comparative analysis of the phylogeny of the related *A. protothecoides* UTEX 25 (S485) and 12 strains of *Prototheca* were found to form a monophyletic clade (Ueno *et al.*, 2005).

2.2. Genotype

As discussed above, delineation of microalgal species based on morphology is problematic due to a potentially strong morphological and physiological similarity among diverse species. Molecular indicators such as plastidic 23S rDNA are more reliable indicators of strain relatedness. As an example, using plastid 23S rDNA sequences shown in Figure 1

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[8] <http://www.atcc.org/ATCCAdvancedCatalogSearch/AllCollectionSearch/tabid/454/Default.aspx>; site accessed November 30, 2011.

[9] Many of these stains have been reclassified as *C. sorokiniana* or *C. fusca* var. *vacuolata.*

[10] Analysis of 18S RNA gene sequences has demonstrated only a weak phylogenetic relationship to *C. protothecoides* var. *acidicola* which is no longer considered a variety of *C protothecoides* (Huss *et al.*, 2002; Ferris *et al.*, 2005)

[11] rRNA = ribosomal ribonucleic acid

[12] SSU = small subunit of ribulose bis-phosphate carboxylase-oxygenase

[13] LSU = large subunit of ribulose bis-phosphate carboxylase-oxygenase

[14] rDNA = ribosomal deoxyribonucleic acid

(Solazyme, 2011a), Solazyme demonstrated that its *C. protothecoides* S106 (UTEX 250 (Solazyme source); also known as (aka) SAG 211-7C and CCAP 211/7C; original deposition CCAP(1952-5)) possesses a genotype identical to that of Solazyme designated strain *C. protothecoides* S485 (UTEX 25 (Solazyme source); aka, SAG 211-7A, CCAP211/7A, and ATCC 30407). Strain S485 is known formally as *Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová (1987) and is the type strain for *A. protothecoides*. *C. protothecoides* S106 was also demonstrated to share approximately 87.5% homology with another *C. protothecoides* strain, S102 (UTEX B 25 (Solazyme source); aka original deposition CCAP (1952-5)) which is called *C. protothecoides* Krüger (1892) and *A. protothecoides*. In conclusion, based on plastid 23S rDNA sequencing, *C. protothecoides* S106 is identical (*i.e.* 100% homology) to *A. protothecoides* S485 (type strain of *Auxenochlorella protothecoides*) and only somewhat closely related (approximately 87.5% homology) to *C. protothecoides* S102.

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S102 23S   TGTTGAAGAATGAGCCGGCGACTTAGAAAACGTGGCAAGGTTAAGGAAACGTATCCGGAG
S106 23S   TGTTGAAGAATGAGCCGGCGACTTAGAAAAAGTGGCGTGGTTAAGGAAAAAT-TCCGAAG
S485 23S   TGTTGAAGAATGAGCCGGCGACTTAGAAAAAGTGGCGTGGTTAAGGAAAAAT-TCCGAAG

S102 23S   CCGAAGCGAAAGCAAGTCTGAACAGGGCG----------------------ATTAAGTCA
S106 23S   CCTTAGCGAAAGCGAGTCTGAATAGGGCGATCAAATATTTTAATATTTACAATTTAGTCA
S485 23S   CCTTAGCGAAAGCGAGTCTGAATAGGGCGATCAAATATTTTAATATTTACAATTTAGTCA

S102 23S   TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAGCTTGGGTGACAC
S106 23S   TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAACTTGGGTGATAC
S485 23S   TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAACTTGGGTGATAC

S102 23S   CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT
S106 23S   CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT
S485 23S   CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT

S102 23S   GAAATACCAGTCGAACTCGGAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCGG
S106 23S   GAAATACCAGTCGAACCCGGAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAG
S485 23S   GAAATACCAGTCGAACCCGGAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAG

S102 23S   TTCATA-AGGCTGTCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGCGAAAGCGGTACCA
S106 23S   TACATCTAGTCTATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGTGAAAACGGTACCA
S485 23S   TACATCTAGTCTATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGTGAAAACGGTACCA

S102 23S   AATCGTGGCAAACTCTGAATACTAGATATG-CTATTTATGGGCCAGTGAGACGGTGGGGG
S106 23S   AATCGTGGCAAACTCTGAATACTAGAAATGACGGTGTA-GT---AGTGAGACTGTGGGGG
S485 23S   AATCGTGGCAAACTCTGAATACTAGAAATGACGGTGTA-GT---AGTGAGACTGTGGGGG

S102 23S   ATAAGCTTCATCGTCGAGAGGGAAACAGCCCAGATCACTAGCTAAGGCCCCAAAATGATC
S106 23S   ATAAGCTCCATTGTCAAGAGGGAAACAGCCCAGACCACCAGCTAAGGCCCCAAAATGGTA
S485 23S   ATAAGCTCCATTGTCAAGAGGGAAACAGCCCAGACCACCAGCTAAGGCCCCAAAATGGTA

S102 23S   GTTAAGTGACAAAGGAGGTGAGAATGCAGAAACAACCAGGAGGTTTGCTTAGAAGCAGCC
S106 23S   ATGTAGTGACAAAGGAGGTGAAAATGCAAACACAACCAGGAGGTTGGCTTAGAAGCAGCC
S485 23S   ATGTAGTGACAAAGGAGGTGAAAATGCAAACACAACCAGGAGGTTGGCTTAGAAGCAGCC

S102 23S   ACCCTTTAAAGAGTGCGTAATAGCTCACTG
S106 23S   ATCCTTTAAAGAGTGCGTAATAGCTCACTG
S485 23S   ATCCTTTAAAGAGTGCGTAATAGCTCACTG
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Figure 1. Plastid 23S rDNA sequences for Solazyme designated *C. protothecoides* strains S102, S106 (Solazyme strain of interest) and S485 (*Auxenochlorella protothecoides* type strain) (Solazyme, 2011a)

Similar comparison using partial plastidic 23S rDNA sequencing was made for Solazyme's *C. protothecoides* S106 strain against 21 *C. vulgaris* strains obtained from five different depository collections (Solazyme, 2011a). As indicated below, each test strain is identified by its Solazyme designation, common name and strain source; identical strains and other current or previous names are included as parenthetical information for ease of reference:

(1) S183: *C. vulgaris*, UTEX 395 (formerly *C. pyrenoidosa* (Starr and Zeikus 1987));
(2) S184: *C. vulgaris* (type strain), UTEX 259, (CCAP 211/11B; aka *Chlorella candida*; *Chlorella miniata*; *Chlorella vulgaris* var. *vulgaris* f. *vulgaris*; original deposition CCAP 1952-5);
(3) S185: *C. vulgaris*, UTEX 1809 (formerly *C. salina* (Starr and Zeikus 1987));
(4) S187: *C. vulgaris*, UTEX 2714 (*Chlorella vulgaris* Bashan);
(5) S188: *C. vulgaris*, UTEX 30 (CCAP 211/12; SAG 211-12; ATCC 16487; aka *C. vulgaris* Beijerinck f. *viridis* ((Chodat) Fott and Nováková)));
(6) S190: *C. vulgaris*, UTEX 396 (formerly *C. vulgaris* var. *viridis* (Starr and Zeikus 1987));
(7) S191: *C. vulgaris*, UTEX 26 (formerly *Chlorella pyrenoidosa* (Starr & Zeikus 1987); aka *C. photophila* (Shihira & Krauss 1965); *C. emersonii*; *C. fusca* var. *vacuolata*));
(8) S192: *C. vulgaris*, UTEX 265 (CCAP 211/11J; SAG 211-11J; *Chlorella vulgaris* Beijerinck 1890 (Rodhe); aka *C. simplex* (Shihira & Krauss 1965) and *Chlorella vulgaris* var. *vulgaris* f. *vulgaris*; original designation CCAP 1630);
(9) S246: *C. vulgaris*, SAG 30.80 (*Chlorella vulgaris* Beijerinck (Senger 1965); formerly *C. saccharophila*);
(10) S257: *C. vulgaris*, SAG 211.11T (*Chlorella vulgaris* Beijerinck (Ruschmann));
(11) S300: *C. vulgaris,* CCAP 211/19 (*Chlorella vulgaris* Beijerinck 1890 (von Witsch 1946/7));
(12) S301: *C. vulgaris,* CCAP 211/11S (*Chlorella vulgaris* Beijerinck 1890 (Pirson));
(13) S302: *C. vulgaris,* CCAP 211/11Q (*Chlorella vulgaris* Beijerinck (Czurda));
(14) S303: *C. vulgaris,* CCAP 211/11P (SAG 211-11P; *Chlorella vulgaris* Beijerinck (Algéus 1942 Strain B);
(15) S344: *C. vulgaris,* CCAP 211/81 (*Chlorella vulgaris* Beijerinck 1890 (Krienitz (1979); original designation CCAP A36);
(16) S345: *C. vulgaris*, CCAP 211/80 (*Chlorella vulgaris* Beijerinck 1890 (Krienitz (1979); original designation CCAP A35);
(17) S410: *C. vulgaris,* CCALA[15] 263 (*C. vulgaris* Beijerinck (Hindak 1967/61));
(18) S412: *C. vulgaris,* CCALA 266 (*C. vulgaris* Beijerinck (Marsalek 1985/57));

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[15] Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň, Czech Republic; <http://www.butbn.cas.cz/ccala/index.php?page=sr&cbl=Algae>; site accessed April 14, 2011.

(19) S413: *C. vulgaris,* CCALA 268 (*C. vulgaris* Beijerinck (Gaffron/Bethesda C 1.3.1.));
(20) S497: *C. vulgaris,* CAUP[16] H1993 (*C. vulgaris* Beijerinck (Punčochářová 1981/22)); and
(21) S498: *C. vulgaris,* CAUP H1996 (*C. vulgaris* Beijerinck f. *globosa* (Andreeva 1961); *C. vulgaris* var. *vulgaris* f. *globosa*).

Distance values (D values), a numerical metric used to indicate genetic relatedness, were generated from the sequence data (Solazyme, 2011a); lower D-values indicate sequences that are more closely related with "0.000" indicating identical sequences (*i.e.*, 100% homology). Higher values, on the other hand, indicate more divergent sequences. Isolates with minor genetic variation (high homology) are generally accepted as varying by a value of only about 0.005% (Rawat, *et al.* 2005). For perspective, strains having less than 95.5% homology (based on 16S rRNA sequence identity) are routinely regarded as different species (Stackebrandt and Goebel, 1994). Based on an evaluation of the numeric metric used to assess relatedness (Table 1), the partial plastidic 23S rDNA sequence for Solazyme strain S106, the *C. protothecoides* strain of interest, had D values ≥ 0.080 relative to the 21 *C. vulgaris* strains examined. When the D values for Solazyme strain S184 (the type strain for *C. vulgaris*) are considered, it was determined that S184 is (1) identical to 15 of the other 21 *C. vulgaris* strains tested (D value = 0.000 for all),[17] (2) very closely or reasonably closely matched to three strains (D values from 0.002 to 0.034),[18] and (3) more distantly related to the remaining three strains (D values ≥ 0.090),[19] S106 being counted among this last group. An additional comparison of the 23S sequence of *C. protothecoides* S106 using nBLAST analysis to that of GenBank accession No.[20] L43357 *C. vulgaris* Beijerinck (NIES[21]-2170, formerly in the IAM[22] collection as C-27; formerly classified as *C. ellipsoidea* Gerneck) determined 87% homology (FDA, 2011). In conclusion, *C. protothecoides* S106 is distantly related to many of the *C. vulgaris* strains tested, including S184 (UTEX 259), but it was also observed that the *C. vulgaris* type strain S184 is less closely related to some *C. vulgaris* isolates (*e.g.*, S412 and S498) than to S106.

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[16] Culture Collection of Algae of Charles University in Prague (CAUP), Praha, Czech Republic; <http://botany.natur.cuni.cz/algo/caup-list.html>; site accessed April 14, 2011.
[17] S183, S185, S188, S190, S192, S246, S257, S300, S302, S303, S344, S345, S410, S413, and S497
[18] S187, S197, and S301
[19] S106, S412, and S498
[20] No. = number
[21] National Institute for Environmental Studies, Tsukuba, Japan.
[22] Institute of Applied Microbiology, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences University of Tokyo, Tokyo, Japan.

Table 1. Tabulated Distance values for 23S rDNA sequences of *C. protothecoides* S106 (Solazyme strain of interest) compared to S183, S184 (*C. vulgaris* type strain), S185, S187, S188, S190, S191, S192, S246, S257, S300, S301, S302, S303, S344, S345, S410, S412, S413, S497, and S498. (Lower values indicate sequences that are more closely related, with "0.000"indicating identical sequences. Higher values indicate more divergent sequences.) (Solazyme, 2011a)

	S106 23S	S183 23S	S184 23S	S185 23S	S187 23S	S188 23S	S190 23S	S191 23S	S192 23S	S246 23S	S257 23S	S300 23S	S301 23S	S302 23S	S303 23S	S344 23S	S345 23S	S410 23S	S412 23S	S413 23S	S497 23S	S498 23S
S106 23S		0.090	0.090	0.090	0.080	0.090	0.090	0.095	0.090	0.090	0.090	0.090	0.082	0.090	0.092	0.090	0.090	0.090	0.150	0.090	0.090	0.186
S183 23S	0.090		0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S184 23S	0.090	0.000		0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S185 23S	0.090	0.000	0.000		0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S187 23S	0.080	0.030	0.030	0.030		0.030	0.030	0.033	0.030	0.030	0.030	0.030	0.006	0.030	0.031	0.030	0.030	0.030	0.110	0.030	0.030	0.148
S188 23S	0.090	0.000	0.000	0.000	0.030		0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S190 23S	0.090	0.000	0.000	0.000	0.030	0.000		0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S191 23S	0.095	0.002	0.002	0.002	0.033	0.002	0.002		0.002	0.002	0.002	0.002	0.036	0.002	0.002	0.002	0.002	0.002	0.106	0.002	0.002	0.154
S192 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002		0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S246 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000		0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S257 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000		0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S300 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000		0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S301 23S	0.082	0.034	0.034	0.034	0.006	0.034	0.034	0.036	0.034	0.034	0.034	0.034		0.034	0.034	0.034	0.034	0.034	0.112	0.034	0.034	0.150
S302 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034		0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152
S303 23S	0.092	0.000	0.000	0.000	0.031	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000		0.000	0.000	0.000	0.109	0.000	0.000	0.156
S344 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000		0.000	0.000	0.106	0.000	0.000	0.152
S345 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000		0.000	0.106	0.000	0.000	0.152
S410 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000		0.106	0.000	0.000	0.152
S412 23S	0.150	0.106	0.106	0.106	0.110	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.112	0.106	0.109	0.106	0.106	0.106		0.106	0.106	0.123
S413 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106		0.000	0.152
S497 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000		0.152
S498 23S	0.186	0.152	0.152	0.152	0.148	0.152	0.152	0.154	0.152	0.152	0.152	0.152	0.150	0.152	0.156	0.152	0.152	0.152	0.123	0.152	0.152	

Solazyme's *C. protothecoides* S106 strain was also compared to eight strains in Solazyme's collection that had previously (prior to 1992) been classified as *C. pyrenoidosa* (Solazyme, 2011b). As *C. pyrenoidosa*, these strains had been the subject of several published nutritional studies. With the advent of modern molecular methods of identification, the species in the *Chlorella* genus have undergone significant taxonomic revision. In 1992, many *C. pyrenoidosa* strains were re-evaluated and reclassified as *C. sorokiniana* (Kessler and Huss, 1992) or *C. fusca* var. *vacuolata*. As indicated below, each test strain is identified by Solazyme designation, current classification and strain source; previous names and identical strains and/or other current or former names are also included as parenthetical information:

(1) S129: *C. sorokiniana*, UTEX 1230 as *C. pyrenoidosa* (formerly *C. pyrenoidosa* (Starr & Zeikus 1987); *C. vulgaris* fo. *tertia* and *C. vulgaris* group; ATCC 22521; CCAP 211/8K as *C. vulgaris* f. *tertia;* CCAO 259 as *C. sorokiniana*; SAG 211-8K as *C. sorokiniana*; originally designated Tx 7-11-05);
(2) S134: *C. sorokiniana* UTEX 1666, (formerly *C. pyrenoidosa*);
(3) S135: *C. sorokiniana* UTEX 1670 (formerly *C. pyrenoidosa* (Starr & Zeikus 1987); originally designated B1E-B2P);
(4) S140: *C. sorokiniana* UTEX B1810 (formerly *C. sorokiniana* var. *pacificensis* (Starr & Zeikus 1987));
(5) S167: *C. fusca* var. *vacuolata* UTEX 251 (formerly *C. pyrenoidosa*; SAG 211-8B; CCAP 211/8B; CCAUP H 6401; ATCC 11469);
(6) S168: *C. fusca* var. *vacuolata* UTEX 252 (formerly *C. pyrenoidosa*; aka *Scenedesmus* sp.; *Chlorella emersonii* var. *emersonii*; SAG 211-8C as *C. fusca*; CCAP 211/8C as *Chlorella emersonii* var. *globosa* (Shihira & Kraus (1965));
(7) S545: *C. sorokiniana* UTEX 1664; and
(8) S547: *C. sorokiniana* UTEX 1668 (formally *C. pyrenoidosa* (Starr and Zeikus 1987).

Evaluation of the D values generated from partial plastidic 23S rDNA sequencing (Table 2) indicated that Solazyme strain S106, the *C. protothecoides* strain of interest, had D values ≥ 0.076 (≤ 88% homology), showing slightly greater homology than was seen between S106 and the 21 previously examined *C. vulgaris* isolates. In addition, Solazyme strains S129, S134 (UTEX 1666), S135 (UTEX 1670), S545 (UTEX 1664), and S547 (UTEX 1668) were determined to be identical (D value = 0.000 for all), which is not surprising, considering that UTEX strains 1664 through 1671 are mutants of UTEX 1663, a sub-isolate of Tx-7-11-05 (UTEX 1230 or S129). As with the *C. vulgaris* isolates examined above, strain S106 is less closely related to most of the strains previously classified as *C. pyrenoidosa* than they are to one another. The exception is strain S168, whose 23S sequence shows less homology to the other strains in this set than S106 shows to the other strains.

In conclusion, partial plastidic 23S rDNA sequences were used by Solazyme to assess the genetic relatedness between S106, *C. protothecoides* and two genera of *Chlorella* previously studied and used as foods or dietary supplements, namely *C. vulgaris* and *C. pyrenoidosa*. Algal strain S106 was found by Solazyme researchers to be less closely related to these isolates than it was with S485 (type strain for *A. protothecoides*), against which S106 demonstrated 100%

homology. The researchers also noted that two of the *C. vulgaris* isolates, S412 and S498 were less closely related to S184 (UTEX 259), the *C. vulgaris* type strain, than was S106 and that S168, previously classified as *C. pyrenoidosa*, was more distantly related to the other *C. pyrenoidosa* isolates than was S106.

Table 2. Tabulated Distance values for 23S rDNA sequences of *C. protothecoides* S106 (Solazyme strain of interest) compared to other Chlorella strains S129, S134, S135, S140, S167, S168, S545, and S547. (Lower values indicate sequences that are more closely related, with "0.000"indicating identical sequences. Higher values indicate more divergent sequences.) (Solazyme, 2011b)

	S106 23S	S129 23S	S134 23S	S135 23S	S140 23S	S167 23S	S168 23S	S545 23S	S547 23S
S106 23S		0.076	0.076	0.076	0.086	0.090	0.182	0.076	0.076
S129 23S	0.076		0.000	0.000	0.022	0.049	0.158	0.000	0.000
S134 23S	0.076	0.000		0.000	0.022	0.049	0.158	0.000	0.000
S135 23S	0.076	0.000	0.000		0.022	0.049	0.158	0.000	0.000
S140 23S	0.086	0.022	0.022	0.022		0.041	0.164	0.022	0.022
S167 23S	0.090	0.049	0.049	0.049	0.041		0.142	0.049	0.049
S168 23S	0.182	0.158	0.158	0.158	0.164	0.142		0.158	0.158
S545 23S	0.076	0.000	0.000	0.000	0.022	0.049	0.158		0.000
S547 23S	0.076	0.000	0.000	0.000	0.022	0.049	0.158	0.000	

2.2.1. History of use

Microalgae, such as *Chlorella* spp., have been an accepted part of the human diet for hundreds of years, especially among the populations of Asia and sub-Saharan Africa (Kay, 1991; Ravishankar *et al.*, 2006). Currently, wild stocks of various microalgae are harvested as food sources not only in Asia (*e.g.*, China, Japan, Burma, Thailand, India, Mongolia, and Siberia), but also in Central and South America (*e.g.*, Mexico, Bolivia, Ecuador, and Peru), Pacific island nations such as Fiji, and Hawaii in the U.S. (Kay, 1991). Of the microalgae that are commercially cultured, those most popular in the U.S. include, but are not limited to, the green microalgae *Chlorella* spp. and *Scenedesmus* spp., and the cyanobacteria *Spirulina* spp. and *Aphanizomenon flos-aquae* spp. (Kay, 1991). In the early 1970s, U.S. companies cultivating microalgae for human and animal use included Earthrise Farms[23] in California, Cell Tech[24] in Oregon and Cyanotech Corporation[25] in Hawaii (formerly, Washington) (Kay, 1991). Internationally, Sun Chlorella[26] in Japan and SOSA Texcoco/Spirulina Mexicana[27] in Mexico also grew microalgae during this same period (Kay, 1991).

