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ORIGINAL SUBMISSION

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Wilmington, DE 19880-0356

August 30, 2010

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Division of
Biotechnology and
GRAS Notice Review

Office of Food Additive Safety (HFS-225)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Pkwy
College Park, MD 20740

RE: **GRAS EXEMPTION CLAIM: EPA-Rich Triglyceride Oil**

Dear Sir or Madam,

E.I. du Pont de Nemours and Company ("DuPont") is hereby submitting the attached GRAS Exemption claim for Eicosopentaenoic acid-rich oil from *Yarrowia lipolytica* in triglyceride form ("EPA-rich triglyceride oil") for use as a food ingredient. EPA-rich triglyceride oil has been determined by DuPont to be Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. This determination is based on scientific procedures and has been evaluated by experts qualified by scientific training and experience to assess the safety of EPA-rich triglyceride oil under the conditions of its intended use in food. Therefore, the use of EPA-rich triglyceride oil in food as described in this GRAS Exemption Claim is exempt from the requirement of premarket approval.

The attached GRAS Notification provides a review of the information related to intended uses, manufacturing and safety of the DuPont EPA-rich triglyceride oil product. Included with this GRAS Notification submission are four ***(4)*** hard copies of the GRAS Notification dossier, which include the signed Expert Panel Consensus Statement.

DuPont looks forward to the Agency's review of the accompanying dossier and is prepared to respond to any questions that arise in the review process.

Respectfully,

(b) (6)

Matthias Liebergesell, Ph.D.
DuPont

I. GRAS EXEMPTION CLAIM

A. Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)]

As defined herein EPA-rich triglyceride oil has been determined by E.I. du Pont de Nemours and Company ("DuPont") to be Generally Recognized As Safe (GRAS), consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use in food. Therefore, the use of EPA-rich triglyceride oil in food as described below is exempt from the requirement of premarket approval.

Signed,

(b) (6) [redacted] 8-30-2010

Date

Matthias Liebergesell, Ph.D.
DuPont Applied BioSciences
Rt 141 & Powder Mill Road
Wilmington, DE 19803

B. Name and Address of Notifier

Matthias Liebergesell
DuPont Applied BioSciences
Rt 141 & Powder Mill Road
Wilmington, DE 19803

C. Common Name of the Notified Substance

EPA-rich triglyceride oil

D. Conditions of Intended Use in Food

EPA-rich triglyceride oil, from a genetically derived strain of *Yarrowia lipolytica*, is intended for use as a direct food ingredient in the food categories listed in 21 CFR 184.1472(a)(3) (not to be used in infant formula) and not to be combined or augmented with any other food ingredient containing EPA and/or another omega-3 fatty acid, docosahexaenoic acid (DHA) at incorporation levels which would result in a consumer intake that exceeds a maximum intake of 3.0 g/day.

E. Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30, the composition of EPA-rich triglyceride oil intended for use in foods by DuPont, as defined herein has been determined to be GRAS based on scientific procedures. This determination is based on data that are generally available in the public domain for omega-3 containing oils, supporting safety study information for EPA-rich triglyceride oil, and is supported by a consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of EPA-rich triglyceride oil as a component of food[1] [see Appendix 1, entitled, "**EXPERT PANEL CONSENSUS STATEMENT CONCERNING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF EPA-RICH TRIGLYCERIDE OIL**"].

F. Availability of Information

The data and information that serve as the basis for the GRAS determination, as well as the information that has become available since the GRAS determination, will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times as arranged DuPont Applied BioSciences, Rt 141 & Powder Mill Road, Wilmington, DE 19803 (contact: Matthias Liebergesell, email: Matthias.Liebergesell@usa.dupont.com)

II. DETAILED INFORMATION ABOUT THE IDENTITY OF THE SUBSTANCE

A. Identity

EPA-rich triglyceride oil is produced from a fermentation process using a genetically engineered strain of *Yarrowia lipolytica*, a dimorphic ascomycetous yeast that is ubiquitous in nature. *Y. lipolytica* is non-toxigenic and non-pathogenic and specific virulence factors suggestive of pathogenicity have not been identified. The process used to produce EPA-rich triglyceride oil involves fermentation, dewatering the *Yarrowia* yeast biomass and then drying it. The EPA-rich triglyceride oil may be produced with an EPA content ranging from 35 to 55% by using strains with different copy numbers and promoters. No antibiotic resistance marker was used in the construction of the production organism.