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[23] <http://www.earthrise.com/farm.html>; site accessed February 1, 2012.
[24] Currently SimpleXity Health, Inc. (<http://www.simplexityhealth.com>; site accessed February 1, 2012).
[25] <http://www.cyanotech.com>; site accessed February 1, 2012).
[26] <http://www.sunchlorella.com>; site accessed February 1, 2012.
[27] The world's first large plant for processing microalgae (*Spirulina* spp.) was built on Lake Texacoco in the 1970s. Although Mexican Spirulina was first imported into the U.S. for use in health food products in 1979, in 1982 importation was blocked by U.S. authorities due to quality concerns related to pollution of the lake system. In addition to health food products, Spirulina was also used in animal and aquaculture feeds. Spirulina Mexicana has

Modern interest in *Chlorella* as a protein source for humans and animals and consumption of various *Chlorella* preparations began to increase about 50 years ago when large-scale culture production became technically feasible. Early manufacturing facilities were constructed both in the U.S. in 1951 (first pilot plant) and Taiwan in the 1960s (Ravishankar *et al.*, 2006). Even though *Chlorella* is not presently considered a food in the U.S.[28] and consumption is not as high in the U.S. as it is among the major consuming populations of China, Korea, and Japan, *Chlorella* spp. are still widely available in U.S. markets as dietary supplements (Day *et al.*, 2009). At least twelve different brands of chlorella products are available in forms that include powder, tablet and capsule with recommended dosages ranging from 200 mg/day up to 10 g/day (Hendler and D. Rorvik,2008; FDA, 2011). National retailers Vitamin Shoppe[29] and GNC[30] both carry *Chlorella* products.

Among the many strains in the *Chlorella* genus, those that have been most commonly used in foods and dietary supplements belong to the *C. vulgaris*, *C. pyrenoidosa*, *C. fusca*[31], *C. kessleri*,[32] and *C. sorokiniana*[32] species. Neither literature nor electronic searches have revealed any documentation of *C. protothecoides* (or *A. protothecoides*) strains or of HLAF being used in human or animal foods.

2.2.2. Current uses

As economically promising sources of lutein (xanthophyll), certain *C. protothecoides* strains such as UTEX 29 are being investigated as potential replacements for French marigold (*Tagetes patula*), the petals of which are currently harvested and extracted for the carotenoid antioxidant (Wei *et al.*, 2008; Fernández-Sevilla *et al.*, 2010). Lutein, a natural constituent of many foods (*e.g.*, oranges, lettuce, broccoli, spinach, eggs) is also included in feed as an indirect colorant in animal tissues and is frequently included as an ingredient in foods and cosmetics, and as a component in drug products and dietary supplements.

C. protothecoides including UTEX 25, the *A. protothecoides* type strain, are currently being developed for large scale cultivation of high quality[32] biodiesel feedstock (Xiong *et al.*, 2008; Xiong *et al.*, 2010; Sirisansaneeyakul *et al.*, 2011).

C. protothecoides is used to assess the toxicity of effluent from bleached kraft pulp mills by the National Pulp Mills Research Program[33] in Australia (Neil *et al.*, 2009) and is one of three aquatic organisms (the water flea *Ceriodaphnia* cf. *dubia* and ciliated protozoan *Tetrahymena*

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been closed for several years (<http://www.spirulinasource.com/earthfoodch6c.html>; site accessed February 1, 2012).

[28] As of this writing, the U.S. FDA is considering two GRAS notifications for the usage of *Chlorella* spp. in food.

[29] <http://www.vitaminshoppe.com>; site accessed February 1, 2012.

[30] <http://www.gnc.com>; site accessed February 1, 2012.

[31] Prior to the advent of gene-based identification methods, most strains currently designated as *C. fusca*, *C. kessleri*, and *C. sorokiniana* were at one point assigned to *C. pyrenoidosa*.

[32] Biomass requirements include the efficient production of specific lipid content and profiles

[33] National Pulp Mills Research Program, Commonwealth Scientific and Industrial Research Organisation (CSIRO) Division of Coal and Energy Technology in Australia

thermophila, being the other two) used to monitor the toxicity of pit lake[34] waters during *in situ* remediation trials (Neil *et al.*, 2009).

Microalgae, *Chlorella* spp. among them, although not specifically *C. protothecoides* or *A. protothecoides*), are also cultivated as feedstock for mariculture (Brown and Jeffery, 1992).

In similar fashion, although dietary supplements containing *Chorella* spp. are widely available in the U.S. (Day *et al.*, 2009) and *Chlorella* continues to appear in the Utah Natural Products Alliance (UNPA) "New old dietary ingredients list" from the list's first release in 1997[35] to its current revised form (dated October 29, 2011 (UNPA, 2011)) neither *C. protothecoides* nor *A. protothecoides* has been specified or documented as a species of use.

Although chlorella species *C. vulgaris* and *C. pyrenoidosa* have been and are currently used as foods and ingredients in dietary supplements, electronic searches of the U.S. and European regulatory website and the trade and scientific literature have not revealed any documentation of *C. protothecoides* (or *A. protothecoides*) strains or of HLAF having been used in human or animal foods.

2.3. Proposed use or uses

HLAF, a golden yellow high lipid flour composed of the milled dried biomass of the green microalgae *Chlorella protothecoides* S106, will replace a portion of the cream, milk, eggs/egg yolks, and/or butter/shortening in baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups. The levels of intended use range from 2000 ppm[36] (μg/g) in vegetable gravies to 120,000 ppm in mayonnaise.

2.4. Mechanism of action

HLAF will replace a portion of the cream, milk, eggs/egg yolks, and/or butter/shortening in a variety of conventional foods, including baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups. As a replacement source of macronutrients and energy, the lipids, proteins, fiber and carbohydrates found in HLAF will be digested through the same normal physiological processes by which other dried, ground plant materials common to the human diet are digested and utilized.

2.5. Regulatory status

HLAF has not been approved for use in food by FDA[37], FEMA[38], or USDA[39]. GRAS Notification 000384 for the use of "algalin oil" from *C. protothecoides* as a food oil in a variety of foods excluding meat and poultry products was received by FDA on May 2, 2011 and filed on

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[34] Lake formed by a mining void that has filled with ground and/or surface water.
[35] Purpose of the list was to identify those dietary ingredients that had been marketed in the U.S. prior to implementation *of Dietary Supplement Health and Education Act of 1994* (DSHEA, 1994)
[36] ppm = parts *per* million
[37] FDA = United States Food and Drug Administration
[38] FEMA = Flavor Extract Manufactures Associations
[39] USDA = United States Department of Agriculture

June 15, 2011; determination is currently pending.[40] In Europe, according to the Novel Food Regulation (EC) N° 258-97, HLAF is not a novel food but a traditional food ingredient (TNO, 2012). The EU's Scientific Committee on Food does not have an established Acceptable Daily Intake (ADI) for HLAF in humans.

3. DESCRIPTION, SPECIFICATIONS AND MANUFACTURING PROCESS

3.1. Description and Specifications

HLAF is a golden yellow high lipid powder composed of the milled dried biomass of the microalgae *Chlorella protothecoides* S106. The physical and chemical properties of HLAF are provided in Table 3. The specifications for HLAF are provided in Table 4.

Table 3. Physical and chemical properties of HLAF

Characteristic	Value
Synonyms	Algal flour, Algalin flour, Whole algalin flour
Appearance	Golden yellow powder
Moisture Content	≤ 10 %
Fiber Content	10 – 50 %
Ash Content	< 10 %
Protein Content	2 – 15 %
Fat Content [a]	40 – 70 %

[a] Major fatty acid present is oleic acid (≥ 25 %) (Day *et al.*, 2009).
HLAF= High Lipid Algal Flour

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[40] <http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=384>; Formal status last confirmed July 10, 2012.

Table 4. Specifications for HLAF

Analysis	Method	Specification	Batch Analysis Results (n = 3) Range	Average
Appearance	C-M-00023-000 Appearance [a]	Golden yellow powder	Conforms	Conforms
Moisture Content	AOAC 930.15	≤ 10 %	2.0 – 2.5%	2.3%
Fiber Content	AOAC 991.43	10 – 50 %	29.6 – 33.6%	31.5%
Ash Content	AOAC 942.05	< 10 %	1.5 – 3%	2.0%
Protein Content	AOAC 990.03	2 – 15 %	4.4 – 7.1%	6.0%
Fat Content	AOAC 954.02	40 – 70 %	49.9 – 53.8%	52.0%
Oleic Acid Content [b]	Q-019-00 Rev 00 Calculations for Various Analytical Data [c]	≥ 25 %	32.2 – 33.7%	33.1%
Sucrose	AOAC 980.13	0 – 10 %	5.9 – 6.5%	6.2%
Heavy metals				
Lead	EPA 3050/6020 USP 730	< 0.5 ppm	0.067 – 0.193 ppm	0.136 ppm
Arsenic	EPA 3050/6020 USP 730	< 0.2 ppm	0.033 – 0.05ppm	0.0423 ppm
Mercury	EPA 3050/6020 USP 730	< 0.1 ppm	< 0.005ppm	< 0.005 ppm
Cadmium	EPA 3050/6020 USP 730	< 0.1 ppm	< 0.001 – 0.0038 ppm	0.0027 ppm
Chromium	EPA 3050/6020 USP 730	< 2 ppm	0.073 – 0.597 ppm	0.409 ppm
Microbiological Limits				
Aerobic Plate Count	AOAC 990.12	< 10,000 cfu/g	100 – 420 cfu/g	240 cfu/g
Coliform	AOAC 966.24	≤ 5 cfu/g	< 3 cfu/g	< 3 cfu/g
E. coli	USP 32, NF 27, 2009	Negative in 10 g	Negative	Negative
Staphylococci	USP 32, NF 27, 2009	Negative in 10 g	Negative	Negative
Salmonella	AOAC 2004.03	Negative in 25 g	Negative	Negative
Pseudomonas aeruginosa	USP 32, NF 27, 2009	Negative in 10 g	Negative	Negative
Yeast	Chapter 18, FDA-BAM, 7th ed.	< 100 cfu/g	< 10 cfu/g	<10 cfu/g
Mold	Chapter 18, FDA-BAM, 7th ed.	< 100 cfu/g	< 10 – 10 cfu/g	< 10 cfu/g

[a] C-M-00023-000- Appearance (Solazyme, internal method, Communicated by e-mail March 30, 2011; Revised by e-mail April 9, 2012)
[b] Main fatty acid present; for minor constituents see Day *et al.* (2009).
[c] Q-019-00 Rev 00 Calculations for Various Analytical Data (Solazyme, internal method, Communicated by e-mail March 30, 2011)
AOAC = Association of Analytical Chemists; cfu = colony-forming units; EPA = Environmental Protection Agency; FDA-BAM = Food and Drug Administration Bacteriological Analytical Manual; g = gram; n = number; HLAF= High Lipid Algal Flour; ppm = parts *per* million; USP = United States Pharmacopeia

Pheophorbides (Pheides) are naturally occurring degradation products of chlorophyll that form during "de-greening" events (Jassby, 1988). Ingestion of pheophorbide-A (PheideA) is known to induce photosensitive dermatitis in humans (Jitsukawa *et al.*, 1984; Jassby, 1988).

When Solazyme's ground yellow high-lipid *C. protothecoides* biomass[41,42] was assayed, the concentration for PheideA was determined to be below the detection limit of 0.5 ppm (FDA, 2011; UBE, 2009[43]), a concentration considerably lower than the limit of 1.2 mg/g established by the Japanese Public Health Ministry in 1981 (Becker, 1994; FDA, 2011).

Algal and cyanobacterial toxins have a broad range of potencies. Ingestion of one or more of these toxins by a human can cause a range of effects from mild illness to death. Solazyme's ground yellow high-lipid *C. protothecoides* biomass was analyzed by high performance liquid chromatography with fluorescence or mass spectrometric detection for all groups of algal and cyanobacterial toxins that have been identified in the published literature and mentioned in international food regulations (Day *et al.*, 2009; FDA, 2011). No toxins were detected.[44] Assayed toxins included amnesic shellfish poisoning toxins (domoic acid), paralytic shellfish poisoning toxins (*N*-sulfocarbamoyl toxins C1-4, B1, B2; decarbamoylgonyautoxins 1-4; gonyautoxins 1-4; decarbamoylsaxitoxin; saxitoxin; and neosaxitoxin), diarrhetic shellfish poisoning toxins (okadaic acid, dinophysistoxins, pectenotoxins, yessotoxins, azaspiracides, and gymnodimines), neurotoxic shellfish poisoning toxins (brevetoxins), and the cyanobacterial toxins (microcystins MC-RR, -LR, -YR, -LA, -LF, and –LW), nodularin, anatoxin-a, cylindrospermopsins, and β-methylamino- *L*-alanine (BMAA).

3.2. Manufacturing Process

HLAF is manufactured by fermenting and harvesting cultures of *C. protothecoides* S106. A pure, clonally isolated culture is initially used to prepare a master seed bank from which working seed vials are prepared. Three samples from the master and each working seed bank are characterized by molecular genotyping to demonstrate that they are genetically identical (*i.e.*, 100% homology between the six chromosomal footprints and 100% homology between their 23S ribosomal deoxyribonucleic acid (DNA) sequences).[45] For a production lot, a cryo-preserved working seed vial is thawed and the contents used to inoculate a flask culture, which is transferred into larger flasks at mid-log phase, and then to standard, industrial seed fermenters. Throughout the aseptic fermentation process, pH, temperature, agitation and aeration rates are controlled, and glucose or sucrose and nutrients are added (Appendix 1). Lipid production is induced by limiting inorganic nitrogen during the fermentation process. Following completion of fermentation, the cells are inactivated by pasteurization, and separated from the culture broth by centrifugation. The separated cells are washed with water to remove the medium and other non-biomass related material, concentrated, and milled (>30% cells disrupted). After milling, the pH is adjusted to neutral and food-grade antioxidants are added. The biomass, including undisrupted cells and cell walls, is then homogenized, dried and packaged. If needed, food grade flow agents

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[41] The *C. protothecoides* used in this material was produced through a process similar to that used in the production of HLAF.

[42] The material studied by Day *et al.* (2009) and the subject of GRN000384 (FDA, 2011) has been confirmed by Solazyme to be the same material as that of the current GRAS (E-mail communication, dated April 18, 2012).

[43] BM182 = HLAF sample designation

[44] Limits of detection ranged from 0.1–2.5 μg/g (Day *et al.*, 2009)

[45] When tested, the six 23S ribosomal DNA sequences also demonstrated 100% identity to the 23S reference sequence for the original *C. protothecoides* S106 isolate.

(Appendix 1) may be added to assist processing through drying and packaging; at present the use of such agents is not part of the standard process. All ingredients used during manufacture are safe and suitable (Appendix 1). A graphical depiction of the manufacturing process is presented in Figure 2.

The final product, HLAF, is available in quantities of 15 – 25 kg packed in a bag-in-box arrangement and stored at temperatures < 25 °C.

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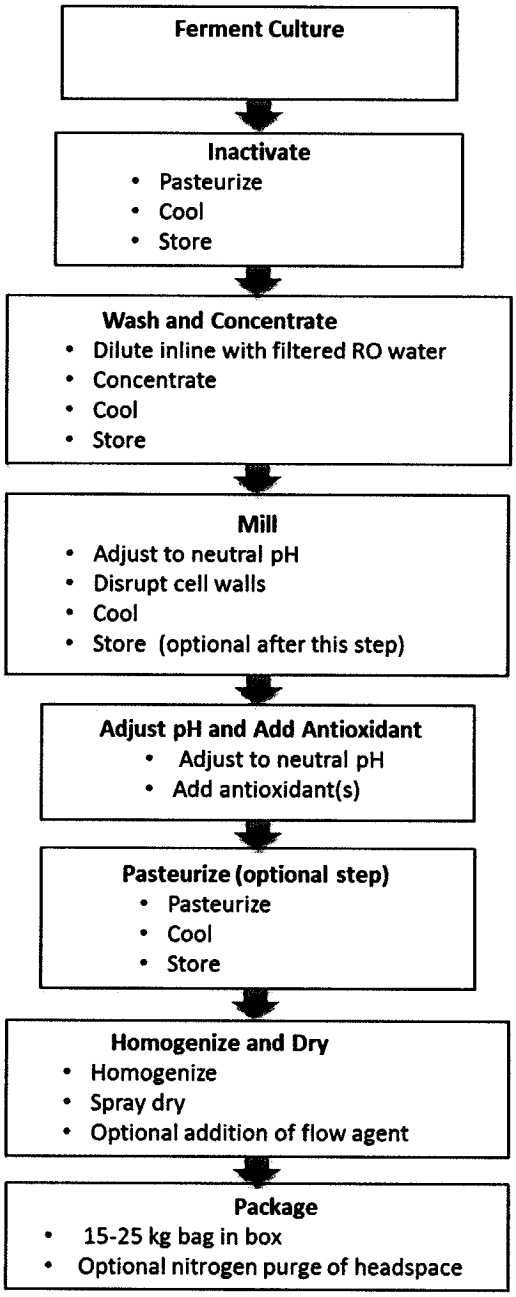

Figure 2. HLAF production schematic

3.3. Stability

HLAF is stable for 12 months, the latest time point tested, when stored at -20 °C (Table 5). HLAF is stable for nine months when stored at controlled room temperature, 23 ± 3 °C (Table 6); after 12 months at this temperature, peroxide values (indicative of lipid peroxidation) exceeded the specification of 5 meq/kg in some samples. HLAF is stable for one month when stored at 40 °C (Table 7); after three months at this temperature, peroxide values exceeded the specification of 5 meq/kg in some samples. HLAF was stored in sealed foil pouches for all stability evaluations.

Table 5. Stability of HLAF stored at -20 °C

Lot	Appearance (Golden yellow powder)	Lipid content (≥ 50%)	Peroxide Value (≤ 5.0 meq/kg)
HLAF 353			
0 month	Conforms	53.8	0.1
6 months	Conforms	54.6	4.8
12 months	Conforms	58.7	2
HLAF 354			
0 month	Conforms	53.3	0.8
6 months	Conforms	51.0	1.6
12 months	Conforms	55.7	0.1
HLAF 379			
0 month	Conforms	51.4	0.4
6 months	Conforms	55.5	0.1
12 months	Conforms	55.6	0.01

HLAF= High Lipid Algal Flour

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Table 6 Stability of HLAF stored at 23 ± 3 °C

Lot	Appearance (Golden yellow powder)	Lipid content (≥ 50%)	Peroxide Value (≤ 5.0 meq/kg)
HLAF 353			
0 month	Conforms	53.8	0.1
3 month	Conforms	56.0	1.8
6 months	Conforms	53.4	4.0
9 months	Conforms	58.9	4.6
12 months	Conforms	58.2	3.6
HLAF 354			
0 month	Conforms	53.3	0.8
3 month	Conforms	53.7	2.0
6 months	Conforms	53.0	4.8
9 months	Conforms	56.1	5.0
12 months	Conforms	55.0	*7.6*[a]
HLAF 379			
0 month	Conforms	51.4	0.4
3 month	Conforms	50.4	1.6
6 months	Conforms	54.0	0.4
9 months	Conforms	56.9	2.6
12 months	Conforms	55.2	2.8

[a]Use of *italics* indicates peroxide values > 5meq/kg; HLAF= High Lipid Algal Flour

Table 7. Stability of HLAF at 40 °C

Lot	Appearance (Golden yellow powder)	Lipid content (≥ 50%)	Peroxide Value (≤ 5.0 meq/kg)
HLAF 353			
0 month	Conforms	53.8	0.1
1 month	Conforms	54.3	2.0
3 month	Conforms	56.6	4.2
6 months	Conforms	54.8	*25.6*
9 months	Conforms	59.2	*56.0*
12 months	*Bleached* [a]	57.8	*390.0*
HLAF 354			
0 month	Conforms	53.3	0.8
1 month	Conforms	52.2	2.0
3 month	Conforms	55.2	*20.0*
6 months	Conforms	51.8	*120.0*
9 months	*Bleached*	55.1	*160.0*
12 months	*Bleached*	53.7	*66.0*
HLAF 379			
0 month	Conforms	51.4	0.4
1 month	Conforms	51.6	2.0
3 month	Conforms	52.7	3.4
6 months	Conforms	54.7	*7.8*
9 months	Conforms	56.1	*30.4*
12 months	*Bleached*	54.4	*290*

[a] Use of *italics* indicates parameters outside of specifications (*i.e.*, "Bleached", rather than "golden yellow" appearance; peroxide values > 5 meq/kg); HLAF= High Lipid Algal Flour

4. ESTIMATED DAILY INTAKE

The intake profile (amount and frequency) by individuals in USDA's What We Eat in America (WWEIA) Continuing Survey of Food Intakes by Individuals 2003-2004 (Dwyer *et al.*, 2003)[46] was used to calculate the estimated daily intake (EDI) of HLAF for individuals consuming the food groups selected for the addition of HLAF *per* this GRAS evaluation (*i.e.*, "eaters only"). The individual foods selected for addition of HLAF are provided in Appendix 2. HLAF will only be added to foods for which a standard of identity does not exist.

The means and 90th percentile EDIs were calculated only for HLAF intake following addition of HLAF to the selected food groups. The means and 90th percentile EDIs were not calculated for current HLAF intake from natural sources as the information on which exposure could be calculated was not located through a comprehensive search of the published literature. HLAF added to the selected foods at the levels specified in Table 8 would provide a mean and 90th percentile HLAF consumption of 4.70 and 10.52 g/day, respectively (Table 9)

All food categories designated by Solazyme have been utilized in the calculations as appropriate; however, certain categories designated by Solazyme may contain foods for which a standard of identity exists, which prohibit the addition of ingredients to the food not identified as mandated or optional ingredients under the regulation. Therefore, addition of HLAF to a food for which a standard of identity exists would demand that the food product be named other than that as indicated under the standard of identity or a waiver of that standard be obtained.

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[46] USDA (2006) What We Eat In America, NHANES 2003-2004; Documentation and Data Files. U.S. Department of Agriculture, Agriculture Research Service; http://www.ars.usda.gov/Services/docs.htm?docid=15044; site visited September 28, 2011.

Table 8. Food groups selected for HLAF supplementation*

Food Category	Intended use level (ppm)
Baked Goods	
Biscuits	50000
Breakfast flour-based foods	30000
Brownies	50000
Cakes	50000
Cookies	50000
Muffins	50000
Beverages Non-alcoholic	
Grain based drinks	50000
Soy based drinks	10000
Breakfast Cereals	
Cold whole grain based cereals	5000
Cheese	
Imitation cheese	10000
Processed cheese and cheese spreads	10000
Non dairy	
Cream substitute and whipped topping	10000
Whipped topping	10000
Creamer	20000
Egg Products	
Scrambled egg	20000
Egg substitute	50000
Scrambled egg, cholesterol-free, w/ cheese	10000
Fats and Oils	
Imitation butter spreads	80000
Mayonnaise	120000
Salad dressing	85000
Frozen Dairy	
Frozen Yogurt	10000
Ice-cream	40000
Ice-cream fat free	20000
Ice-cream sugar free	50000
Sherbet	40000
Pudding	30000
Custard	40000
Vegetable Gravy	2000
Sauces	50000
Honey mustard	20000
Soy based sauce	10000
Meal Replacement	
Breakfast bar	20000
Granola bar	20000
Protein bar	20000
Instant breakfast drinks	20000
Milk	
Soy	10000
Milk Product	
Yogurt	10000
Snack Food	

Food Category	Intended use level (ppm)
Crackers	30000
Salty snacks from grain products	30000
Vegetable and seafood soups	10000
Sweet sauces	5000

*The food categories correspond to those listed in 21 CFR §170.3(n).
HLAF = High Lipid Algal Flour; ppm = parts *per* million

Table 9. Predicted intake of HLAF following supplementation of selected foods at the indicated levels (Table 8) for individuals consuming selected supplemented foods

	Per User (mg/day)	
HLAF intake from:	**Mean**	**90th Percentile**
Possible maximum consumption with HLAF as an added ingredient to food	4699	10,522

HLAF = High Lipid Algal Flour

5. ABSORPTION, DISTRIBUTION, METABOLISM AND ELIMINATION (ADME)

The lipids, proteins, and carbohydrates found in HLAF (a high lipid *Chlorella protothecoides* S106 flour) are expected to be digested, absorbed, metabolized and excreted through the same normal physiological processes by which other plant materials common to the human diet are digested and utilized for macronutrients.

6. SAFETY EVALUATION

6.1. Acute Studies

No acute toxicity studies related to *C. protothecoides* or *A. protothecoides* were discovered in the scientific literature.

6.2. Short Term Repeated-Dose Studies

In a 28-day repeated-dose toxicity study (Day *et al.*, 2009) HSD:SD® rats (*n* = 10/sex/group) were each provided diets *ad libitum* containing 0 ppm (placebo control), 25,000 ppm (low-dose), 50,000 ppm (mid-dose) and 100,000 ppm (high-dose) ground yellow high-lipid[47] *C. protothecoides* S106 biomass. The test diets for the Day *et al.* (2009) study were formulated using the AIN-93G Rodent Diet (Research Diets, Inc., New Brunswick, NJ) as the basal diet to which sufficient test substance was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose groups. The low-, mid-, and high dose diets were equivalent to 1794, 3667, and 7557 mg/kg bw[48]/day, respectively in males and 1867, 3918, and 8068 mg/kg bw/day, respectively, in females. Following the treatment period, the rats from each group were terminated on Day 31 (males) or Day 32 (females). This study was performed in compliance with OECD[49] Guidelines for the Testing of Chemicals,

[47] 48% lipid
[48] bw = body weight
[49] OECD = Organisation for Economic Co-operation and Development

Section 4 (Part 407): Health Effects, *Repeated Dose 28-day Oral Toxicity Study in Rodents* (1995) and with Good Laboratory Practice (GLP) in accordance with OECD Principles of Good Laboratory Practice (as revised in 1997)[50] and US FDA GLP: 21 CFR 58, 1987.

The daily administration of the ground yellow high-lipid *C. protothecoides* biomass material at dietary concentrations up to 100,000 ppm in the feed was well-tolerated by the rats in the 28-day study. Consumption of this high-lipid algal biomass was not found to affect health or growth as measured by viability, appearance, behavior, body weight, body weight gain, food consumption, or food efficiency. No treatment-related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, or histopathology of animals in any group. Although statistical significance was shown for several parameters, none were attributable to ingestion of the test substance because the changes were noted only sporadically, did not demonstrate a dose-response relationship, were within the ranges historically observed in the age and strain of rats used in this study, and/or were also observed in the control group. These observations are summarized below:

(a) One control female died from suspected accidental overdose of anesthesia on Day 29 during orbital sinus bleeding. This occurrence was an isolated incident and not related to the test substance. (b) Incidental findings during clinical observation included a scab on the head of one mid-dose male (Days 2-6) and an abrasion on the nose of one low-dose female (Day 3). (c) A minor unilateral observation was also made in one control female during ophthalmologic examination on Day 25. This finding was sporadic and not associated with the test substance. (d) A minimally significant increase in food consumption was observed in high-dose males during Week 2 ($P < 0.05$) compared to the control group. Although food consumption by high-dose males did not differ significantly from the control group during the other three weeks, overall food consumption (Days 0-28) was also statistically higher for high-dose males compared to the controls ($P < 0.05$). Because increased consumption was slight and not accompanied by corresponding changes in body weight or food efficiency, the finding was not toxicologically significant and not necessarily related to the test substance. (e) A statistically significant decrease in mean food efficiency was reported in mid-dose males during Week 3 ($P < 0.05$) compared to control males. This decrease was short term and, therefore, judged to be incidental and not related to the test substance. (f) Statistically significant decreases in mean corpuscular hemoglobin concentration (MCHC) and absolute basophil concentrations were noted for the males of the high-dose and low-dose groups, respectively, compared to the control group ($P < 0.05$ for both). Because these Day 29 hematology findings were not accompanied by any other clinical or histopathologic change and no dose-dependent relationship was demonstrated, the findings were judged to be not attributable to treatment. (g) A significant increase in the absolute large unstained cell concentration in mid-dose group females compared to the control group ($P < 0.05$) was not found to be toxicologically relevant because the change was sporadic (not present at Day 29) and did not demonstrate a dose-dependent relationship. (h) Clinical chemistry results revealed statistically significant increases in creatinine in mid-dose males and triglycerides in

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[50] OECD Environmental Health and Safety Publication, Series on Principles of Good Laboratory Practice and Compliance Monitoring – Number 1. Environment Directorate, Paris 1998.

high-dose males on Day 15 and blood urea nitrogen in mid-dose males on Day 29 compared to the control group ($P < 0.05$ for all). Cholesterol in high-dose females was significantly elevated compared to the control group ($P < 0.05$) on Day 15. Because these findings did not present with consistency, dose-response relationship, or corresponding clinical or histopathological changes, they were not related to the test substance. (i) Clinical chemistry results from Days 15 and 29 revealed statistically significant increases in alkaline phosphatase in high-dose group males ($P < 0.05$ for both). These findings did not demonstrate a dose-response relationship and were not present in females, nor were they accompanied by corresponding clinical or histopathologic change, nor were there corresponding changes in liver or kidney weight; they were, therefore, judged not to be toxicologically relevant. (j) Findings for Day 29 urinalysis included a statistically significant increase in specific gravity and a decrease in urine volume in mid-dose females compared to controls ($P < 0.05$ for both). These changes were not dose-related nor were they accompanied by clinical or histopathologic change, nor was liver or kidney weight affected. The changes were judged not to be treatment related. (k) Macroscopic observation revealed a statistically significant increase in absolute adrenals ($P < 0.05$), adrenals-to-body ($P < 0.01$) and adrenals-to-brain ($P < 0.05$) weights in the mid-dose males compared to the control group values. Due to the absence of clinical or histopathological changes, these observations were incidental.

Under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for ground yellow high-lipid *C. protothecoides* biomass in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponded to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats.

The findings of a 28-day trial of dietary *C. protothecoides* in the Syrian golden hamster (sex not specified; $n = 15$/group) were reported by Harding and Jones (2008) in an unpublished report. Control animals received a hyperglycemic-hypercholesterolemic diet *ad libitum*; the treatment groups received the same diet supplemented with 2.5% or 5.0% (*w/w*)[51] *C. protothecoides* (equivalent to ~3000 or ~6000 mg/kg bw/day, respectively). No statistically significant effect was observed in body weight, food intake, body composition (*e.g.*, percentage body fat), or plasma triglycerides, cholesterol, protein, or albumin for the treatment groups compared to the control group. Compared to the control group, plasma glucose was significantly reduced in both low- and high-dose groups ($P < 0.05$ for both), but without affecting triglycerides or total cholesterol. Although plasma insulin concentrations for the treatment group did not differ significantly compared to the control group, plasma insulin in the 2.5% group was significantly elevated compared to plasma insulin in the 5.0% group ($P < 0.05$). Oxygen consumption was also found to be significantly greater in the 2.5% group than in the control group ($P < 0.05$), but without affecting production of carbon dioxide. The authors concluded that *C. protothecoides* in a hyperglycemic-hypercholesterolemic diet decreased plasma glucose independent of plasma insulin and further determined that "consumption of *C. protothecoides* at 2.5% and 5% of total diet (w/w) [~3000 and ~6000 mg/kg bw/day] appeared to be safe as there was no difference between groups in the liver production total plasma protein or albumin" (Harding and Jones, 2008).

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[51] w/w = weight/weight

In summary, although statistically significant effects were noted for several endpoints in a 28-day repeated-dose study in rats (Day *et al.*, 2009), these were not found to be related to administration of the test substance, a ground yellow high-lipid[52] *C. protothecoides* S106 biomass material. Under the conditions of the study, the NOAEL for high-lipid *C. protothecoides* biomass in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponded to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats. In an unpublished 28-day repeated-dose study in hamsters, consumption of *C. protothecoides* up to ~6000 mg/kg bw/day was determined by the authors to be safe (Harding and Jones, 2008). The short-term repeated-dose toxicity studies in rodents are summarized in Table 10.

Table 10. 28-Day short term repeated-dose toxicity studies in rodents

Duration	Species (#/dose group)	Dose/Route	Results/Notes	Reference
28 days	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm in diet	Dietary ground yellow high-lipid *C. protothecoides* biomass was well-tolerated; NOAEL was 7557 mg/kg bw/day in males and 8068 mg/kg bw/day in females. No reported adverse effects.	Day *et al.* (2009)
28 days	Syrian golden hamster (15/group; sex not specified)	0, 2.5% and 5.0% *C. protothecoides* in diet (equivalent to 0, ~3000, and ~6000 mg/kg bw/d)	Plasma glucose significantly reduced in low- and high-dose groups compared to the control ($P < 0.05$ for both). Plasma insulin for treatment groups did not differ significantly compared to control, but plasma insulin in low-dose group was significantly elevated compared to high-dose group ($P < 0.05$). Oxygen consumption significantly greater in low-dose group than in control group ($P < 0.05$). Consumption of *C, protothecoides* at up to ~6000 mg/kg bw/day was well tolerated.	Harding and Jones (2008)

C. Protothecoides = *Chlorella protothecoides*; F = Female; HLAF= High Lipid Algal Flour; M = Male; NOAEL = No Adverse Effect Level; ppm = parts *per* million; # = number

6.3. Subchronic Studies

In a 13-week repeated-dose subchronic toxicity study (Szabo *et al*, 2012), HSD:SD® rats (n = 10/sex/group) were provided *ad libitum* diets containing 0 ppm (Group 1 placebo control), 25,000 ppm (Group 2), 50,000 ppm (Group 3) and 100,000 ppm (Group 4) HLAF (equivalent to 0, 1249, 2478, and 4807 mg/kg bw/day, respectively in males and 0, 1413, 2739, and 5366 mg/kg bw/day, respectively, in females). The test diets were formulated using the DIO Rodent® basal diet to which sufficient HLAF was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose groups. Following the treatment period, the rats from each group were terminated on Day 93 (males) or Day 94 (females). The subchronic repeated-dose toxicity study in rodents is summarized in Table 11.

[52] 48% lipid

This study was performed in compliance with OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Part 408): Health Effects, *Repeated Dose 90-Day Oral Toxicity Study in Rodents* (1998) and with GLP in accordance with OECD Principles of Good Laboratory Practice (as revised in 1997) [49] and US FDA GLP: 21 CFR 58, 1987. The study protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC) of PSL[53], Dayton, NJ, which is fully accredited by Accreditation of Laboratory Animal Care International (AAALAC International) (Accredited Unit No. 000939).

The daily administration of HLAF at dietary concentrations up to 100,000 ppm was well-tolerated by the rats in the 13-week study. Consumption of HLAF was not found to affect health or growth as measured by viability, condition, behavior, body weight, body weight gain, food consumption, or food efficiency. No treatment related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, or histopathology of animals in any group. Although statistical significance was shown for several parameters, none were attributable to ingestion of HLAF because the changes were within the ranges historically observed in the age and strain of rats used in this study, did not demonstrate a dose-dependent relationship, were noted only sporadically, and/or were also observed in the control group. These observations are summarized below:

(a) A firm, round, subcutaneous lesion on the left side of the neck (cranial) area in one Group 2 female was first noted and palpated on Day 70 during cage-side observation. Monitored daily from Day 70 to Day 94 when the animal was terminated, the mass was collected for pathological evaluation and later identified as an adenocarcinoma of mammary gland origin. The lesion was an isolated finding limited to this one female. (b) A statistically significant decrease in food consumption was observed in Group 4 males on Days 14-21, 35-56, and 84-91 ($P < 0.05$ for all) and overall, Days 0-91 ($P < 0.05$) as compared to the control group. Because decreased consumption occurred during three discrete weeks out of 13 weeks, was minimal on an individual basis regarding the Day 0-91 assessment and was not accompanied by decreases in body weight or food efficiency, the finding was not adverse. (c) A statistically significant increase in mean food efficiency in Group 3 females on Days 21-28 ($P < 0.05$) and overall 0-91 Days ($P < 0.01$), compared to control females was not dose-related and was therefore considered not to be treatment-related. (d) A statistically significant decrease in mean daily food consumption was reported for Group 4 females compared to the control group ($P < 0.01$) on Days 63-70. This decrease was short term and, therefore, judged to be incidental and not related to the test substance. (e) A vitreal hemorrhage obscuring the optic nerve was reported in the right eye of a Group 1 (control) female on Day 90. The finding was interpreted to result from a sporadically occurring retinal vascular abnormality or hyaloid artery remnant. (f) Statistically significant increases in platelet concentration were noted for the females of Group 2 and Group 4 compared to the control group ($P < 0.05$ for all). Because these Day 91 hematology findings were not accompanied by any other clinical or histopathologic change and no dose-response relationship was demonstrated, the findings were judged to not be attributable to HLAF treatment. (g) A significant increase in the absolute reticulocytes in Group 3 females compared

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[53] PSL = Product Safety Labs

to the control group ($P < 0.05$) was not found to be toxicologically relevant because the change was sporadic, was not accompanied by any other clinical or histopathologic change and were not dose-dependent. (i) Clinical chemistry results from Day 91 revealed statistically significant increases in alkaline phosphatase in Group 2 and Group 3 males, decreased aspartate aminotransferase in Group 2 females, and an increase in total protein in Group 2 females compared to their respective control groups ($P < 0.05$ for all). These findings did not demonstrate a dose-response relationship, nor were they accompanied by corresponding clinical or histopathologic change; they were, therefore, judged not to be toxicologically relevant. (j) A firm, white lesion in the right kidney of a Group 4 male corresponded to a neoplasm having morphologic features consistent with a renal mesenchymal tumor. The tumor was isolated finding, limited to this one male. (k) The enlarged, pale, mottled thymus noted in a Group 2 male lacked any histologic correlate and was spontaneous in origin. (l) In comparison to controls, Group 3 male relative adrenal-, brain-, and testis-to-body weight ratios were significantly decreased, while the kidney-to-brain weight ratio was significantly increased ($P < 0.05$ for all). In Group 2 and 3 females, absolute liver weights, liver-to-body weight and liver-to-brain weight ratios were significantly increased when compared to the control group ($P < 0.01$ for all Group 2 parameters; $P < 0.05$ for Group 3). All changes were incidental and not toxicologically relevant as no change demonstrated a dose-dependent relationship or was found in the high-dose group.

In summary, although statistically significant effects were noted for several endpoints, these were sporadic and not toxicologically relevant. Under the conditions of this study, the NOAEL for HLAF in the diet is 100,000 ppm, the highest dietary concentration provided in the study, which corresponds to a dietary NOAEL of 4807 mg/kg bw/day in male rats and 5366 mg/kg bw/day in female rats.

Table 11. 90-Day subchronic repeated-dose toxicity study in rodents

Duration	Species (#/dose group)	Dose/Route	Results/Notes	Reference
13 weeks	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm HLAF in diet (equivalent to 0, 1249, 2478, and 4807 mg/kg bw/day, respectively in males and 0, 1413, 2739, and 5366 mg/kg bw/day, respectively, in females)	Dietary HLAF was well-tolerated; NOAEL was 4807 mg/kg bw/day in males and 5366 mg/kg bw/day in females; No adverse effects	Szabo *et al.* (2012).

F = Female; HLAF = High Lipid Algal Flour; M = Male; NOAEL = No Adverse Effect Level; ppm = parts *per* million; # = number

6.4. Other Studies

6.4.1. Pathogenicity in rodents

To assess potential pathogenicity of the source organism *C. protothecoides* S106 utilized in the production of HLAF, a study designed to monitor the possible survival or propagation of Microbial Pest Control Agents (MPCA) was conducted using the rat model (Solazyme Roquette

Nutritionals, 2012a). The in-life dosing and tissue collection portion of the study was based on US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 3a: *Short-Term Toxicity Studies with Rodents* (2003) and US EPA Health Effects Test Guidelines, OPPTS[54] 885.3050, Acute Oral Toxicity/Pathogenicity (1996); and conducted under GLP.[55] The tissue culture and analysis portion of the MPCA study was also conducted under GLP [56] in accordance with the EPA Microbial Pesticide Test Guidelines, OPPTS 885.3050, "Acute Oral Toxicity/ Pathogenicity", US Environmental Protection Agency, Prevention, Pesticides and Toxic Substances (7101). EPA 712-C-96-315, February 1996.

In order to evaluate whether or not *C. protothecoides* S106 microalgae could survive or propagate in the rat, 32 HSD:SD® rats (12 weeks old; mean weight ±20% for each sex) were randomly assigned to either the treatment (n = 12/sex) or vehicle control (n = 4/sex) group. Either live microalgal cells (~2.5 x 10^8 CFU[57]/rat) in 2 ml vehicle (Defined EBO2[58] minus Co^{2+} and nitrogen) or 2 ml vehicle alone was administered *via* oral gavage to each rat as an acute dose. All treatment animals (24 rats) and half of the vehicle control animals (2 males, 2 females) were housed in one room. The remaining vehicle control animals (2 males, 2 females) were housed in a separate room. All rats were provided 2016CM Harlan Teklad Global Rodent Diet® (Harlan Teklad, Inc., Indianapolis, IN) and filtered tap water *ad libitum*. All rats were monitored for viability twice *per* day with cage-side observations twice the first day and once *per* day thereafter. Body weights for control rats were recorded during acclimation and on Days 1, 3, 7, 14 and 21 with terminal weights recorded on Day 22. Body weights for all treatment rats were recorded during acclimation and on Day 1, with terminal weights recorded for the first subgroup (n = 3/sex) on Day 4. Body weights for the second subgroup (n = 3/sex) were recorded on Day 7 with terminal weights recorded on Day 8. Third subgroup body weights (n = 3/sex) were recorded on Days 7 and 14 with terminal weights recorded on Day 15. Fourth subgroup body weights (n = 3/sex) were recorded on Days 7, 14 and 21 with terminal weights recorded on Day 22. Tissue[59], blood (*i.e.*, EDTA[60]/citrate buffered blood and plasma), and fecal samples were collected over a period of three weeks post-dosing (Days 3 (feces only), 4, 8, 15, and 22), transferred (under controlled conditions of 2-8 °C) from PSL (Dayton, NJ) to BSL Bioservice (Planegg, Germany) for analysis.

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[54] OPPTS = Office of Prevention, Pesticides, and Toxic Substances
[55] US EPA GLP: Toxic Substances Control Act (TSCA): 40 CFR 792, 1989 and US FDA GLP: 21 CFR 58, 1987
[56] Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on June 20, 2002 (BGBl. I Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407); the OECD Principles of Good Laboratory Practice (as revised in 1997); and OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998
[57] CFU = Colony Forming Units
[58] Defined EB02 = A proprietary media formulation developed by Solazyme containing: 3 g/L K_2HPO_4, 5.66 g/L $Na_2HPO_4 \cdot 7H_2O$, 1.2 g/L citric acid monohydrate, 1 g/L $(NH_4)_2SO_4$, 0.23 g/L $MgSO_4 \cdot 7H_2O$, 0.03 g/L $CaCl_2 \cdot 2H_2O$, 1 mL trace elements minus Co^{2+}, 0.225 ml/L antifoam (Sigma 204), 1.5 ml DAS vitamins, glucose to 40 g/L. Defined EB02 minus Co^{2+} and nitrogen was prepared as above except ammonium sulfate was omitted.
[59] Brain, lung, liver, spleen, kidney, and lymph nodes
[60] EDTA = Ethylenediaminetetraacetic acid

No mortalities occurred during the in-life portion of the study. No effect in clinical signs, mean body weight or mean body weight gain were found in the treatment groups, compared to the control groups. In addition, no differences were identified in any monitored parameter between the control groups (housed with or isolated from the test group). Although two incidental findings (a small-appearing kidney in one control male and one treatment female) were reported at necropsy, no macroscopic differences were observed among the groups.