B. Method of Manufacture

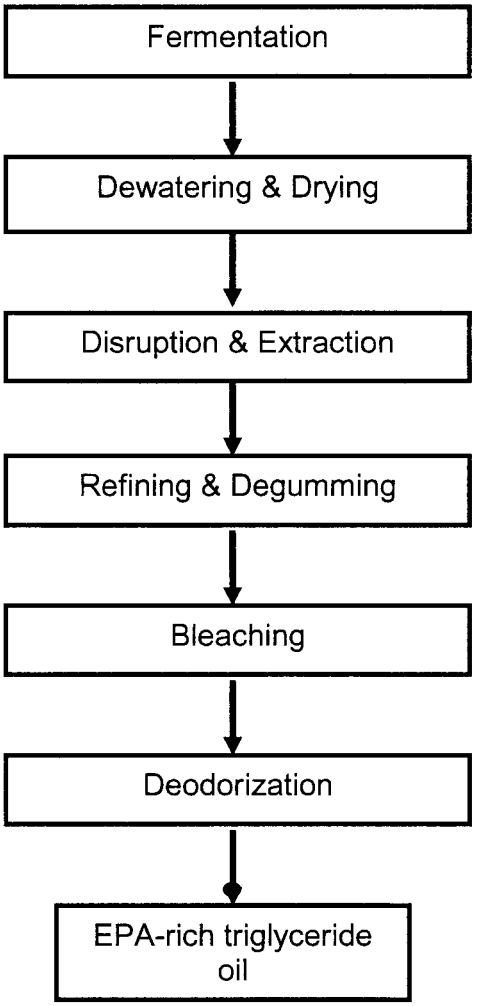
1. Overview

The process used to produce EPA-rich triglyceride oil involves fermentation, dewatering the *Yarrowia* yeast biomass and then drying it. The dried yeast biomass is disrupted to release the

[1] The Panel consisted of Ernst Schafner, M.D., Joseph F. Borzelleca, Ph.D., Michael W. Pariza, Ph.D., and David Bechtel, Ph.D., DABT.

oil, which is then refined using processes commonly practiced in the seed oil and fish oil industries. The process is conducted in a nitrogen-rich environment, and natural antioxidants (e.g., tocopherols, ascorbyl palmitate, rosemary extract, and ascorbic acid) are added at various steps of the process to ensure the oxidative stability of the EPA-rich triglyceride oil. Any DNA from the production organism is removed from the oil during the purification process. Analyses of non-consecutive batches demonstrate that the manufacturing process results in product that consistently meets product specifications. An overview of the manufacturing process for EPA-rich triglyceride oil is shown below.

Figure 1: Process Flow Diagram



2. Details on the Manufacturing Process for EPA-rich triglyceride oil

a EPA-Rich Triglyceride Oil Production Organism

Yarrowia lipolytica can accumulate oil to at least 30% dry cell weight (dcw) as an energy reserve when starved for nitrogen in the presence of excess glucose, but the only polyunsaturated fatty acid it synthesizes is linoleic acid (C18:2n-6). DuPont enabled the synthesis of EPA (C20:5n-3) by integrating into the *Y. lipolytica* genome single or multiple copies of a variety of well-characterized genes involved in efficient fatty acid synthesis and specifically in omega-3 fatty acid synthesis. Information about these genes is presented in Appendix 4. All genes were synthesized *in vitro* according to respective sequences from source microorganisms and have been sequenced and, in several cases, codon-optimized for expression in *Yarrowia*. The EPA-rich triglyceride oil may be produced with an EPA content ranging from 35 to 55% by using strains with different copy numbers and promoters. Genetic transformation occurred by integration of linear DNA into the genome by homologous or non-homologous recombination and by using selectable markers that complemented auxotrophic mutations. A combination of targeted and random insertion was used to incorporate all of the DNA modifications.

b Fermentation and Biomass Harvesting

Inocula are prepared from frozen cultures of *Yarrowia lipolytica* in a shake flask. After an incubation period, the culture is used to inoculate a seed fermentor. When the seed culture reaches a target cell density, it is then used to inoculate the main fermentor. The culture medium consists of glucose, various nitrogen sources, trace metals and vitamins. Critical process variables, including temperature, pH, dissolved oxygen and glucose concentration, are monitored and controlled per standard operating conditions to ensure consistent process performance and final EPA-rich triglyceride oil quality.