Tissue, blood, plasma, and fecal samples were analyzed for the presence of *C. protothecoides* cells using a surface spread method with plate counting.[61] All sample and control plates were prepared in triplicate with each plate being analyzed in duplicate. After incubation (3-8 days at 30±2 °C), all plates prepared from tissue, blood, and feces samples in addition to negative control (basal medium) and positive control plates (basal medium inoculated with 1:100, 1:1000 and 10,000 dilutions of 2-4 day old *C. protothecoides* S106 cultures) were analyzed by plate counting for CFU/plate of the microalgae. Plate counting determined that algal colony counts were below the limits of detection[62] for all treatment and control tissue, blood (except for unreadable plates from three control animal samples), plasma, and feces samples for all collection days. Of all plates prepared for analysis, one sample from each of three control animals (two female Day 22 heparinized bloods and one male Day 22 brain) could not be read due to overgrowth by molds and red-colony bacteria. Although the analyses of nearly all fecal samples[63] from all days were also affected by rapid overgrowth of molds, each of the fecal plates could still be analyzed and each reported an algal count below the limit of detection. No presence or growth of *C. protothecoides* S106 was observed on the negative control plates (< 1 CFU/ml negative control inoculum, equivalent to < 1 CFU/plate). The results of the positive controls, expressed as CFU/ml positive control inoculum, (equivalent to CFU/plate), were directly comparable to the CFU/mL of the 2-4 day *C. protothecoides* S106 cultures.

In summary, no adverse or toxic effects related to treatment was observed in any rat and, further, no viable count of the *C. protothecoides* S106 was detected in any examined tissues or fluids collected during a three-week post-treatment period to the administration of live algal culture. Under the conditions of this study, *C. protothecoides* S106 was not acutely toxic, pathogenic or toxigenic. This study is summarized in Table 12.

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[61] Prior to initiation of the pathogenicity study in the rats, the surface spread method was validated for quantification of *C. protothecoides* S106 in the spiked tissues (brain, lung, liver, spleen, kidney and lymph nodes), blood and feces of control animals (Solazyme, 2012b). The surface spread method is the method of choice for quantification of colony forming units of aerobic microorganisms (USP <61>).

[62] All limits of detection (LODs) were normalized to the volume (blood) or weights (tissues and feces) of the samples: <5 CFU/ml plasma and heparinized blood; <14 - < 20 CFU/g brain; <15 - <19 CFU/g lung; <14 - <19 CFU/g liver; <14 - <20 CFU/g spleen; <14 - <19 CFU/g kidney; <14 - <18 CFU/g lymph node; <14 - <31 CFU/g feces for 29 of 32 feces samples with the LOD for one sample at < 54 CFU/g, for a second at < 67 CFU/g, and for a third at < 115 CFU/g (all extremely small, highly diluted samples from Day 15).

[63] All treatment fecal samples from Days 4, 8, 15, and 22; seven out of eight control fecal samples from Day 22.

6.4.2. Chlordecone detoxification in rodents

The roles that three different algal strains (*C. protothecoides* 902[64], *P. zopfii* 822[65], and *C. vulgaris* 1206[66]) could play in the detoxification of chlordecone-poisoned rats were explored and compared by Pore *et al.* (1984) in a series of inter-related studies. In previous work (Conte and Pore, 1973; Pore, 1984), both the living cells and cell walls of *C. protothecoides* 902 had been shown to have a high binding affinity for chlordecone, similar to that of cholestyramine.[67] All animals in the current study were female SD rats (175-199 g; 43-51 days of age). All rats received 2.8-5.2 μCi of ^{14}C chlordecone[68] plus ~0.05 mg unlabeled chlordecone in corn oil *via* intraperitoneal injection on Day 0. For the initial ingestion study, a subset of the rats (n = 6) received a diet supplemented with 4 g/day (20-23 g/kg bw/day) of freeze-dried *C. protothecoides* 902 starting on Day 4 and continuing through Day 17; the corresponding control group (n = 6) received the control diet without algal supplementation. As determined by scintillation counting of feces (the primary means of chlordecone excretion) collected on Days 4-13, the half-life ($t_{½}$) of chlordecone in the rat was 19.0 days for the *C. protothecoides*-treated group and 35.5 days for the control animals. All rats in the initial study were sacrificed on Day 17. In confirmation of the feces-based findings, the residual ^{14}C-chlordecone radioactivity of abdominal adipose samples[69] from *C. protothecoides*-treated rats was significantly lower than radioactivity in the control group ($P < 0.01$).

In a series of shorter four-day studies, the $t_{½}$ of chlordecone in the rat (as determined by radioactivity in the feces) was 18.7 days for the *C. protothecoides*-treated group (n = 6), significantly less than the 40.3 days for the control group (n = 6) ($P < 0.01$). When *C. protothecoides* cell walls (isolated by hydrolysis in sulfuric acid from 4/g/day equivalent of *C. protothecoides* 902) were fed to chlordecone-treated rats (n = 6), the $t_{½}$ was 19.4 days, comparable to the freeze-dried intact cells. In comparison, neither administration (4 g/day) of intact *Prototheca zopfii* nor of intact *C. vulgaris* exhibited a similar effect on the chlordecone $t_{½}$ in the rat; measured half-lives (based on radioactivity of feces) were 32.7 days for *C. vulgaris* and 29.5 days for *P. zopfii*. The authors proposed the difference in chlordecone $t_{½}$ was due to the presence of relatively large amounts of sporopollenin[70] (2% of cell dry weight) in the outer trilaminar layer of *C. protothecoides* cell walls. No sporopollenin is present in the cell walls of *C. vulgaris*; intermediate amounts are present in *P. zopfii* cell walls. The authors did not report

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[64] 902 *Chlorella protothecoides* BTR (Biotech Research, Inc.) 902; reported depository entries includes ATCC 75667

[65] *Prototheca zopfii* Krüger (Cooke 1962/62-344); aka *P. stagnora* 62-344, and *P. moriformis*; depository designations include ATCC 16527 and UTEX 1442; ATCC Biosafety Level 1 is required for this organism.

[66] No additional strain information is available.

[67] Cholestyramine is an ion exchange resin and the primary material used to treat cases of chlordecone intoxication. Cholestyramine binds to chlordecone in the intestine (during primary poisoning or later when absorbed chlordecone is secreted back into the intestine) which prevents reabsorption and allows for elimination with the feces. Without assistance, chlordecone is neither detoxified by the body nor is it effectively eliminated.

[68] 10 mCi/mmol ^{14}C chlordecone

[69] Retro-kidney and mesentary

[70] Rare but natural oxidative carotenoid polymers that occur in a few microorganisms and plants

any adverse effects in the rats that were associated with consumption of living *C. protothecoides* 902 cells or isolated cell walls.

In summary, exposure of chlordecone-treated rats to either living cells or the cell walls of *C. protothecoides* 902 (20-23 g/kg bw/day) resulted in a significantly decreased chlordecone $t_{½}$ compared to the control group. An intermediate effect was observed when intact *P. zopfii* was consumed, but no effect occurred when rats ate intact *C. vulgaris*. The authors believed the reduction of ^{14}C-chlordecone was due to the high amounts of sporopollenin (~2% dry cell weight) in the *C. protothecoides* cell walls. *C. vulgaris* cell walls contained no sporopollenin and *P. zopfii* contained an intermediate amount. The authors did not report any adverse effects associated with consumption of the living cells or the cell walls of the algae. This study is summarized in Table 12.

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Table 12. Other studies in the rodent model

Method	Concentration	Subjects (#/group)	Results	Reference
Pathogenicity study, 21-d	0 or ≥ $2.5x10^8$ CFU live *C. protothecoides* S106 /rat in vehicle (Defined EB02 minus Co^{2+} and nitrogen) *via* oral gavage (Day 0); Treatment rats (3M,3F/ time point) sacrificed on Days 4, 8, 15 and 22; Control rats sacrificed on Day 22	Hsd:SD® rats (12M/12F treatment group; 4M/4F vehicle control group)	No mortalities; No effect on clinical signs, bw, bw gain or macroscopic examination at necropsy; Algal counts below the limit of detection in tissue, plasma, heparinized blood, and fecal samples; *C. protothecoides* S106 was not acutely toxic or pathogenic	Solazyme Roquette Nutritionals, (2012a)
Detoxification study, 17-d	0 or 4 g/d (0 or 20-23 g/kg bw/d) live *C. protothecoides* 902 in feed after rats received 2.8-5.2 μCi of ^{14}C chlordecone plus ~0.05 mg unlabeled chlordecone in corn oil *via* I.P. injection (Day 0)	Female SD rats (6/group)	Chlordecone $t_{½}$ in rats fed live 902 was significantly reduced (18.7-19.0 d), compared to the control group (35.5 d) ($P < 0.01$)	Pore *et al.* (1984)
Detoxification studies, 4-d	Live *P. zopfii* 822, live *C. vulgaris* 1206 or cell walls of *C. protothecoides* 902 in feed after rats received 2.8-5.2 μCi of ^{14}C chlordecone plus ~0.05 mg unlabeled chlordecone in corn oil *via* I.P. injection (Day 0)	Female SD rats (6/group)	Chlordecone $t_{½}$ in rats fed 902 cell walls (19.4 d) was comparable to $t_{½}$ in live-902 group; Chlordecone $t_{½}$ in rats fed live 822 or live 1206 were 29.5 and 32.7 d, respectively, and did not differ from the control. The authors did not report any adverse effects to consumption of live cells or cell walls.	Pore *et al.* (1984)

CFU = Colony Forming Units; Defined EBO = A proprietary media formulation developed by Solazyme containing: 3 g/L K_2HPO_4, 5.66 g/L $Na_2HPO_4 \cdot 7H_2O$, 1.2 g/L citric acid monohydrate, 1 g/L $(NH_4)_2SO_4$, 0.23 g/L $MgSO_4 \cdot 7H_2O$, 0.03 g/L $CaCl_2 \cdot 2H_2O$, 1 mL trace elements minus Co^{2+}, 0.225 ml/L antifoam (Sigma 204), 1.5 ml DAS vitamins, glucose to 40 g/L. Defined EB02 minus Co^{2+} and nitrogen was prepared as above except ammonium sulfate was omitted; F= female; I.P.= Intraperitoneal; M=Male; $t_{½}$ = half life

6.5. Genotoxicity

The mutagenic potential of HLAF was evaluated by bacterial reverse mutation assay (*i.e.* Ames test) using standard plate incorporation (Experiment I) and pre-incubation (Experiment II) methods with and without S9 metabolic activation[71] (Szabo *et al.*, 2012). Tester strains included *Salmonella typhimurium* TA100 and TA1535 and *Escherichia coli* WP2uvrA for detection of base-pair substitutions and *S. typhimurium* TA98 and TA1537 for detection of frame-shift mutations. The assays were performed in compliance with the international guidelines of (1) the Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial

[71] S9 metabolic activation = The supernatant fraction of rat liver homogenate (derived from male Wistar rats pretreated with the inducers phenobarbital and β-naphthoflavone) mixed with Cofactor-1.

Reverse Mutation Test", adopted July 21, 1997 and (2) Commission Regulation (EC) No. 440/2008 B.13/14, "Mutagenicity – Reverse Mutation Test using Bacteria", dated May 30, 2008. The assays were also conducted under GLP conditions.[55] Based on pre-experiments to evaluate cytotoxicity, 5000 µg/plate HLAF was selected as the highest dose for all test strains and conditions, except for TA 1537 in Experiment II without activation, where 1000 µg/plate was selected as the highest dose due to cytotoxic effects of the test substance. For the 5000 µg/plate series, five lower dose levels (2500, 1000, 316, 100 and 31.6 µg/plate) were prepared and tested; for the 1000 µg/plate series, six lower dose levels (316, 100, 31.6, 10.0, 3.16 and 1.00 µg/plate) were prepared. In the absence of activation, sodium azide served as positive control for *S. typhimurium* TA100 and TA1535, 4-nitro-*o*-phenylene-diamine for *S. typhimurium* TA98 and TA1537 and methylmethanesulfonate for *E. coli* WP2uvrA. In the presence of activation, the positive control for all bacterial strains was 2-aminoanthracene. The negative control for all tests was distilled water and the solvent control was dimethyl sulfoxide.

Precipitation of HLAF was visually observed in all test strains at concentrations of 2500 µg/plate and higher in Experiment I and II in the absence of S9 mix and in all test strains at a concentration of 5000 µg/plate in Experiment I and II in the presence of S9. In Experiment I cytotoxic effects of the test substance were observed in test strain TA 1537 at concentrations of 316 µg/plate and higher without metabolic activation and at concentrations of 2500 µg/plate with metabolic activation. In Experiment II these same effects were observed in test strains TA 98 and TA 100 at concentrations of 316 µg/plate and higher without metabolic activation and in test strain TA 1537 at concentrations of 10.0 µg/plate and higher without metabolic activation and concentrations of 316 µg/plate and higher with metabolic activation. Regardless of the presence or absence of metabolic activation, dose-dependent increases in the number of revertant colonies of greater than twice the negative control values were not observed in any strain treated with HLAF in either experiment. Under the conditions of this study, HLAF was not a mutagen.

The clastogenic potential of HLAF was evaluated in an *in vivo* bone marrow chromosome aberration test in the mouse (Szabo *et al*, 2012). The assays were performed under GLP conditions[55] and in compliance with international guidelines.[72] Prior to the main experiment, a range-finding study (OECD guideline 475) was used to determine the maximum tolerated dose (MTD) of HLAF. Due to a lack of observed toxicity, the MTD defaulted to 2000 mg/kg bw, the highest dose evaluated for the assay (in accordance with OECD guideline 475). This dose was then selected for use in the main study. Prepared in cottonseed oil one hour before treatment, HLAF at a dose of 2000 mg/kg bw was administered *via* gavage to ten male and ten female NMRI mice (7-13 weeks old) in a single application at study initiation. Negative control mice (n = 10/sex) were given the vehicle in similar volumes (10 ml/kg bw). Positive control animals (n = 5/sex) received a single dose of 40 mg/kg bw of cyclophosphamide in physiological saline *via* i.p. injection. Bone marrow cells were harvested 24 hours and 48 hours post-administration for the treatment and negative control groups (n = 5/sex/group/time) and 24 hours post-administration for the positive control group. After bone marrow cells were fixed and

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[72] The Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 475, "Mammalian Bone Marrow Chromosome Aberration Test'', adopted July 21, 1997 and Commission Regulation (EC) No. 440/2008 B.11, "Mutagenicity –Mammalian Bone Marrow Chromosome Aberration Test", dated May 30, 2008.

stained, 100 metaphases[73] were scored for cytogenic damage to determine the incidence of structural chromosomal aberrations (*i.e.*, breaks, fragments, deletion exchanges, chromosomal disintegrations and gaps). In addition, approximately 1000 cells were evaluated for cytotoxicity to determine the mitotic index (% of cells in mitosis). Although the positive controls (male and female groups) exhibited biologically and statistically significant increases in aberrant cell values ($P < 0.01$ for both *vs.* corresponding negative control), no dose dependent, biologically relevant or statistically significant increase in the incidence of aberrant cells or in the mitotic index was observed in the 24 hour- or 48 hour-dose groups compared to their corresponding negative control groups. The findings (aberrant cell values and mitotic indices) for all groups except for those of the positive control were in the range of the historic negative controls. Under the conditions of this study, HLAF was not clastogenic.

In summary, HLAF did not exhibit mutagenicity or clastogenicity under the conditions of, respectively, the bacterial reverse mutation and *in vivo* bone marrow chromosome aberration assays. The genotoxicity studies are summarized in Table 13.

Table 13. Genotoxicity studies

Assay	Test system	Concentrations	Results	Reference
Ames assay	*S. typhimurium* TA98, TA100, TA1535, and TA1537; and *E. coli* WP2uvrA	Experiment I (standard plate incorporation): Up to 5000 µg/plate in DMSO for all test strains and conditions (with and without S9 activation) Experiment II (pre-incubation): Up to 5000 µg/plate in DMSO for all test strains and conditions except for TA 1537 without S9 activation where 1000 µg/plate was highest dose due to cytotoxic effects.	Not mutagenic	Szabo *et al.* (2012).
In vivo bone marrow chromosome aberration assay	NMRI Mouse (10M/10F/group)	0 and 2000 mg/kg bw in cottonseed oil *via* gavage; single dose	Not clastogenic	Szabo *et al.* (2012).

DMSO = Dimethyl sulfoxide

6.6. Carcinogenesis

No studies addressing carcinogenicity were discovered in the scientific literature for *C. protothecoides* or *A. protothecoides* .

6.7. Observations in Humans

Although HLAF is not intended to be consumed as a standalone food, the ingredient has a slightly sweet, fatty taste with a creamy mouth-feel texture. When used at its intended levels in

[73] For animals that showed a distinct positive result in fewer than 100 metaphases, 50 metaphases were examined (in accordance with OECD Guideline 475).

food, taste of the final food is not adversely affected. In creamy products such as ice cream, HLAF provides a more indulgent mouth-feel.

6.7.1. Allergenicity Potential

A search of the scientific literature did not indicate any association of allergy or allergic response to *C. protothecoides* or to any *Auxenochlorella* or *Prototheca* species. Published studies of allergy instead have primarily been limited to *Chlorella* species[74] *C. vulgaris*, *C. pyrenoidosa*, *C. saccharophila* and *C. homosphaera* (Tiberg *et al.*, 1990a; Tiberg *et al.*, 1990b). Although allergy to *Chlorella* has generally presented as hypersensitization resulting from exposure to algae-infested waters or respiratory allergy to airborne[75] algae (Bernstein and Safferman, 1966; Bernstein and Safferman, 1973; Tiberg *et al.*, 1995; Chrisostomou *et al.*, 2009), there is a published case study of oral allergy in an 11-year-old boy (Yim *et al.*, 2007). Three months into a daily regimen of Chlorella food supplements (200 mg/tablet, 10 tablets/day), acute tubulointerstitial nephritis[76] was diagnosed in the child. Renal function improved with cessation of the *Chlorella*[77] supplements and a six-month regimen of corticosteroids. A follow-up skin prick test (100 mg and 200 mg *Chlorella* in 5 ml distilled water) six months after steroid therapy failed to produce positive wheal reactions for the 100 mg or 200 mg test amounts. In a previous study by Tiberg and Einarsson (1989), the allergenic potencies of eight strains within four species of *Chlorella* (*C. vulgaris*, C. *homosphaera*, C. *saccharophila*, and *C.fusca*) were compared in an attempt to define a representative extract preparation for the genus. Due to the highly variable allergenic activity of the eight strains, the authors concluded that development of a representative extract preparation for the genus was unlikely and that "selection of a strain is a crucial step for the production of an allergen extract from *Chlorella*."

The potential for the induction of an allergenic response from HLAF when consumed as a food has been investigated *via* dermal sensitization of human subjects (Szabo *et al*, 2012). The study design was in accordance with the applicable guidelines for the protection of human subjects for research as outlined in 21 CFR 50 and in accordance with the accepted standards for Good Clinical Practices (GCPs), International Conference on Harmonization (ICH Expert Working Group, 1996) and the standard practices of Thomas J. Stephens and Associates, Inc. (Carrollton, TX). Performance of a dermal patch test in humans as a means of predicting food allergy by the occurrence of contact dermatitis is a noninvasive approach that holds low risk for the safety of the participants. Written informed consent complying with 21 CFR §50.25 was obtained from each subject prior to study enrollment. A total of 130 healthy subjects enrolled in

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[74] When considering species identified in articles published prior to 2000, the possibility of reclassification is a concern especially for *C. vulgaris* and *C. pyrenoidosa* species. Verification against the culture collections of the depositories is recommended.

[75] Aerosolized or associated with house dust

[76] Tubulointerstitial nephritis is a common cause of acute renal failure from immune-mediated tubulointerstitial injury; hypersensitivity reactions can induce allergic response in the renal interstitium (Yim *et al.*, 2007).

[77] Composition of the tablets was not defined beyond the genus.

the Human Repeat Insult Patch test (HRIPT) for HLAF and 110 healthy human subjects[78] (28 men, 82 women; average subject age of 45.01 ± 13.01 years) completed the study.

The HRIPT developed by Rizer and Nozawa (Trookman *et al.*, 2011) consisted of two phases, an induction phase and a challenge phase. The three-week induction phase included nine patch periods. During each period, sufficient HLAF test material to cover the test site (approximately 2 cm^2 in area) was applied under a patch to healthy skin on the upper back of each subject. The patch was removed 48 hours after application or about two hours before a study visit. Induction sites were graded 48 to 72 hours after each application using a standardized skin irritation scale (Berger and Bowman, 1982). A minimum of 12 days after application of the last induction patch, challenge patches were applied to the original test site and to a naïve site, also on the upper back. These were removed 48 hours after application or about two hours before the study visit. Challenge sites were graded 48 hours and 96 hours post-application. Occlusive patches (non-woven cotton pads covered by Blenderm tape and held securely to skin on all sides with a porous, hypoallergenic tape) were used for the first two patch periods in the induction phase; semi-occlusive patches (non-woven cotton pads covered and held securely to skin on all sides with a porous, hypoallergenic tape) were used for all later patch periods in the induction phase and for the entire challenge phase.

Of the 130 subjects who enrolled, one was discontinued for noncompliance, 16 for missing more than one study visit, and one withdrew for a cardiac condition unrelated to the test material. Two additional subjects were withdrawn after grading of the first and second occlusive patches determined moderate site reactions to the test material. One subject was scored during the first grading *(i.e.*, evaluation of the first patching) as having severe erythema (very intense redness) with edema (swelling), bullae[79] and spreading at the application site; this subject withdrew from the study and the reaction was followed to resolution. The second subject was scored during the first grading as having severe edema with bullae at the application site. For this subject, an alternate site was used for the second patching and both the original and alternate sites were evaluated during the second grading. At the second grading, the original site scored "severe" for erythema with edema and papules[80] and the alternate site scored as "severe" edema with bullae; the subject was then withdrawn and the reactions were followed to resolution.

Because increasing instances of site irritation were broadly observed among the study participants at the second grading (37%; Table 14), semi-occlusive patches were substituted for occlusive patches for the remainder of the study. At the ninth (final) grading in the induction phase, the test sites of four participants (3.6%) exhibited a mild erythema[81] (faint to definite pink) while the test sites of 106 participants (96.4%) presented with no visible erythema. After the

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[78] The 110 subjects completing the study identified their ethnicity/race as follows: 11 African Americans (10.0%), 1 Asian (0.9%), 76 Caucasians (69.1%), 13 Hispanics (11.8%), 2 Native Americans (1.8%), 1 Pacific Islander (0.9%), and 6 subjects of mixed ethnicity (5.5%).

[79] Vesicles (small, circumscribed elevations having translucent surfaces so that fluid is visible (*i.e.*, blister-like)) with a diameter > 0.5 cm; vesicles may coalesce to form one or a few large blisters that fill the patch site.

[80] Small red solid elevations; when touched the affected surface has a granular feeling.

[81] Because redness can result from local irritation, mild erythema alone is not treated as potentially indicative of allergenic response.

change to semi-occlusive patching, no cumulative irritation was observed in the induction phase. The results of the challenge phase demonstrated that the test material did not induce an allergic response in the study group. At the 96-hour grading in the challenge phase, the original application sites of three subjects (2.7%) and naïve site of one subject (0.9%) exhibited mild erythema. No other subjects demonstrated irritation.

Regarding the two subjects withdrawn during the occlusive portion of the test (one after the first and one after the second patch period), the total evidence suggests that these individuals are more likely to have experienced irritation, rather than allergic response. Although pre-existing skin sensitization is strongly suggested by the rapidity (first of nine 48-h patch periods), severity (edema and papules/bullae), and persistence of the responses (3-4 weeks to resolve) in both subjects, neither of the responses was confirmed (*via* oral food challenge) to result from a true allergic response. Because erythema is known to result from local irritation (Cudowska and Kaczmarski, 2005), gradings of mild erythema (faint to definite pink) without infiltration are usually regarded as negative and a positive reaction is assessed only if erythema with infiltration or papules is present. Distinguishing irritation from allergy can, however, be challenging for test materials with inherent irritant potential (McNamee *et al.*, 2008).[82] The use of occlusive patching which is recognized to be an exaggerated exposure condition (and is frequently used for this very reason) would exacerbate irritant effects (McNamee *et al.*, 2008). In this study, the relatively new HLAF material was applied as a dry solid to subjects' backs under occlusive patching without dilution or extraction.[83] At the end of the second patch period (occlusive) more than one-third (37%) of study volunteers exhibited some degree of patch site irritation (*i.e.*, erythema) with three individuals also presenting with papules and a fourth with edema. The use of semi-occlusive patching during the remainder of the study was found to resolve nearly all irritation concerns in the 110 remaining subjects for the remaining weeks of the study (Table 14.). At the end of the induction phase, only four subjects presented with mild erythema at the application site. At the end of the challenge phase, three presented with mild erythema at the original site and only one at the alternative (naïve) site. As an additional consideration, while atopic patch tests (APTs) have been shown to generate negative predictive values of about 90%[84] and have been found particularly useful in predicting late phase (T-cell-mediated) reactions[85] (Cudowska and Kaczmarski, 2005; Chung *et al.*, 2010) positive predictive values for this APT have been substantially lower (50% and 57% compared to DBPCFC[86] for milk and egg allergy, respectively, (Chung *et al.*, 2010); 29% for milk, casein, egg white and yolk (Peron *et al.*, 2011)). A tendency for false positives (20% compared to DBPCFC for milk allergy in older children; (Cudowska and Kaczmarski, 2005) is a recognized limiting factor of APT.

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[82] Wheat gluten is an example of a test material suspected of inducing false positive (irritant) reactions (McNamee *et al.*, 2008; Turjanmaa *et al.*, 2006).

[83] When a test material is a potential irritant, dilution or extraction is recommended as a means of decreasing the incidence of false positive responses (Turjanmaa *et al.*, 2006; McNamee *et al.*, 2008)

[84] 95.9% and 88.9% compared to double-blind placebo-controlled food challenge (DBPCFC) for milk and egg allergy, respectively;(Chung *et al.*, 2010); and 90% for milk, casein, egg white and yolk; (Peron *et al.*, 2011).

[85] In comparison, skin prick tests (SPT) and food-specific IgE antibodies *via* serological assay are indicative of early (IgE-mediated) food allergy reaction (Cudowska and Kaczmarski, 2005).

[86] DBPCFC = Double-Blind Placebo-Controlled Food Challenge

In summary, although mild-moderate irritation was identified in several subjects, HLAF did not, under the conditions of this test, induce contact sensitization (allergic contact dermatitis) in any subject completing the study which suggests a low likelihood of food allergy.

Table 14. The score frequencies for the test material at each grading time point during the induction and challenge phases.

Score	*Induction Phase*									*Challenge Phase*			
	G1	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**G8**	**G9**	**48 O**	**96 O**	**48 A**	**96 A**
0	81	68	99	106	98	106	106	105	106	106	107	103	109
0P	0	0	1	0	3	0	0	0	0	0	0	0	0
0R	0	1	0	0	0	0	0	0	0	0	0	0	0
1	27	36	9	3	9	4	4	3	4	4	3	7	1
1P	2	3	1	1	0	0	0	2	0	0	0	0	0
2	0	1	0	0	0	0	0	0	0	0	0	0	0
2E	0	1	0	0	0	0	0	0	0	0	0	0	0
Total	110	110	110	110	110	110	110	110	110	110	110	110	110

0 = No visible erythema; **1** = Mild erythema (faint pink to definite pink); **2** = Moderate erythema (definite redness); 48 = 48-hour observation; 96 = 96-hour observation; A = Alternate (naïve) site; E = Edema; G = Grading; O = Original site; P = papules, R = Removal of the patch at other than the assigned time

6.7.2. Infection

Even though microalgae are ubiquitous organisms, algal infections are rare in humans and other mammals. Of those that do occur, the most common are protothecosis and chlorellosis, pseudofungal diseases caused, respectively, by opportunistic members of the achlorphyllous *Prototheca* spp. and green *Chlorella* spp. (Ramírez-Romero *et al.*, 2010). The two *Prototheca* species known to be disease agents are *P. wickerhamii*, the cause of most human protothecosis and *P. zopfii,* the usual causative agent in animals (Chandler *et al.*, 1978; Jagielski and Lagneau, 2007). Thus far, the *Chlorella* species responsible for incidents of chlorellosis have not yet been identified. Chlorellosis is usually differentiated from protothecosis by the color of lesions and/or affected organs. Protothecosis lesions tend to be ivory, white or cream-colored like the achlorophyllous causative organism; chlorellosis lesions, on the other hand, are generally bright green or emerald in color (Chandler *et al.*, 1978).

Protothecosis typically manifests in animals and humans as localized (*i.e.*, cutaneous or articular) or disseminated systemic infection.[87] In the cow, protothecosis is commonly diagnosed as the cause of bovine mastitis. Although infection in cats and goats is also usually localized and cutaneous, infection in dogs can be severe, involving the eyes, brain, and internal organs (Jagielski and Lagneau, 2007; Satoh *et al.*, 2010). Infections in humans usually occur from contamination of wounds or other breaks in the skin with dirty water. On a yearly basis, 2-5 cases of infection are typically reported, with 108 total cases of human protothecosis reported within a 25 year period (Krcméry Jr., 2000). Protothecosis in humans most commonly presents as skin infection (Torres *et al.*, 2003). Individuals who become ill with systemic infection are

[87] The infection of internal organs that often results in altered immune response.

nearly always debilitated or immunocompromised (Torres *et al.*, 2003; Jagielski and Lagneau, 2007; Ramírez-Romero *et al.*, 2010; Satoh *et al.*, 2010).

In comparison, chlorellosis has been reported in a variety of domestic and wild animals, including dogs, cattle, sheep, gazelle, dromedaries and beavers, but in only one human. The single reported incident of human infection presented as localized cutaneous lesions in surgical wounds exposed to river water (Ramírez-Romero *et al.*, 2010); the causative species was not identified. Although infection in the human patient was mild, chlorellosis can cause systemic disease in livestock (Chandler *et al.*, 1978; Ramírez-Romero *et al.*, 2010). Most bovine infections are mild (*i.e.*, infected lymph nodes found during slaughterhouse inspection); chlorellosis in sheep, however, is often enteric and frequently the cause of morbidity and mortality (Chandler *et al.*, 1978; Ramírez-Romero *et al.*, 2010). Although infection usually occurs through environmental contamination of open wounds, infection in animals has also been associated with drinking sewage water or stagnant water, or grazing pasture irrigated with untreated sewage water (Ramírez-Romero *et al.*, 2010).

In summary, one incident of chlorellosis has been documented in a human. The infection arose from the exposure of surgical wounds to contaminated water and was limited to cutaneous lesions. Because lesional organisms are rarely cultured for genotyping, there is no supportive evidence that *C. protothecoides* was or was not the opportunistic pathogen in the one human patient or in the animals that are occasionally infected. Because only one case of chlorellosis in humans was reported compared to 108 reported cases of human protothecosis, it is highly unlikely that *C. protothecoides* has or will be the cause of human disease.

6.7.3. Photosensitive dermatitis

In 1977, at least 23 residents of Tokyo were diagnosed with photosensitive dermatitis[88] (Tamura *et al.*, 1979; Jassby, 1988). Investigation revealed that each patient had consumed the same brand of *Chlorella* tablet ("Kenbi Chlorella") and, further, that the tablets contained PheideA and its ester in high quantities (up to 8.2 mg/g total PheideA; (Jassby, 1988)). A subsequent feeding trial in mice confirmed the association when administration of suspect tablets induced the phototoxic response and administration of *Chlorella* tablets having lower concentrations of Pheides (< 10% of suspect concentrations) did not. The researchers were also able to establish a linear dose-response relationship between inflammation severity and the Pheide content of the administered tablets (Jitsukawa *et al.*, 1984; Jassby, 1988).

Pheophorbide-associated phototoxicity occurs after ingested Pheides enter the circulatory system and distribute throughout the body. When Pheides that have deposited in the skin or other near-surface tissues are exposed to light, oxygen is generated and the fatty acids of nearby cell membranes are subsequently oxidized which leads to cell rupture, damage to dermal capillaries and escalating inflammation (Jitsukawa *et al.*, 1984; Jassby, 1988). A change in the manufacturing process (the use of ethanol during granule formation of the dried *Chlorella* prior to pelleting) was determined to have been the cause of the high PheideA concentrations

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[88] Skin inflammations characterized by rash and itchiness that develop on exposure to light. Usual presentation is cutaneous lesions on the face and dorsa (backs) of the hands. In severe cases, lesions necrotize and scar.

(Jitsukawa *et al.*, 1984) in the Tokyo incident. The concentrations of existing and potential total PheideA permitted in algae preparations were limited, respectively, to 0.8 mg/g and < 1.2 mg/g by the Japanese Public Health Ministry in 1981 (Jassby, 1988).

Because Pheides are natural degradation products of chlorophylls (Chls), they are present in all photosynthetic plants. In nature, Chl catabolites, including the phototoxic Pheides, typically increase in concentration during leaf senescence, fruit ripening or other incidents of "de-greening" (Jassby, 1988; Hörtensteiner *et al.*, 2000). During "de-greening" events, Pheides form from Chl through the step-wise loss of the phytol residue *via* chlorophyllase followed by the loss of the central magnesium atom *via* magnesium dechelatase (Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000). As mentioned previously, certain manufacturing steps can affect Pheide formation in Chl-containing microalgae products including the use of ethanol, acetone or methanol during processing which can enhance chlorophyllase activity and production of Pheides. On the other hand, heating Chl-containing materials to 100 °C for three minutes is sufficient to inactivate the chlorophyllase enzyme (Jassby, 1988). To ensure against Pheide formation in preparations of microalgae that contain Chl, enzyme inactivation is often combined with an avoidance of processing steps using ethanol, acetone or methanol (Jassby, 1988).

The mechanism of Chl breakdown has been extensively studied in *C. protothecoides* (Hörtensteiner *et al.*, 1998; Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000), the bleaching or de-greening of which under high glucose/nitrogen deficient conditions has been compared to the Chl catabolism of senescing plants (Hörtensteiner *et al.*, 2000), albeit with some substantial differences:

(1) Higher plants contain exclusively Chl-a. Although Chl-a is the major Chl in all algae; species within Division Chlorophyta, such as *C. protothecoides* also contain Chl-b (Becker, 1994).
(2) Because *C. protothecoides* contains both Chl-a and Chl-b, colored catabolites of Chl degradation form for both Chls (Hörtensteiner *et al.*, 1998).
(3) In higher plant species the majority of intermediate and final Chl catabolites are colorless, but in *C. protothecoides*, the main intermediate catabolites are the green-colored Pheides (PheideA and PheideB) with the final catabolites being the red-colored Red Chl Catabolites (RCC-a and RCC–b) (Hörtensteiner *et al.*, 1998; Hörtensteiner, 1999).
(4) After rapid formation from the green-colored Pheides in a two-step ring-opening of the porphyrin macrocycle (Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000), algal RCCs are excreted into the medium. In higher plants, RCC-a (there is no RCC-b) is further degraded into a variety of Fluorescent and Nonfluorescent Chlorophyll Catabolites (FCCs and NCCs) (Hörtensteiner, 1999) which are then deposited into the vaucoles of senescent mesophyll cells (Hörtensteiner *et al.*, 1998).

Ordinary commercial *Chlorella* products generally contain 2-3% dry weight Chl content (Jassby, 1988). *C. protothecoides* Krüger ACC[89] 25, however, has been shown to have no detectable Chl and diminished chloroplast structures[90] when "glucose-bleached" in high glucose/low nitrogen medium (Grant and Hommersand, 1974). HLAF is generated from *C. protothecoides* S106, a strain that has been shown by Solazyme to be identical to *C. protothecoides* UTEX 25 which is also known as *C. protothecoides* Krüger ACC NO. 25.[91] After growth in (and isolation from) the nitrogen-limited medium of the Solazyme manufacturing process, Chl is not present and chloroplasts are minimized in the algae and subsequent HLAF ingredient.

7. EVALUATION

HLAF is a high lipid flour composed of the dried milled biomass of the microalgae *Chlorella protothecoides* S106. HLAF can be used as a substitute for cream, milk, eggs/egg yolks, and/or butter/shortening in baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups.

HLAF is stable at ambient temperatures (23 ± 3 °C) for up to nine months, up to 12 months at -20 °C, and up to one month when stored at 40 °C.

Although statistically significant effects were noted for several endpoints in a published 28-day short-term repeated-dose study in rats, these were not found to be related to administration of a ground yellow high-lipid *C. protothecoides* biomass material. Under the conditions of the study, the NOAEL for the biomass material in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats. In support of these findings, an unpublished 28-day short-term repeated-dose study in hamsters demonstrated that consumption of *C. protothecoides* at up to ~6000 mg/kg bw/day was safe.

Although statistically significant effects were noted for several endpoints in a published 13-week subchronic toxicity study in rats, these were sporadic, not toxicologically relevant, and not test article-related. Under the conditions of this study, the NOAEL for HLAF in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 4807 mg/kg bw/day in male rats and 5366 mg/kg bw/day in female rats.

No viable counts of *C. protothecoides* S106 were detected in any of the examined tissues from a three-week pathogenicity study in which rats were acutely dosed with live *C. protothecoides* S106 (> 2.5×10^8 CFU/rat). Under the conditions of this study, *C. protothecoides* S106 was not pathogenic.

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[89] Algal Culture Collection at Indiana University, Bloomington, IN.

[90] As measured by absorbance, Chl returns to normal levels within 48 hr of providing a "greening" medium (little/no glucose, high nitrogen) (Grant and Hommersand, 1974).

[91] UTEX 25 was initially designated as ACC No. 25 when the collection was held by Indiana University. In 1976 the ACC at Indiana University was transferred to the University of Texas and became the core of the UTEX collection. <http://www.sbs.utexas.edu/utex/insideUtex.aspx>; site accessed December 12, 2011.

In a study exploring the effect of consuming live cells or the isolated cell walls of *C. protothecoides* 902 (20-23 g/kg bw/day) on the $t_{½}$ of chlordecone in C^{14}-chlordecone-treated rats, no adverse effects associated with consumption of the living cells or the cell walls of the algae were reported by the authors.

The results of the bacterial reverse mutation and *in vivo* bone marrow chromosome aberration assays indicate that HLAF is neither mutagenic nor clastogenic.

Under the conditions of the HRIP test in healthy male and female human subjects, HLAF did not induce contact sensitization (allergic contact dermatitis) in any subject completing the study, although mild-moderate irritation was identified in several subjects. The results of the study therefore indicate a low likelihood of food allergy.

Human infection with *C. protothecoides* is extremely unlikely. Only one case of human chlorellosis has been documented in the literature and this infection was localized and cutaneous, the result of surgical wounds exposed to contaminated water.

Due to the use of nitrogen-limited medium in the Solazyme manufacturing process, *C. protothecoides* S106 cells are effectively de-greened. Assay of Solazyme's ground yellow high-lipid *C. protothecoides* biomass reported PheideA concentrations below the detection limit of 0.5 ppm, concentrations that are well below the permitted limit of 0.8 mg/g PheideA in algal preparations imposed by the Japanese Public Health Ministry. The results of the conducted assay indicate that insufficient PheideA is present in HLAF for photosensitivity to occur.

The NOAEL for HLAF in a 13-week subchronic toxicity study in male and female rats was 100,000 ppm, the highest dietary concentration provided. The dietary NOAEL for HLAF in male and female rats was 4807 and 5366 mg/kg bw/day, respectively (equivalent to 288,408 mg/day in a 60 kg human).

The estimated daily intake of HLAF is based on the 90th percentile HLAF consumption levels from foods supplemented with HLAF (10.52 g/day). The estimated daily intake of HLAF when added to foods was determined to be 10.52 g/day. Thus, the potential theoretical maximum HLAF consumption at the 90th percentile may reach 10.52 mg/day. This theoretical intake level represents a conservative estimate because it is unlikely that an individual would consume HLAF from conventional foods at the 90th percentile level.

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8. CERTIFICATION

The undersigned authors of this document—a dossier in support of GRAS status determination for use of HLAF—hereby certify that, to the best of their knowledge and belief, this document is a complete and balanced representation of all available information, favorable as well as unfavorable, known by the authors to be relevant to evaluation of the substance described herein.

(b) (6)

24 JUL 2012

Nancy J. Szabo, Ph.D.
Senior Staff Toxicologist

Date

(b) (6)

24 July 2012

George A. Burdock, Ph.D.
Diplomate, American Board of Toxicology,
Fellow, American College of Nutrition
President, Burdock Group

Date

9. CONCLUSION

After critically evaluating the information available, as detailed within this dossier, the Expert Panel has determined that, based on common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food, there is reasonable certainty that HLAF, produced in accordance with current Good Manufacturing Practice (cGMP) and meets the defined specifications, is safe under the intended conditions of use, and is therefore Generally Recognized As Safe (GRAS), by scientific procedures, when used as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups, so that total daily consumption of HLAF from all sources is up to 10.52 g HLAF/ day for 90th percentile consumers. In particular, the Expert Panel has evaluated the proposed use of HLAF at specified levels in the foods listed in Appendix 2 of this document and has concluded that such use results in an estimated intake that is below 10.52 g/day HLAF and is therefore Generally Recognized As Safe (GRAS).