The fermentation is a 2-stage fed-batch process. In the first stage, the yeast are cultured under conditions that promote rapid growth to a high cell density, and in the second stage, they are starved for nitrogen and continuously fed glucose to promote lipid and EPA accumulation. The yeast biomass is dewatered and washed to remove salts and residual medium, and to minimize lipase activity. Drying follows to reduce moisture to a few percent to ensure oil stability during short term storage and transportation.

c Oil Extraction and Refining

Dry yeast biomass is mechanically disrupted in the presence of a food grade non-polar solvent, isohexane, to extract EPA-rich triglyceride oil from the biomass. The liquid phase containing isohexane and EPA-rich triglyceride oil is separated from the disrupted cells, and the isohexane is evaporated, which yields "crude oil". The crude oil is refined and degummed to remove free fatty acids, phospholipids, soaps and trace metals. Acid-activated clay is used during the bleaching process to remove color compounds and minor oxidation products, followed by deodorization to remove volatile, odorous, and additional color compounds.

The oil process is carried out in a nitrogen-rich environment, and natural antioxidants such as tocopherols, ascorbyl palmitate, rosemary extract, and ascorbic acid are added at various steps in the process to ensure the oxidative stability of the EPA-rich triglyceride oil.

3. Product Characterization

Product specifications for the EPA-rich triglyceride oil derived from the process described above are as follows:

Test	Specification
Fatty Acids	
Eicosapentaenoic acid (EPA)	350 - 550 mg FA/g oil (35 to 55% of total lipids)
Docosahexaenoic acid (DHA)	< 10 mg FA/g oil
Peroxide Value	<3 meq/kg
P-Anisidine Value	<12
Totox	<18
% Max Unsaponifiables	<2%
Color	Clear, slight yellow
Acid Value	< 6 mg KOH/g
Total Plate Count	< 2000 CFU/mL
E. coli	< 5 CFU/mL
Salmonella	Absent
Yeast and molds	< 100 CFU/mL
S. aureus	< 5 CFU/mL
Coliforms	< 5 CFU/mL

Product specification analysis of three non-consecutive batches of the 35%, as well as 50% EPA-rich triglyceride oil is shown in Appendix 2. These data demonstrate that the manufacturing process described herein results in product that consistently meets product specifications

The fatty acid profile of three non-consecutive batches of the 50%, as well as 35% EPA-rich triglyceride oil is shown in Table 1 below.

Table 1 Fatty Acid Profile

Fatty Acid Profile	mg FA/g oil					
	DS09434A	DS09021A	DS09026A	F12	F14	F17
	50% EPA			35% EPA		
C14 Myristic	0.3	0.3	0.3	0.5	0.5	0.5
C15 Pentadecanoic	0.6	0.5	0.6	0.4	0.5	0.5
C16 Palmitic	15.7	15.0	16.0	21.7	24.3	24.3
C16:1n7 Palmitoleic	3.4	3.6	3.3	5.9	6	6.1
C17 Margaric	2	1.8	1.9	1.5	1.7	1.8
C18 Stearic	17.1	15.7	16.7	23.3	26.2	25.4
C18:1n9 Oleic	40	36.2	38.8	56.3	57.4	57.1
C18:1 Octadecenoic	2.55	2.9	3.0	4.5	4.4	4.6
C18:2 Linoleic	139.7	145.6	143.4	186.4	184.7	187
C18:3n6 gamma-Linolenic	1.95	1.9	1.9	1.4	1.3	1.1
C18:3n3 alpha-Linolenic	12.4	10.6	10.5	36.4	35.7	35.3
C18:4 Octadecatetraenoic	0.9	1.6	0.9	1.5	1.4	1.5
C20 Arachidic	0.8	0.8	0.7	1	1.1	1
C20:1 Eicosenoic	1.15	1.1	1.0	3.4	3.4	3.4
C20:2n6 Eicosadienoic	14.7	15.9	18.1	29.2	29.5	30.4
C20:3n6 Homogamma Linolenic	14	17.7	16.0	13.6	13.2	13.3
C20:4n6 Arachidonic	5.1	6.2	6.4	2.9	2.7	2.9
C20:4n3 Eicosatetraenoic	0	9.5	7.4	12.8	13.1	13.1
C20:5n3 Eicosapentaenoic	**507.4**	**513.2**	**518.5**	**357.9**	**352.1**	**357.1**
C22 Behenic	1.2	1.3	1.1	1.3	1.4	1.3
C22:2n6 Docosadienoic	1.35	1.4	0.5	2.6	2.4	2.5
C22:5n3 Docosapentaenoic	10.2	9.2	10.9	15.2	15	15.8
C22:6n3 Docosahexaenoic	0	0.4	0.0	1	1.1	1.3
C24 Lignoceric	2.6	2.2	2.0	7.6	9	8.2
Others	60	61.9	65.5	68.4	71.1	71.9
Total Fatty Acids	861.6	876.7	885.4	866.3	869.4	877.9
Total Saturates	40.3	37.5	39.3	57.3	64.7	63
Total Monounsaturates	47.3	43.8	46.0	71.1	72.1	72.1
Total Polyunsaturates	714.1	733.5	734.6	669.5	661.5	670.9
Total Omega 3	537.4	544.6	538.3	432.8	427	433
Total Omega 6	176.7	188.9	186.4	236.4	234.2	237.6
Total Omega 9	41.4	37.3	39.7	61	62	61.7