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

10. SIGNATURES

(b) (6)

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

23 July 2012
Date

(b) (6)

Eric A. Johnson, Ph.D.
Professor
Department of Bacteriology
Food Research Institute
University of Wisconsin
Madison, Wisconsin

July 18, 2012
Date

(b) (6)

Gary M. Williams, M.D.
Professor of Pathology,
Department of Pathology and Clinical Public Health
New York Medical College
Valhalla, New York

19 July 2012
Date

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12. APPENDIX 1

Appendix 1. Materials used in the growth medium, fermentation process, recovery process, and antioxidants in the manufacture of HLAF

Appendix 1 redacted at the request of FDA (Conference call dated August 29, 2012, regarding findings of initial review)

Appendix 1 redacted at the request of FDA
(Conference call dated August 29, 2012,
regarding findings of initial review)

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(Conference call dated August 29, 2012,
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regarding findings of initial review)

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(Conference call dated August 29, 2012,
regarding findings of initial review)

13. APPENDIX 2

Food Product List	Parts *per* million (ppm)
BISCUIT MIX, DRY	50000
BISCUIT, BAKING POWDER OR BUTTERMILK TYPE, NFS	50000
BISCUIT DOUGH, RAW	50000
BISCUIT DOUGH, FRIED	50000
BISCUIT, BAKING POWDER OR BUTTERMILK, FROM MIX	50000
BISCUIT,BAKING PWR/BUTTER MILK,REFRIG DOUGH,LOWFAT	50000
BISCUIT, BAK POWDER OR BUTTERMILK, FROM REFRG DOUGH	50000
BISCUIT, BAKING POWDER/BUTTERMILK TYPE, COMMERCIALLY BAKED	50000
BISCUIT, BAKING POWDER OR BUTTERMILK, HOMEMADE	50000
BISCUIT, WHOLE WHEAT	50000
BISCUIT, CHEESE	50000
BISCUIT, CINNAMON-RAISIN	50000
PANCAKES, PLAIN (INCLUDE PANCAKES, NFS)	30000
PANCAKES, REDUCED CALORIE, HIGH FIBER	30000
PANCAKES, W/ FRUIT (INCLUDE BLUEBERRY PANCAKES)	30000
PANCAKES W/ CHOC CHIPS	30000
PANCAKES, BUCKWHEAT	30000
PANCAKES, CORNMEAL	30000
PANCAKES, WHOLE WHEAT	30000
PANCAKES, SOURDOUGH	30000
PANCAKES, RYE	30000
FLOUR & WATER PATTY (INCLUDE CHINESE PANCAKE)	30000
WAFFLE, PLAIN	30000
WAFFLE, WHEAT, BRAN, OR MULTIGRAIN	30000
WAFFLE, FRUIT	30000
WAFFLE, NUT & HONEY (INCL EGGO)	30000
WAFFLE, CORNMEAL	30000
WAFFLE, 100% WHOLE WHEAT OR 100% WHOLE GRAIN	30000
WAFFLE, OAT BRAN	30000
WAFFLE, MULTI-BRAN (INCLUDE EGGO NUTRIGRAIN)	30000
WAFFLE, PLAIN, FAT FREE	30000
WAFFLE, PLAIN, LOWFAT	30000
FRENCH TOAST, PLAIN (INCLUDE ROMAN MEAL)	30000
FRENCH TOAST STICKS, PLAIN	30000
BREAD FRITTERS, P.R. (TORREJAS, GALICIAN FRITTERS)	30000
CREPE, PLAIN (INCLUDE FRENCH PANCAKE)	30000
COOKIE, BROWNIE, NS AS TO ICING	50000
COOKIE, BROWNIE, W/O ICING	50000
COOKIE, BROWNIE, W/ ICING	50000
COOKIE, BROWNIE, W/ CREAM CHEESE FILLING, W/O ICING	50000
COOKIE, BROWNIE, W/ PEANUT BUTTER FUDGE ICING	50000
BROWNIE, DIET, NS AS TO ICING	50000

Food Product List	Parts *per* million (ppm)
COOKIE, BROWNIE, LOWFAT, W/ ICING	50000
COOKIE, BROWNIE, LOWFAT, W/O ICING	50000
COOKIE, BROWNIE, FAT FREE, NO CHOL, W/ ICING	50000
COOKIE, BROWNIE, FAT FREE, W/O ICING	50000
COOKIE, BUTTERSCOTCH, BROWNIE	50000
COOKIE, CAROB & HONEY BROWNIE	50000
CAKE, BATTER, CHOCOLATE, RAW	50000
CAKE, BATTER, RAW, NOT CHOCOLATE	50000
CAKE, NS AS TO TYPE, W/ OR W/O ICING	50000
CAKE, ANGEL FOOD, NS AS TO ICING	50000
CAKE, ANGEL FOOD, W/O ICING	50000
CAKE, ANGEL FOOD, W/ ICING	50000
CAKE, ANGEL FOOD, W/ FRUIT & ICING/FILLING	50000
CAKE, ANGEL FOOD, CHOC, WITHOUT ICING	50000
CAKE, APPLESAUCE, NS AS TO ICING	50000
CAKE, APPLESAUCE W/O ICING	50000
CAKE, APPLESAUCE W/ ICING	50000
CAKE, APPLESAUCE, DIET, WITHOUT ICING	50000
CAKE, BANANA, NS AS TO ICING	50000
CAKE, BANANA, W/O ICING	50000
CAKE, BANANA, W/ ICING	50000
CAKE, BLACK FOREST (CHOC-CHERRY)	50000
CAKE, BOSTON CREAM PIE	50000
CAKE, BUTTER, NS AS TO ICING	50000
CAKE, BUTTER, W/O ICING	50000
CAKE, BUTTER, W/ ICING	50000
CAKE, CARROT, NS AS TO ICING	50000
CAKE, CARROT, NO ICING	50000
CAKE, CARROT, W/ ICING	50000
CARROT CAKE, DIET	50000
CAKE, COCONUT, W/ ICING	50000
CHEESECAKE	50000
CHEESECAKE, DIET	50000
CHEESECAKE, W/ FRUIT	50000
CHEESECAKE, DIET, WITH FRUIT	50000
CHEESECAKE-TYPE DESSERT, MADE W/ YOGURT, W/ FRUIT	50000
CHEESECAKE, CHOCOLATE	50000
CHEESECAKE, CHOCOLATE, REDUCED FAT	50000
CAKE, CHOC, MADE W/ MAYONNAISE, NS AS TO ICING	50000
CAKE, CHOC, MADE W/ MAYONNAISE, W/O ICING	50000
CAKE, CHOC, MADE W/ MAYONNAISE, W/ ICING OR FILLING	50000
CAKE,CHOC,DEVIL'S FOOD/FUDGE,STNDRD MIX,NS ICING	50000
CAKE, CHOC, DEVIL'S FOOD/FUDGE, HOMEMADE, NS ICING	50000

Food Product List	Parts *per* million (ppm)
CAKE,CHOC,DEVILS FOOD/FUDGE,STNDRD MIX, W/O ICING	50000
CAKE,CHOC,DEVIL'S FOOD/FUDGE, W/O ICING, HOMEMADE	50000
CAKE,CHOC,DEVIL'S FOOD/FUDGE,STNDRD MIX,W/ICING	50000
CAKE,CHOC,DEVIL'S FOOD/FUDGE,W/ICING, HOMEMADE	50000
CAKE, GERMAN CHOC, W/ ICING AND FILLING	50000
CAKE, CHOC, W/ ICING, DIET	50000
CAKE,CHOC/DVL FD, PUDD MIX, LITE RECIPE, W/ ICING	50000
CAKE,CHOC,DEVIL FOOD/FUDGE,PUD TYPE.NO CHOL,NS ICIN	50000
CAKE,CHOC,DEVIL FOOD/FUDGE,PUD TYPE,NO CHOL,NO ICIN	50000
CAKE,CHOC,DEVIL FOOD/FUDGE,PUD TYPE,NO CHOL,	50000
CAKE, CHOC,DEVIL'S FOOD/FUDGE,PUDDING MIX, NS ICING	50000
CAKE,CHOC,DEVIL'S FOOD/FUDGE,PUDDING MIX, W/O ICING	50000
CAKE, CHOC,DEVIL'S FOOD/FUDGE, PUDDING MIX, W/ICING	50000
CAKE, POOR MAN'S (SPICE-TYPE), W/O ICING	50000
CAKE, CREAM, W/O ICING OR TOPPING	50000
CAKE, CUPCAKE, NS AS TO TYPE AND ICING	50000
CAKE, CUPCAKE, NS AS TO TYPE, W/O ICING	50000
CAKE, CUPCAKE, NS AS TO TYPE, W/ICING	50000
CAKE, CUPCAKE, CHOCOLATE, NS AS TO ICING	50000
CAKE, CUPCAKE, CHOCOLATE, W/O ICING	50000
CAKE, CUPCAKE, CHOCOLATE, W/ ICING OR FILLING	50000
CAKE, CUPCAKE, NOT CHOCOLATE, NS AS TO ICING	50000
CAKE, CUPCAKE, NOT CHOCOLATE, W/O ICING OR FILLING	50000
CAKE, CUPCAKE, NOT CHOCOLATE, W/ ICING OR FILLING	50000
CAKE, CUPCAKE, NOT CHOC, W/ ICING, LOWFAT, NO CHOL	50000
CUPCAKE, NOT CHOCOLATE, W/ FRUIT & CREAM FILLING	50000
CUPCAKE,CHOC,W/ORW/O ICING,FRUIT/CREAM FILL,LOWFAT	50000
CAKE,DOBOS TORTE(NON-CHOC CAKE W/CHOC FILL & ICING)	50000
CAKE, FRUITCAKE, LIGHT/DARK, HOLIDAY TYPE CAKE	50000
CAKE, PLUM PUDDING	50000
CAKE, GINGERBREAD, W/O ICING	50000
CAKE, GRAHAM CRACKER, W/O ICING	50000
CAKE, ICE CREAM & CAKE ROLL, CHOCOLATE	50000
CAKE, ICE CREAM & CAKE ROLL, NOT CHOCOLATE	50000
CAKE,FROZEN YOGURT & CAKE LAYER,NOT CHOC,W/ ICING	50000
CAKE,FROZEN YOGURT & CAKE LAYER,CHOCOLATE,W/ ICING	50000
CAKE, ICE BOX, W/ FRUIT & WHIPPED CREAM	50000
CAKE, JELLY ROLL	50000
CAKE, LEMON, NS AS TO ICING	50000
CAKE, LEMON, W/O ICING	50000
CAKE, LEMON, W/ ICING	50000
CAKE, LEMON, LOW FAT, NS AS TO ICING	50000
CAKE, LEMON, LOW FAT, W/O ICING	50000

Food Product List	Parts *per* million (ppm)
CAKE, LEMON, LOW FAT, W/ ICING	50000
CAKE, MARBLE, NS AS TO ICING	50000
CAKE, MARBLE, W/O ICING	50000
CAKE, MARBLE, W/ ICING	50000
CAKE, NUT, NS AS TO ICING	50000
CAKE, NUT, W/O ICING	50000
CAKE, NUT, W/ ICING	50000
CAKE, OATMEAL, W/O ICING	50000
CAKE, OATMEAL, W/ICING	50000
CAKE, PEANUT BUTTER, WITH ICING	50000
CAKE,PINEAPPLE,FAT FREE,W/ ICING	50000
CAKE, POPPYSEED, W/O ICING	50000
CAKE, POUND, W/O ICING	50000
CAKE, POUND, W/ ICING	50000
CAKE, POUND, CHOCOLATE	50000
CAKE, POUND, CHOC, FAT FREE, NO CHOL	50000
CAKE, POUND, P.R. (PONQUE)	50000
CAKE, POUND, FAT FREE, NO CHOL	50000
CAKE, POUND, REDUCED FAT, NO CHOLESTEROL	50000
CAKE,PUMPKIN,NS AS TO ICING	50000
CAKE, PUMPKIN, W/O ICING	50000
CAKE,PUMPKIN,W/ ICING	50000
CAKE, RAISIN-NUT, W/O ICING	50000
CAKE, RAISIN-NUT, W/ ICING	50000
CAKE, RAVANI (MADE W/ FARINA)	50000
CAKE, RICE FLOUR, W/O ICING	50000
CAKE, QUEZADILLA, EL SALVADORIAN STYLE	50000
CAKE, SOY FLOUR, W/O ICING	50000
CAKE, SPICE, NS AS TO ICING	50000
CAKE, SPICE, W/O ICING	50000
CAKE, SPICE, W/ ICING	50000
CAKE, SPONGE, NS AS TO ICING	50000
CAKE, SPONGE, W/O ICING	50000
CAKE, SPONGE, W/ ICING	50000
CAKE, SPONGE, CHOCOLATE, W/O ICING	50000
CAKE, SPONGE, CHOCOLATE, W/ ICING	50000
CAKE, SWEETPOTATO, W/ ICING (INCL WITH GLAZE)	50000
CAKE, RUM FLAVORED W/O ICING	50000
CAKE, TORTE	50000
CAKE, CHIFFON, NS AS TO ICING	50000
CAKE, CHIFFON, W/O ICING	50000
CAKE, CHIFFON, W/ ICING	50000
CAKE, CHIFFON, CHOCOLATE, W/O ICING	50000

Food Product List	Parts *per* million (ppm)
CAKE, CHIFFON, CHOCOLATE, W/ ICING	50000
CAKE, UPSIDE DOWN (ALL FRUITS)	50000
CAKE, WHITE, STANDARD MIX, NS AS TO ICING	50000
CAKE, WHITE, HOMEMADE, NS AS TO ICING	50000
CAKE, WHITE, STANDARD MIX, W/O ICING	50000
CAKE, WHITE, W/O ICING, HOMEMADE	50000
CAKE, WHITE, STANDARD MIX, W/ ICING	50000
CAKE, WHITE, W/ ICING, HOMEMADE	50000
CAKE, WHITE, PUDDING-TYPE MIX, NS AS TO ICING	50000
CAKE, WHITE, PUDDING-TYPE MIX, W/O ICING	50000
CAKE, WHITE, PUDDING-TYPE MIX, W/ ICING	50000
CAKE, WHITE, EGGLESS, LOWFAT	50000
CAKE, WHOLE WHEAT, W/ FRUIT & NUTS, W/O ICING	50000
CAKE, YELLOW, STANDARD MIX, NS AS TO ICING	50000
CAKE, YELLOW, HOMEMADE, NS AS TO ICING	50000
CAKE, YELLOW, STANDARD MIX, W/O ICING	50000
CAKE, YELLOW, W/O ICING, HOMEMADE	50000
CAKE, YELLOW, STANDARD MIX, W/ ICING	50000
CAKE, YELLOW, W/ ICING, HOMEMADE	50000
CAKE, YELLOW, PUDDING MIX, NS AS TO ICING	50000
CAKE, YELLOW, PUDDING MIX, W/O ICING	50000
CAKE, YELLOW, PUDDING MIX, W/ ICING	50000
CAKE, SHORTCAKE, BISCUIT, W/ WHIPPED CREAM & FRUIT	50000
CAKE, SHORTCAKE, BISCUIT, W/ FRUIT	50000
CAKE, SHORTCAKE, SPONGE, W/ WHIPPED CREAM & FRUIT	50000
CAKE, SHORTCAKE, SPONGE, W/ FRUIT	50000
CAKE, SHORTCAKE, W/ WHIP TOPPING & FRUIT, DIET	50000
CAKE, ZUCCHINI, NS AS TO ICING	50000
CAKE, ZUCCHINI, W/O ICING	50000
COFFEE CAKE, NFS	50000
COFFEE CAKE, CRUMB OR QUICK-BREAD TYPE	50000
COFFEE CAKE,CRUMB/QUICK BREAD TYPE,RED FAT,NO CHOL	50000
COFFEE CAKE, CRUMB OR QUICK-BREAD TYPE, W/ FRUIT	50000
COFFEECAKE, CRUMB OR QUICK-BREAD TYPE, CHEESE FILLD	50000
COFFEE CAKE, QUICK-BREAD TYPE, CUSTARD FILLED	50000
CAKE MADE W/ GLUTINOUS RICE	50000
CAKE OR PANCAKE MADE W/ RICE FLOUR &/OR DRIED BEANS	50000
CAKE MADE W/ GLUTINOUS RICE & DRIED BEANS	50000
FUNNEL CAKE	50000
COOKIE, BATTER / DOUGH, RAW, NOT CHOCOLATE	50000
COOKIE, NS AS TO TYPE	50000
COOKIE, ALMOND	50000
COOKIE, APPLESAUCE	50000

Food Product List	Parts *per* million (ppm)
COOKIE, BISCOTTI	50000
COOKIE, BUTTERSCOTCH CHIP	50000
COOKIE, CARAMEL COATED, W/ NUTS	50000
COOKIE, CAROB	50000
COOKIE, CHOCOLATE CHIP	50000
COOKIE, CHOCOLATE CHIP W/ RAISINS	50000
COOKIE, CHOC CHIP, HOMEMADE OR PURCHASED AT BAKERY	50000
COOKIE, CHOC CHIP, REDUCED FAT	50000
COOKIE,RICH,CHOCOLATE CHIP,W/ CHOCOLATE FILLING	50000
COOKIE, CHOCOLATE CHIP SANDWICH	50000
COOKIE, CHOCOLATE, MADE WITH RICE CEREAL	50000
COOKIE, CHOCOLATE, MADE W/ OATMEAL & COCONUT	50000
COOKIE, CHOCOLATE FUDGE	50000
COOKIE, CHOCOLATE, W/ CHOC FILLING/COATING, FAT FREE	50000
COOKIE, CHOCOLATE-COVERED, MARSHMALLOW	50000
COOKIE, CHOCOLATE-COVERED, MARSHMALLOW PIE	50000
COOKIE, CHOC,CHOC SANDWICH/CHOCOLATE-COATED/STRIPED	50000
COOKIE,CHOC-COVER,SUGAR WAFFER,CREME/CARAMEL FILLED	50000
COOKIE, CHOCOLATE SANDWICH, REDUCED FAT	50000
COOKIE, CHOCOLATE SANDWICH, CHOCOLATE COVERED	50000
COOKIE, CHOCOLATE, SANDWICH, W/ EXTRA FILLING	50000
COOKIE, CHOCOLATE & VANILLA SANDWICH	50000
COOKIE, CHOCOLATE WAFER	50000
COOKIE, GRAHAM CRACKER SANDWICH W/CHOC&MARSHMELLOW	50000
COOKIE, GRAHAM CRAX W/ MARSHMALLOW	50000
COOKIE, BAR, W/ CHOCOLATE, NUTS, & GRAHAM CRACKERS	50000
COOKIE, COCONUT	50000
COOKIE, COCONUT & NUT	50000
COOKIE, FRUIT-FILLED	50000
COOKIE, FRUIT-FILLED BAR, FAT FREE	50000
COOKIE, DATE BAR	50000
COOKIE, FIG BAR	50000
COOKIE, FIG BAR, FAT FREE	50000
COOKIE, FORTUNE	50000
COOKIE, CONE SHELL, ICE CREAM TYPE,WAFER / CAKE	50000
COOKIE, CONE SHELL, ICE CREAM TYPE, BROWN SUGAR	50000
COOKIE, GINGERSNAPS	50000
COOKIE, GRANOLA	50000
COOKIE, LADY FINGER	50000
COOKIE, LEMON BAR	50000
COOKIE, MACAROON	50000
COOKIE, MARSHMALLOW, W/ COCONUT	50000
COOKIE, MARSHMALLOW, W/ RICE CEREAL (NO-BAKE)	50000

Food Product List	Parts *per* million (ppm)
COOKIE, MARSHMALLOW, W/ RICE CEREAL & CHOC CHIPS	50000
COOKIE, MARSHMALLOW & PEANUT BUTTER, W/ OAT CEREAL (NO-BAKE)	50000
COOKIE, MARSHMALLOW PIE, NONCHOCOLATE COATING	50000
COOKIE, MERINGUE	50000
COOKIE, MOLASSES	50000
COOKIE, LEBKUCHEN	50000
COOKIE, MULTIGRAIN, HIGH FIBER	50000
COOKIE, OATMEAL	50000
COOKIE, OATMEAL, W/ RAISINS OR DATES	50000
COOKIE, OATMEAL, W/ FRUIT FILLING	50000
COOKIE,OATMEAL,FAT FREE,W/ RAISINS(INCL W/ DATES)	50000
COOKIE, OATMEAL, RED FAT, W/ RAISINS	50000
COOKIE, OATMEAL SANDWICH, W/ CREME FILLING	50000
COOKIE, OATMEAL, W/ CHOCOLATE CHIPS	50000
COOKIE, OATMEAL SANDWICH, W/ PEANUT BUTTER & JELLY FILLING	50000
COOKIE,OATMEAL,W/ CHOC & PEANUT BUTTER (NO-BAKE)	50000
COOKIE, OAT BRAN	50000
COOKIE, PEANUT BUTTER (INCLUDE PB WAFER)	50000
COOKIE, PEANUT BUTTER W/ OATMEAL	50000
COOKIE, PEANUT BUTTER, W/ CHOCOLATE (INCL NASSAU)	50000
COOKIE, PEANUT BUTTER W/ RICE CEREAl (NO-BAKE)	50000
COOKIE, PEANUT	50000
COOKIE, W/ PEANUT BUTTER FILLING, CHOCOLATE-COATED	50000
COOKIE, PFEFFERNUSSE	50000
COOKIE, PIZZELLE (ITALIAN STYLE WAFER)	50000
COOKIE, PUMPKIN	50000
COOKIE, RAISIN	50000
COOKIE, RAISIN SANDWICH, CREAM-FILLED	50000
COOKIE, RUM BALL (NO-BAKE)	50000
COOKIE, SANDWICH TYPE, NOT CHOCOLATE OR VANILLA	50000
COOKIE, SHORTBREAD	50000
COOKIE, SHORTBREAD, REDUCED FAT	50000
COOKIE, SHORTBREAD, WITH CHOCOLATE FILLING	50000
COOKIE, BUTTER OR SUGAR	50000
COOKIE, BUTTER/SUGAR COOKIE, W/ FRUIT AND/ OR NUTS	50000
COOKIE, SUGAR WAFER	50000
COOKIE, TEETHING, BABY FOOD	50000
COOKIE, TOFFEE BAR	50000
COOKIE, VANILLA SANDWICH	50000
COOKIE, VANILLA SANDWICH, REDUCED FAT	50000
COOKIE, RICH, ALL CHOCOLATE, W/ CHOC FILLING OR CHOC CHIPS	50000
COOKIE, BUTTER/SUGAR, W/ CHOCOLATE ICING / FILLING	50000
COOKIE, BUTTER/SUGAR, W/ ICING/FILLING OTHER THAN CHOC	50000

Food Product List	Parts *per* million (ppm)
COOKIE, VANILLA WAFFLE CREME	50000
COOKIE, TEA, JAPANESE	50000
COOKIE, VANILLA WAFER, NS AS TO TYPE	50000
COOKIE, VANILLA WAFER, REDUCED FAT	50000
COOKIE, VANILLA W/ CARAMEL, COCONUT, CHOC COATING	50000
COOKIE, WHOLE WHEAT, DRIED FRUIT & NUTS	50000
COOKIE, RUGELACH	50000
COOKIE, DIETETIC, CHOCOLATE CHIP	50000
COOKIE, LEMON WAFER, LOWFAT	50000
COOKIE, DIETETIC, OATMEAL W/ RAISINS	50000
COOKIE, DIETETIC, SANDWICH TYPE	50000
COOKIE, DIETETIC, SUGAR OR PLAIN	50000
COOKIE, P.R. (MANTECADITOS POLVORONES)	50000
MUFFIN, ENGLISH (INCLUDE SOUR DOUGH)	50000
MUFFIN, ENGLISH, TOASTED	50000
MUFFIN, ENGLISH, W/ RAISINS	50000
MUFFIN, ENGLISH, W/ RAISINS, TOASTED	50000
MUFFIN, ENGLISH, CHEESE	50000
MUFFIN, ENGLISH, CHEESE, TOASTED	50000
MUFFIN, ENGLISH, W/ FRUIT OTHER THAN RAISINS	50000
MUFFIN, ENGLISH, W/ FRUIT OTHER THAN RAISINS, TSTD	50000
MUFFIN, ENGLISH, 100% WHOLE WHEAT	50000
MUFFIN, ENGLISH, 100% WHOLE WHEAT, TOASTED	50000
MUFFIN, ENGLISH, 100% WHOLE WHEAT, W/ RAISINS	50000
MUFFIN, ENGLISH, WHOLE WHEAT, W/ RAISINS, TOASTED	50000
MUFFIN, ENGLISH, WHEAT BRAN	50000
MUFFIN, ENGLISH, WHEAT BRAN, TOASTED	50000
MUFFIN, ENGLISH, WHEAT BRAN, W/ RAISINS	50000
MUFFIN, ENGLISH, WHEAT BRAN, W/ RAISINS, TOASTED	50000
MUFFIN, ENGLISH, WHEAT OR CRACKED WHEAT	50000
MUFFIN, ENGLISH, WHEAT OR CRACKED WHEAT, TOASTED	50000
MUFFIN, ENGLISH, "WHOLE" WHEAT	50000
MUFFIN, ENGLISH, "WHOLE" WHEAT, TOASTED	50000
MUFFIN, ENGLISH, WHEAT OR CRACKED WHEAT W/ RAISINS	50000
MUFFIN, ENGLISH, WHEAT W/ RAISINS, TOASTED	50000
MUFFIN, ENGLISH, "WHOLE" WHEAT, W/ RAISINS	50000
MUFFIN, ENGLISH, "WHOLE" WHEAT, W/ RAISINS, TOASTED	50000
MUFFIN, ENGLISH, RYE	50000
MUFFIN, ENGLISH, RYE, TOASTED	50000
MUFFIN, ENGLISH, PUMPERNICKEL	50000
MUFFIN, ENGLISH, PUMPERNICKEL, TOASTED	50000
MUFFIN, ENGLISH, OAT BRAN	50000
MUFFIN, ENGLISH, OAT BRAN, TOASTED	50000

Food Product List	Parts *per* million (ppm)
MUFFIN, ENGLISH, OAT BRAN, WITH RAISINS	50000
MUFFIN, ENGLISH, OAT BRAN, W/ RAISINS, TOASTED	50000
MUFFIN, ENGLISH, MULTIGRAIN	50000
MUFFIN, ENGLISH, MULTIGRAIN, TOASTED	50000
CORNBREAD MUFFIN, STICK, ROUND	50000
CORNBREAD MUFFIN, STICK, ROUND, TOASTED	50000
CORNBREAD MUFFIN, STICK, ROUND, HOMEMADE	50000
MUFFIN, NFS	50000
MUFFIN, FRUIT & OR NUTS	50000
MUFFIN, FRUIT, FAT FREE, CHOL FREE	50000
MUFFIN, CHOCOLATE CHIP	50000
MUFFIN, CHOCOLATE	50000
MUFFIN, WHOLE WHEAT	50000
MUFFIN, WHEAT	50000
MUFFIN, BUCKWHEAT	50000
MUFFIN, WHEAT BRAN (INCLUDE W/ RAISINS & NUTS)	50000
MUFFIN,BRAN,W/ FRUIT, LOWFAT	50000
MUFFIN, BRAN W/ FRUIT, NO FAT, NO CHOLESTEROL	50000
MUFFIN, OATMEAL	50000
MUFFIN, OAT BRAN	50000
MUFFIN, OAT BRAN WITH FRUIT AND/OR NUTS	50000
MUFFIN, PLAIN	50000
MUFFIN, CHEESE	50000
MUFFIN, PUMPKIN, W/ RAISINS	50000
MUFFIN, ZUCCHINI	50000
MUFFIN, CARROT (INCL W/ RAISINS/NUTS)	50000
MUFFIN, MULTIGRAIN, W/ NUTS	50000
MUFFIN, MULTIGRAIN, WITH FRUIT	50000
TOASTER MUFFIN, FRUIT, UNTOASTED	50000
TOASTER MUFFIN, FRUIT, TOASTED	50000
RICE BEVERAGE (INCL RICE TEA)	50000
HORCHATA, P.R. (BEVERAGE)	50000
OATMEAL BEVERAGE, P.R.	50000
OATMEAL BEVERAGE W/ MILK	50000
RICE BEVERAGE, MEXICAN (HORCHATA)	50000
MILK, SOY, READY-TO-DRINK, NOT BABY	10000
MILK, SOY, READY-TO-DRINK, NOT BABY'S, CHOCOLATE	10000
MILK, IMITATION, FLUID, SOY BASED	10000
MILK, SOY, DRY, RECONSTITUTED, NOT BABY	10000
WHOLE WHEAT CEREAL, COOKED, NS AS TO ADDED FAT	5000
WHOLE WHEAT CEREAL, COOKED, NO FAT ADDED	5000
WHOLE WHEAT CEREAL, COOKED, FAT ADDED	5000
WHEAT, CREAM OF, COOKED, REGULAR, FAT ADDED	5000

Food Product List	Parts *per* million (ppm)
WHEAT, CREAM OF, COOKED,QUICK,FAT ADDED IN COOKING	5000
WHEAT HEARTS, COOKED, NS AS TO ADDED FAT	5000
WHOLE WHEAT CEREAL, W/ BARLEY, COOKED, NO FAT ADDED	5000
WHEAT HEARTS, COOKED, NO FAT ADDED	5000
WHOLE WHEAT CEREAL, WHEAT & BARLEY, FAT ADDED	5000
WHOLE WHEAT CEREAL, WHEAT & BARLEY, ADDED FAT NS	5000
WHEAT CEREAL, CHOC FLAVORED, COOKED W/ MILK	5000
WHEAT CEREAL, CHOC FLAVORED, COOKED, NO FAT ADDED	5000
WHEAT CEREAL, CHOC FLAV,COOKED,NS AS TO ADDED FAT	5000
NESTUM, CEREAL	5000
KASHI CEREAL, NS AS TO READY-TO-EAT OR COOKED	5000
OAT CEREAL, NFS	5000
ALL-BRAN CEREAL	5000
ALL BRAN CEREAL W/ EXTRA FIBER	5000
ALPEN CEREAL	5000
AMARANTH FLAKES CEREAL	5000
BRAN CHEX CEREAL	5000
ALL-BRAN BRAN BUDS CEREAL, KELLOGG'S (FORMERLY BRAN BUDS)	5000
COMMON SENSE OAT BRAN CEREAL, PLAIN	5000
FIBER ONE CEREAL	5000
FIBER 7 FLAKES CEREAL, HEALTH VALLEY	5000
BRAN FLAKES CEREAL, NFS (FORMERLY 40% BRAN FLAKES, NFS)	5000
COMPLETE WHEAT BRAN FLAKES, KELLOGG'S (FORM.40% BRAN FLAKES)	5000
NATURAL BRAN FLAKES CEREAL, POST	5000
GREAT GRAINS, RAISIN, DATE, & PECAN,WHOLE GRAIN CEREAL, POST	5000
GREAT GRAINS DOUBLE PECAN WHOLE GRAIN CEREAL, POST	5000
MILLET, PUFFED (CEREAL)	5000
MUESLIX CEREAL, NFS	5000
MUESLI WITH RAISINS, DATES, AND ALMONDS	5000
MULTI BRAN CHEX	5000
MULTI GRAIN CHEERIOS	5000
NATURAL MUESLI, JENNY'S CUISINE	5000
NU SYSTEM CUISINE TOASTED GRAIN CIRCLES CEREAL	5000
RAISIN BRAN CEREAL, KELLOGG	5000
RAISIN BRAN CEREAL, POST	5000
RAISIN BRAN, TOTAL	5000
RICE CHEX CEREAL	5000
RICE FLAKES, NFS	5000
RICE KRISPIES CEREAL	5000
RICE KRISPIES TREATS CEREAL (KELLOGG'S)	5000
PUFFED RICE CEREAL	5000
SHREDDED WHEAT, 100%	5000
BASIC 4 (RTE CEREAL)	5000

Food Product List	Parts *per* million (ppm)
CHEERIOS	5000
CHEX CEREAL, NFS	5000
CRUNCHY CORN BRAN CEREAL, QUAKER	5000
CORN CHEX CEREAL	5000
CORN FLAKES, NFS (INCLUDE STORE BRANDS)	5000
CORN FLAKES, LOW SODIUM	5000
CORN FLAKES, KELLOGG	5000
TOTAL CORN FLAKES	5000
HONEY BUNCHES OF OATS CEREAL	5000
HONEY BUNCHES OF OATS W/ ALMONDS, POST	5000
HONEYCOMB CEREAL, PLAIN	5000
HONEYCOMB CEREAL, STRAWBERRY	5000
HONEY CRUNCH CORN FLAKES CEREAL, KELLOGG'S	5000
HONEY NUT CHEX CEREAL	5000
HONEY NUT CHEERIOS	5000
HONEY NUT SHREDDED WHEAT CEREAL, POST	5000
JUST RIGHT CEREAL	5000
KASHI, PUFFED	5000
100% BRAN CEREAL	5000
100% NATURAL CEREAL, PLAIN, QUAKER	5000
SUN COUNTRY 100% NATURAL GRANOLA, WITH ALMONDS	5000
100 % NATURAL CEREAL, W/ OATS,HONEY & RAISINS,QUAKER	5000
100% NATURAL WHOLEGRAIN CEREAL W/ RAISINS, LOWFAT, QUAKER	5000
WEETABIX WHOLE WHEAT CEREAL	5000
WHEAT CHEX CEREAL	5000
WHEAT GERM CEREAL, PLAIN	5000
WHEAT GERM CEREAL, W/ SUGAR & HONEY	5000
PUFFED WHEAT CEREAL, PLAIN	5000
IMITATION CHEESE, AMERICAN OR CHEDDAR TYPE	10000
IMITATION CHEESE, AMER/CHEDDAR TYPE, LOW CHOLESTERO	10000
IMITATION MOZZARELLA CHEESE (INCL PIZZA MATE)	10000
CHEESE, PROCESSED, AMERICAN & SWISS BLENDS	10000
CHEESE, PROCESSED, AMERICAN/CHEDDAR TYPE	10000
CHEESE, PROCESSED, AMERICAN/CHEDDAR, LOW SODIUM	10000
CHEESE, PROCESSED, AMERICAN/CHEDDAR TYPE, LOWFAT	10000
CHEESE, PROC CHEESE PRODUCT, AMER/CHED, RED FAT	10000
CHEESE, PROCESSED, AMERICAN/CHEDDAR TYPE, NONFAT	10000
CHEESE, PROCESSED CREAM CHEESE PRODUCT, NONFAT	10000
CHEESE, PROCESSED, SWISS	10000
CHEESE, PROCESSED, SWISS, LOW FAT	10000
CHEESE, PROCESSED, CHEESE FOOD	10000
CHEESE, PROCESSED, W/VEGETABLES(INCL PEPPER CHEESE)	10000
CHEESE, PROCESSED, W/ WINE	10000

Food Product List	Parts *per* million (ppm)
CHEESE SPREAD, NFS	10000
CHEESE SPREAD, AMERICAN OR CHEDDAR CHEESE BASE	10000
CHEESE SPREAD, SWISS CHEESE BASE	10000
CHEESE SPREAD, CREAM CHEESE OR NEUFCHATEL BASE	10000
CHEESE SPREAD, PRESSURIZED CAN	10000
CREAM SUBSTITUTE, NS AS TO FROZEN,LIQUID OR POWDER	20000
CREAM SUBSTITUTE, FROZEN	20000
CREAM SUBSTITUTE, LIQUID (INCLUDE COFFEE WHITNER)	20000
CREAM SUBSTITUTE, LIGHT, LIQUID	20000
CREAM SUBSTITUTE, POWDERED	20000
CREAM SUBST, LIGHT, POWDERED (INCL COFFEE MATE, CRE	20000
CREAM SUBSTITURE, FLAVORED LIQUID	20000
CREAM SUBSTITURE, FAT FREE, FLAVORED LIQUID	20000
CREAM SUBSTITURE, SUGAR FREE, FLAVORED LIQUID	20000
CREAM SUBSTITURE, FLAVORED POWDER	20000
CREAM SUBSTITURE, FAT FREE, FLAVORED POWDER	20000
WHIPPED TOPPING, NONDAIRY, NS AS TO CND/FRZ/POWDER	10000
WHIPPED TOPPING, NONDAIRY, PRESSURIZED CAN	10000
WHIPPED TOPPING, NONDAIRY, FROZEN (INCL COOL WHIP)	10000
WHIPPED TOPPING, NONDAIRY, FZN, LOWFAT (INCL COOL)	10000
WHIPPED CREAM SUBST, NONDAIRY, FROM POWDERED MIX	10000
WHIP CREAM SUB,NONDAIRY,LOWFAT,LO SUGAR,FROM MIX	10000
HONEY MUSTARD DRESSING	20000
HONEY MUSTARD DRESSING, REDUCED CALORIE	20000
MUSTARD (INCL HORSERADISH MUSTARD, CHINESE MUSTARD)	20000
EGG, SCRAMBLED, MADE FROM DRY EGGS	20000
EGG SUBSTITUTE, NS AS TO POWDERED, FROZEN OR LIQUID	50000
SCRAMBLED EGGS, FROM POWDERED MIXTURE	20000
SCRAMBLED EGGS, FROM CHOLESTEROL-FREE FROZEN MIX	20000
SCRAMBLED EGG, CHOLESTEROL-FREE, W/ CHEESE	10000
MARGARINE-LIKE SPREAD, STICK, SALTED	80000
MARGARINE-LIKE SPREAD, MADE W/ YOGURT, STICK, SALTED	80000
MARGARINE-LIKE SPREAD, TUB, SALTED	80000
MARGARINE-LIKE SPREAD, LIQUID, SALTED	80000
MARGARINE-LIKE SPREAD, STICK, UNSALTED	80000
MARGARINE-LIKE SPREAD, TUB, UNSALTED	80000
MARGARINE-LIKE SPREAD, WHIPPED, TUB, SALTED	80000
MARGARINE-LIKE SPREAD, TUB, SWEETENED	80000
MARGARINE-LIKE SPREAD, RED CAL, 40% FAT, TUB, SALTED	80000
MARGARINE-LIKE SPREAD,RED CAL,40% FAT,MADE W/ YOGURT,TUB	80000
MARGARINE-LIKE SPREAD, RED CAL, 40% FAT, STICK, SALTED	80000
MARGARINE-LIKE SPREAD, RED CAL, 20% FAT, TUB, SALTED	80000
MARGARINE-LIKE SPREAD, RED CAL, 20% FAT, TUB, UNSALTED	80000

Food Product List	Parts *per* million (ppm)
MARGARINE-LIKE SPREAD, FAT FREE, TUB, SALTED	80000
MARGARINE-LIKE SPREAD, FAT FREE, LIQUID, SALTED	80000
MAYONNAISE, REGULAR	120000
MAYONNAISE, MADE W/ YOGURT (INCLUDE YOGANNAISE)	120000
MAYONNAISE, MADE W/ TOFU	120000
MAYONNAISE, IMITATION	120000
MAYONNAISE, IMITATION, NO CHOLESTEROL	120000
MAYONNAISE-TYPE SALAD DRESSING	120000
MAYONNAISE-TYPE SALAD DRESSING, CHOLESTEROL-FREE	120000
MAYONNAISE, LOW CALORIE OR DIET	120000
MAYONNAISE, LOW CALORIE OR DIET, LOW SODIUM	120000
MAYONNAISE, REDUCED-CALORIE/DIET, CHOLESTEROL-FREE	120000
MAYONNAISE-TYPE SALAD DRESSING, LOW CALORIE	120000
MAYONNAISE-TYPE SALAD DRESSING,DIET,NO CHOLESTEROL	120000
SALAD DRESSING, NFS	85000
CAESAR DRESSING	85000
FETA CHEESE SALAD DRESSING	85000
FRENCH DRESSSING	85000
ITALIAN DRESSING, W/ VINEGAR & OIL	85000
RUSSIAN DRESSING	85000
MILK, VINEGAR & SUGAR DRESSING	85000
POPPY SEED DRESSING	85000
PEPPERCORN DRESSING	85000
CELERY SEED DRESSING	85000
SESAME DRESSING	85000
SWEET & SOUR DRESSING	85000
THOUSAND ISLAND DRESSING	85000
YOGURT DRESSING	85000
SALAD DRESSING, LOW CALORIE, NFS	85000
FRENCH DRESSING, LOW CALORIE	85000
FRENCH DRESSING, REDUCED CALORIE,FAT-FREE,CHOL-FREE	85000
FRENCH DRESSING, REDUCED CALORIE	85000
CAESAR DRESSING, LOW CALORIE	85000
ITALIAN DRESSING, LOW CALORIE	85000
ITALIAN DRESSING, REDUCED CALORIE	85000
ITALIAN DRESSING, REDUCED CALORIE, FAT-FREE	85000
RUSSIAN DRESSING, LOW CALORIE	85000
THOUSAND ISLAND DRESSING, LOW CALORIE	85000
THOUSAND ISLAND DRSG,REDUCED CAL,FAT-FREE,CHOL-FREE	85000
VINEGAR, SUGAR & WATER DRESSING	85000
KOREAN DRESSING OR MARINADE	85000
MILK, VINEGAR & ARTIFICIAL SWEETENER DRESSING	85000
CREAMY DRESSING W/ BUTTERMILK, NS LOW/REDUCED CAL	85000

Food Product List	Parts *per* million (ppm)
CRMY DRSG W/ SOUR CRM &/ BUTTERMILK & OIL, LOW CAL	85000
CRMY DRSG W/ SOUR CRM &/ BUTTERMILK & OIL, RED CAL	85000
CRMY DRSG W/SOUR CRM&/BTTRMILK,RED CAL,NOFAT/CHOL	85000
CRMY DRSG W/SOUR CRM&/BUTTRMILK&OIL,RED CAL,NOCHOC	85000
SALAD DRESSING, LOW CALORIE, OIL-FREE	85000
YOGURT, FROZEN, NS AS TO FLAVOR, NS TO TYPE OF MILK	10000
YOGURT, FROZEN, NOT CHOCOLATE, TYPE OF MILK NS	10000
YOGURT, FROZEN, CHOCOLATE, TYPE OF MILK NS	10000
YOGURT, FROZEN, NS AS TO FLAVOR, LOWFAT MILK	10000
YOGURT, FROZEN, CHOCOLATE, LOWFAT MILK	10000
YOGURT, FROZEN, NOT CHOCOLATE, LOWFAT MILK	10000
YOGURT, FROZEN, NS AS TO FLAVOR, NONFAT MILK	10000
YOGURT, FROZEN, CHOCOLATE, NONFAT MILK	10000
YOGURT,FROZEN,NOT CHOCOLATE,W/ SORBET/SORBET-COATED	10000
YOGURT, FROZEN, NOT CHOCOLATE, NONFAT MILK	10000
YOGURT,FRZ,CHOCOLATE,NONFAT MILK,W/ LOW-CAL SWEET	10000
YOGURT,FRZ,NOT CHOC,NONFAT MILK,W/ LOW-CAL SWEET	10000
YOGURT, FROZEN, NS AS TO FLAVOR, WHOLE MILK	10000
YOGURT, FROZEN, CHOCOLATE, WHOLE MILK	10000
YOGURT, FROZEN, NOT CHOCOLATE, WHOLE MILK	10000
YOGURT, FROZEN, CHOCOLATE-COATED	10000
YOGURT, FROZEN, CAROB-COATED	10000
YOGURT, FROZEN, SANDWICH	10000
YOGURT, FROZEN, CONE, CHOCOLATE	10000
YOGURT, FROZEN, CONE, NOT CHOCOLATE	10000
YOGURT, FROZEN, CONE, NOT CHOCOLATE, LOWFAT MILK	10000
YOGURT, FROZ, CONE, CHOCOLATE, LOWFAT MILK	10000
FAT FREE ICE CREAM, NO SUGAR ADD, CHOC	20000
FAT FREE ICE CREAM, NO SUGAR ADD, FLAVORS OTHER THAN CHOC	20000
FAT FREE ICE CREAM, FLAVORS OTHER THAN CHOC	20000
FAT FREE ICE CREAM, CHOC	20000
FAT FREE ICE CREAM, NS AS TO FLAVOR	20000
ICE CREAM, NFS	40000
ICE CREAM, REGULAR, NOT CHOCOLATE	40000
ICE CREAM, REGULAR, CHOCOLATE	40000
ICE CREAM, RICH, FLAVORS OTHER THAN CHOCOLATE	40000
ICE CREAM, RICH, CHOCOLATE	40000
ICE CREAM, RICH, NS AS TO FLAVOR	40000
ICE CREAM, SOFT SERVE, NOT CHOCOLATE	40000
ICE CREAM, SOFT SERVE, CHOCOLATE	40000
ICE CREAM, SOFT SERVE, NS AS TO FLAVOR	40000
ICE CREAM BAR OR STICK, NOT CHOC- OR CAKE-COVERED	40000
ICE CREAM BAR/STICK, CHOCOLATE COVERED	40000

Food Product List	Parts *per* million (ppm)
ICE CREAM BAR, CHOCOLATE/CARAMEL COVERED, W/ NUTS	40000
ICE CREAM BAR,RICH CHOC ICE CREAM,THICK CHOC COVER	40000
ICE CREAM BAR,RICH ICE CREAM,THICK CHOC COVER	40000
ICE CREAM BAR/STICK,RICH ICE CREAM,CHOC COVER,W/NUT	40000
ICE CREAM BAR/STICK, CHOC ICE CREAM, CHOC COVER	40000
ICE CREAM BAR, CAKE-COVERED	40000
ICE CREAM BAR/STICK W/ FRUIT	40000
ICE CREAM SANDWICH	40000
ICE CREAM COOKIE SANDWICH (INCLUDE CHIPWICH)	40000
ICE CREAM CONE, W/ NUTS, NOT CHOCOLATE	40000
ICE CREAM CONE, CHOC-COVERED, W/ NUTS, NOT CHOC	40000
ICE CREAM CONE, CHOC-COVERED OR DIPPED, NOT CHOC	40000
ICE CREAM CONE, NO TOPPING, NOT CHOCOLATE	40000
ICE CREAM CONE, NO TOPPING, NS AS TO FLAVOR	40000
ICE CREAM CONE, W/NUTS, CHOCOLATE ICE CREAM	40000
ICE CREAM CONE, CHOC-COVERED, CHOC ICE CREAM	40000
ICE CREAM CONE, NO TOPPING, CHOCOLATE ICE CREAM	40000
ICE CREAM CONE, CHOC-COVERED, W/NUT, CHOC ICE CREAM	40000
ICE CREAM SUNDAE CONE (INCL DRUMSTICK, ALL FLAVORS)	40000
ICE CREAM SODA, NOT CHOCOLATE	40000
ICE CREAM SODA, CHOCOLATE	40000
ICE CREAM SUNDAE, TOPPING NS, W/ WHIPPED CREAM	40000
ICE CREAM SUNDAE, FRUIT TOPPING, W/ WHIPPED CREAM	40000
ICE CREAM SUNDAE, PREPACKAGED, NOT CHOCOLATE	40000
ICE CREAM SUNDAE,CHOCOLATE TOPPING,W/ WHIPPED CREAM	40000
ICE CREAM SUNDAE, NOT FRUIT/ CHOC TOP,W/ WHIP CREAM	40000
ICE CREAM SUNDAE, FUDGE TOPPING, W/ CAKE	40000
ICE CREAM PIE, NO CRUST	40000
ICE CREAM PIE,COOKIE CRUST,FUDGE TOPPING,WHIP CREAM	40000
ICE CREAM, FRIED	40000
LIGHT ICE CREAM, NO SUGAR ADD, NS FLAVOR	50000
LIGHT ICE CREAM, NO SUGAR ADD, NOT CHOCOLATE	50000
LIGHT ICE CREAM, NO SUGAR ADD, CHOCOLATE	50000
LT ICE CREAM, NS FLAV (ICE MILK)	40000
LIGHT ICE CREAM,NOT CHOCOLATE (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,CHOCOLATE (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,SOFT SERVE, NS FLAVOR (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,SOFT SERVE, NOT CHOC (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,SOFT SERVE CHOC (TASTEE FRZ, DAIRY QUEEN)	40000
LIGHT ICE CREAM,SOFT SERVE CONE,NOT CHOC (DAIRY QUEEN)	40000
LIGHT ICE CREAM,SOFT SERVE CONE, CHOC (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,SOFT SERVE CONE, NS FLAV(FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,SANDWICH (DAIRY QUEEN) (FORMERLY ICE MILK)	40000

Food Product List	Parts *per* million (ppm)
LIGHT ICE CREAM,BAR/STICK, CHOC-COATED (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,BAR, CHOC COVERED,W/NUTS (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,CONE, NFS (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,CONE, NOT CHOCOLATE (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,CONE, CHOCOLATE (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,SUNDAE,SOFT SERVE,CHOC/FUDGE TOP (ICE MILK)	40000
LIGHT ICE CREAM,SUNDAE,SOFT SERVE,FRUIT TOPPING (ICE MILK)	40000
LIGHT ICE CREAM,SUNDAE,SOFT SERVE,NOT FRUIT/CHOC TOPPING	40000
LIGHT ICE CREAM,SUNDAE,CHOC / FUDGE TOP (W/O WHIP CREAM)	40000
LIGHT ICE CREAM,SUNDAE,FRUIT TOP (W/O WHIP CREAM)(ICE MILK)	40000
LIGHT ICE CREAM,SUNDAE,NO FRUIT/CHOC TOP (W/O WHIP CREAM)	40000
LIGHT ICE CREAM,CREAMSICLE OR DREAMSICLE (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,FUDGESICLE (FORMERLY ICE MILK)	40000
LIGHT ICE CREAM,BAR/STICK, W/ LOW-CAL SWEETENER, CHOC COAT	40000
SHERBET, ALL FLAVORS	40000
PINEAPPLE CUSTARD, P.R. (FLAN DE PINA)	30000
SPANISH CUSTARD, P.R. (NATILLA ESPANOL)	30000
CUSTARD	30000
CUSTARD, PUERTO RICAN STYLE (FLAN)	30000
FRESH CORN CUSTARD, PUERTO RICAN STYLE	30000
PUDDING, NFS	30000
PUDDING, BREAD (INCLUDE W/ RAISINS)	30000
PUERTO RICAN BREAD PUDDING MADE W/ EVAP MILK & RUM	30000
DIPLOMAT PUDDING, P.R. (BUDIN DIPLOMATICO)	30000
PUDDING, MEXICAN BREAD (CAPIROTADA)	30000
PUDDING, MEXICAN BREAD (CAPIROTADA), LOWER FAT	30000
PUDDING, CHOCOLATE, RTE, NS FROM DRY MIX OR CANNED	30000
PUDDING, CHOC, RTE, LO CAL, W/ ART SWTNER, NS DRY/CAN	30000
PUDDING, NOT CHOC, RTE, NS FROM DRY MIX OR CANNED	30000
PUDDING, NOT CHOC, RTE, LO CAL, W/ ART SWTNER	30000
PUDDING, RICE	30000
PUDDING, RICE FLOUR, W/ NUTS (INDIAN DESSERT)	30000
PUDDING, TAPIOCA,MADE FROM HOME RECIPE, MADE W/ MILK	30000
PUDDING, TAPIOCA,MADE FROM DRY MIX,MADE W/ MILK	30000
PUDDING, TAPIOCA,CHOCOLATE,MADE W/ MILK	30000
PUDDING, COCONUT	30000
PUDDING, INDIAN (MILK, MOLASSES, CORNMEAL-BASED)	30000
PUDDING, PUMPKIN	30000
P.R. PUMPKIN PUDDING (FLAN DE CALABAZA)	30000
PUDDING,NOT CHOCOLATE,PREPARED FROM DRY MIX,MILK ADDED	30000
PUDDING,CHOCOLATE,PREPARED FROM DRY MIX,MILK ADDED	30000
PUDDING,NOT CHOC,FROM DRY,LOW CAL,ARTIFICIAL SWEET,W/MILK	30000
PUDDING,CHOC,FROM DRY,LOW CAL,ARTIFICIAL SWEET,MILK ADDED	30000

Food Product List	Parts *per* million (ppm)
PUDDING, CAN, CHOC, REDUCED FAT (INCL JELL-O LIGHT)	30000
PUDDING, CAN, CHOC, FAT FREE	30000
PUDDING, CANNED, NOT CHOC, REDUCED FAT	30000
PUDDING, CANNED, NOT CHOC, FAT FREE	30000
PUDDING, CANNED, NOT CHOCOLATE	30000
PUDDING,CANNED,LOW CAL,W/ARTIFICIAL SWTNR,NOT CHOC	30000
PUDDING, CANNED, CHOCOLATE	30000
PUDDING, CANNED, LO CAL, W/ ART SWTNER, CHOC	30000
PUDDING, CANNED, CHOC & NON-CHOC FLAVORS COMBINED	30000
PUDDING, CANNED, TAPIOCA	30000
PUDDING, CANNED, TAPIOCA, FAT FREE	30000
PUDDING, W/ FRUIT & VANILLA WAFERS	30000
HAUPIA (COCONUT PUDDING)	30000
LEMON-BUTTER SAUCE	50000
HOLLANDAISE SAUCE	50000
BEARNAISE SAUCE	50000
ORANGE SAUCE FOR DUCK	50000
TARTAR SAUCE	50000
HORSERADISH SAUCE	50000
PESTO SAUCE	50000
TARTAR SAUCE, LOW CALORIE	50000
BARBECUE SAUCE (INCLUDE ARBY'S SAUCE)	50000
BARBECUE SAUCE, LOW SODIUM	50000
MUSTARD SAUCE	50000
STEAK SAUCE, TOMATO-BASE (INCLUDE A-1)	50000
COCKTAIL SAUCE	50000
WORCESTERSHIRE SAUCE	50000
HOISIN SAUCE	10000
MISO SAUCE (INCLUDES AE SAUCE)	10000
MISO (FERMENTED SOYBEAN PASTE)	10000
BLACK BEAN SAUCE	50000
PEANUT SAUCE	50000
OYSTER-FLAVORED SAUCE	50000
GREEN TOMATO-CHILE SAUCE, RAW	50000
GREEN TOMATO-CHILE SAUCE, COOKED	50000
TOMATO SAUCE	50000
TOMATO SAUCE, LOW SODIUM	50000
NATURE VALLEY GRANOLA, W/ FRUIT & NUTS	50000
GRANOLA BAR W/ OATS, SUGAR, RAISINS, COCONUT	20000
GRANOLA BAR, OATS, FRUIT, NUTS, LOWFAT	20000
GRANOLA BAR, NONFAT	20000
GRANOLA BAR W/ PEANUTS, OATS, SUGAR, WHEAT GERM	20000
GRANOLA BAR, CHOCOLATE-COATED	20000

Food Product List	Parts *per* million (ppm)
GRANOLA BAR, W/ COCONUT, CHOCOLATE-COATED	20000
GRANOLA BAR W/ NUTS, CHOCOLATE-COATED	20000
GRANOLA BAR, COATED W/ NONCHOCOLATE COATING	20000
GRANOLA BAR, HIGH FIBER, YOGURT COATING, NOT CHOC	20000
GRANOLA BARS, W/ RICE CEREAL	20000
BREAKFAST BAR, NFS	20000
BREAKFAST BAR, CEREAL CRUST W/ FRUIT FILLING, LOWFAT	20000
BREAKFAST BAR, CEREAL CRUST W/ FRUIT FILLING, FAT FREE	20000
BREAKFAST BAR, DATE, W/ YOGURT COATING	20000
HIGH PROTEIN BAR, CANDY-LIKE, SOY & MILK BASE	20000
INSTANT BREAKFAST POWDER, NOT RECONSTITUTED	20000
INSTANT BFAST,PWDR,SWT W/ LO CAL SWT,NOT RECONSTUT	20000
INSTANT BREAKFAST, FLUID, CANNED	20000
INSTANT BREAKFAST, POWDER, MILK ADDED	20000
INSTANT BFAST,PWDR,SWT W/ LO CAL SWT, MILK ADDED	20000
YOGURT, NS AS TO TYPE OF MILK/FLAVOR	10000
YOGURT, PLAIN, NS AS TO TYPE OF MILK	10000
YOGURT, PLAIN, WHOLE MILK	10000
YOGURT, PLAIN, LOWFAT MILK	10000
YOGURT, PLAIN, NONFAT MILK	10000
YOGURT, VANILLA, LEMON, COFFEE, NS AS TO MILK TYPE	10000
YOGURT, VANILLA, LEMON, COFFEE, WHOLE MILK	10000
YOGURT, VANILLA, LEMON, COFFEE, LOWFAT MILK	10000
YOGURT, VANILLA, LEMON, COFFEE, NONFAT MILK	10000
YOGURT, VANILLA, LEMON, COFFEE, NONFAT MILK, LOW CAL SWEET	10000
YOGURT, CHOCOLATE, NS AS TO TYPE OF MILK	10000
YOGURT, CHOCOLATE, WHOLE MILK	10000
YOGURT, CHOCOLATE, NONFAT MILK	10000
YOGURT, FRUIT VARIETY, NS AS TO MILK TYPE	10000
YOGURT, FRUIT VARIETY, WHOLE MILK	10000
YOGURT, FRUIT VARIETY, LOWFAT MILK	10000
YOGURT, FRUIT VARIETY, NONFAT MILK	10000
YOGURT, FRUITED, NONFAT MILK, LOW CAL SWEETENER	10000
YOGURT, FRUIT & NUTS, NS AS TO TYPE OF MILK	10000
YOGURT, FRUIT & NUTS, LOWFAT MILK	10000
CRACKER, NS AS TO SWEET/NONSWEET (INCL CRACKER,NFS)	30000
CRACKER, ANIMAL (INCL ARROWROOT COOKIE)	30000
CRACKERS, GRAHAM	30000
CRACKERS, GRAHAM, CHOCOLATE COVERED	30000
CRACKERS, OATMEAL	30000
CRACKERS, CUBAN	30000
CRACKERS, CUCA	30000
CRACKERS, GRAHAM, W/ RAISINS	30000

Food Product List	Parts *per* million (ppm)
CRACKERS, GRAHAM, LOWFAT	30000
CRACKERS, GRAHAM, FAT FREE	30000
CRACKERS, GRAHAM, SANDWICH-TYPE, WITH FILLING	30000
CRACKERS, MATZO, LOW SODIUM	30000
CRACKERS, SALTINE, LOW SODIUM	30000
CRACKERS, SALTINE, FAT FREE, LOW SODIUM	30000
CRACKERS, TOAST THINS (RYE/WHEAT/WHITE), LOW SODIUM	30000
CRACKER, 100% WHOLE WHEAT,LO SODIUM	30000
CRACKER, SNACK, LOW SODIUM	30000
CRACKER, CHEESE, LOW SODIUM	30000
CRACKER, SNACK, LOWFAT, LOW SODIUM	30000
CRACKER, MULTIGRAIN, SALT-FREE	30000
CRACKER, SNACK	30000
CRACKER, SNACK, REDUCED FAT	30000
CRACKER, SNACK, FAT FREE	30000
CRACKERS, CHEESE	30000
CRACKER, CHEESE, REDUCED FAT	30000
CRACKER, HIGH FIBER, NO ADDED FAT	30000
CRACKERS, MATZO	30000
CRACKERS, MILK	30000
CRACKERS, OAT BRAN (INCLUDE NABISCO OAT THINS)	30000
CRACKERS, OYSTER	30000
CRACKERS, RICE	30000
CRACKERS, SALTINES	30000
CRACKERS, SALTINE, WHOLE WHEAT	30000
CRACKERS, CYLINDRICAL, PEANUT BUTTER-FILLED	30000
CRACKER, SANDWICH-TYPE, NFS	30000
CRACKER,SANDWICH-TYPE,PEANUT BUTTER FILLED	30000
CRACKER,SANDWICH-TYPE, CHEESE-FILLED	30000
CRACKERS, TOAST THINS	30000
CRACKER, WATER BISCUIT	30000
CRACKER, 100% WHOLE WHEAT	30000
CRACKER, 100% WHOLE WHEAT, REDUCED FAT	30000
CRACKER,WHOLE WHEAT & BRAN (INCL WH & BRAN TRISCUIT	30000
CRACKERS, WHEAT	30000
CRACKERS, WHEAT, REDUCED FAT	30000
CRACKER CORN (INCL STONED CORN CRACKER)	30000
SALTY SNACKS, CORN / CORNMEAL BASE, NUT /NUG, TSTD	30000
SALTY SNACKS, CORN OR CORNMEAL, CORN CHIPS, CHEESE	30000
SALTY SNACKS, CORN OR CORNMEAL, CORN PUFFS, TWISTS	30000
SALTY SNACKS, CORN OR CORNMEAL, TORTILLA CHIPS	30000
SALTY SNACKS, CORN/CORN-CHEESE CHIPS, UNSALTED	30000
SALTY SNACKS,CORN / CORNMEAL BASE,TORTILLA CHIPS LT	30000

Food Product List	Parts *per* million (ppm)
SALTY SNACKS, TORTILLA CHIPS, FAT FREE, W/ OLEAN	30000
SALTY SNACKS,CORN/CORNMEAL BASE,TORTILLA,LOWFAT,BKD	30000
SALTY SNACKS,CORN/CORNMEAL,TORTILLA,LOWFAT,BKD,NO SALT	30000
SALTY SNACKS, CORN/CORNML BASE,W/OAT BRAN,TORT CHPS	30000
SALTY SNACKS, CORN BASED/CHEESE PUFFS & TWISTS, LOWFAT	30000
SALTY SNACK MIXTURE,MOSTLY CORN,W/PRETZELS,W/O NUTS	30000
SALTY SNACKS, WHEAT-BASE, HIGH FIBER	30000
SALTY SNACKS, WHEAT-AND CORN-BASED CHIPS	30000
SALTY SNACKS, MULTIGRAIN CHIPS	30000
POTATO SOUP, NS AS TO MADE W/MILK OR WATER	10000
POTATO SOUP, CREAM OF, W/ MILK	10000
POTATO SOUP, PREPARED W/ WATER	10000
POTATO SOUP, INSTANT, MADE FROM DRY MIX	10000
POTATO & CHEESE SOUP	10000
MACARONI & POTATO SOUP	10000
POTATO CHOWDER (INCL CORN CHOWDER)	10000
MUSHROOM SOUP, NFS	10000
MUSHROOM SOUP, CREAM OF, PREP W/ MILK	10000
MUSHROOM SOUP, CREAM OF, PREPARED W/ WATER	10000
MUSHROOM SOUP, CANNED, UNDILUTED (INCL CREAM OF)	10000
MUSHROOM SOUP, W/ MEAT BROTH, PREPARED W/ WATER	10000
MUSHROOM SOUP, CM OF, LOW SOD, PREP W/ WATER	10000
MUSHROOM SOUP, CREAM OF, NS AS TO W/ MILK OR WATER	10000
MUSHROOM W/ CHICKEN SOUP, CREAM OF, PREP W/ MILK	10000
MUSHROOM SOUP, CREAM OF, CAN, RED. SOD., NS W/ MILK/WATER	10000
MUSHROOM SOUP, CREAM OF, CAN, RED. SODIUM, PREP W/ MILK	10000
MUSHROOM SOUP, MADE FROM DRY MIX	10000
MUSHROOM SOUP, CM OF, CAN, RED SOD, PREP W/ WATER	10000
MUSHROOM SOUP, CM OF, CAN, RED SOD, UNDILUTED	10000
FISH CHOWDER (INCL FISHERMAN'S SOUP, SEAFOOD CHOWD)	10000
CRAB SOUP, NS AS TO TOMATO-BASE OR CREAM	10000
CRAB SOUP, TOMATO BASE	10000
CLAM CHOWDER, NS AS TO MANHATTAN OR NEW ENGLAND	10000
CLAM CHOWDER, MANHATTAN (INCLUDE CHUNKY)	10000
TURTLE & VEGETABLE SOUP (INCLUDE SNAPPER SOUP)	10000
FISH & VEGETABLE SOUP,NO POTATOES (SOPA DE PESCADO)	10000
FISH SOUP, W/ POTATOES (SOPA DE PESCADO)	10000
CODFISH, RICE & VEGETABLE SOUP, P.R.	10000
CODFISH SOUP W/ NOODLES, P.R.	10000
CLAM CHOWDER, NEW ENG, NS AS TO MILK OR WATER ADDED	10000
CLAM CHOWDER, NEW ENGLAND, W/ MILK	10000
CLAM CHOWDER, NEW ENGLAND, W/ WATER	10000
CLAM CHOWDER, CANNED, NEW ENGLAND, RED SODIUM, RTS	10000

Food Product List	Parts *per* million (ppm)
CRAB SOUP, CREAM OF, W/ MILK	10000
LOBSTER BISQUE	10000
LOBSTER GUMBO	10000
OYSTER STEW	10000
SALMON SOUP, CREAM STYLE	10000
SHRIMP SOUP, CREAM OF, NS AS TO MILK/WATER ADDED	10000
SHRIMP SOUP, CREAM OF, W/ MILK	10000
SHRIMP SOUP, CREAM OF, W/ WATER	10000
SHRIMP GUMBO	10000
SEAFOOD SOUP W/ POTATOES & VEGETABLES (INCL DK GREEN LEAF)	10000
SEAFOOD SOUP W/ POTATOES & VEGETABLES (EXCL DK GREEN LEAF)	10000
SEAFOOD SOUP W/ VEGETABLES (INCL DK GREEN LEAFY)	10000
SEAFOOD SOUP W/ VEGETABLES (EXCL DK GREEN LEAFY)	10000
MOLASSES	10000
SUGAR, BROWN, LIQUID	5000
SWEET & SOUR SAUCE (INCLUDE VIETNAMESE SAUCE)	5000
FRUIT SAUCE (INCLUDE ALL FRUITS)	5000
RAISIN SAUCE	5000
PLAIN DESSERT SAUCE (INCLUDE VANILLA, RUM SAUCE)	5000
PLUM SAUCE, ORIENTAL STYLE	5000
MILK GRAVY, QUICK GRAVY	2000
GRAVY/SAUCE,CHINESE(SOY SCE,STOCK/BOUILL,CRNSTRCH)	2000

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