Method of Analysis: AOCS Ce 1b-89

In addition, DuPont has fully characterized 3 non-consecutive batches (F12, F14, and F17) of its 35% EPA-rich triglyceride oil for sterols, proximate analysis, organochlorine pesticides, general pesticides, heavy metals, PCBs, residual solvent, and mycotoxins, as shown in Appendix 2. The methods of analysis used have been fully validated in the performing laboratories and were mostly official Association of Official Analytical Chemists (AOAC) and American Oil Chemists Society (AOCS) methods of analysis.

These data are expected to be representative of the EPA-rich triglyceride oil produced using the methods described herein regardless of the EPA content (35 to 55%).

4. BASIS FOR GRAS DETERMINATION

a. Documentation to Support the Safety of EPA-rich triglyceride oil

The determination that EPA-rich triglyceride oil is GRAS is on the basis of scientific procedures; the information supporting the general recognition of the safe use of EPA-rich triglyceride oil includes:

- The role of omega-3 fatty acids in physiology;
- Prior review and safety assessment by FDA of omega-3 fatty acids (*i.e.*, menhaden oil)
- Preclinical and clinical data available in the published scientific literature for other sources and forms of EPA
- Preclinical safety data demonstrating that EPA-rich triglyceride oil exhibits low toxicity at levels well-above the estimated intakes from the intended uses specified herein;
- Supporting evidence for the safety of EPA from human investigations, including a 6 week trial with the EPA-rich triglyceride oil; and,
- Batch analysis data demonstrating that the manufacturing process results in a product that reproducibly meets specifications.

A summary of these data is presented herein. Moreover, these data were reviewed by a panel of experts, qualified by scientific training and experience to evaluate the safety of EPA-rich triglyceride oil as a component of food, who concluded that the proposed uses of EPA-rich triglyceride oil are safe and suitable and would be GRAS based on scientific procedures [see Appendix 1, entitled, "**EXPERT PANEL CONSENSUS STATEMENT CONCERNING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF EPA-RICH TRIGLYCERIDE OIL**".

b. Estimated Intake of EPA-rich triglyceride oil

The FDA critically evaluated available information on the safety of the omega-3 fatty acids, EPA and DHA, and identified three potential safety issues, namely prolongation of bleeding time, modification of glycemic control, and elevation of LDL cholesterol (US FDA, 1993). In its 1997 menhaden oil decision, the FDA concluded that combined consumption of up to 3 g of DHA + EPA/person/day poses no significant risk for bleeding time, produces no clinically significant effect on glycemic control, and is safe with respect to the effect on LDL cholesterol (FDA, 1997). In its final rule affirming menhaden oil as GRAS, the FDA (2005) published maximum use-levels to ensure that the total daily intake of combined DHA and EPA does not exceed 3.0 g/person.

EPA-rich triglyceride oil, from a genetically derived strain of *Yarrowia lipolytica*, is intended for use as a direct food ingredient in the food categories listed in 21 CFR 184.1472(a)(3) (not to be used in infant formula) and not to be combined or augmented with any other food ingredient containing EPA and/or another omega-3 fatty acid, docosahexaenoic acid (DHA) at incorporation levels which would result in a consumer intake that exceeds a maximum intake of 3.0 g/day. The EPA-rich triglyceride oil is produced with an EPA range of 35 to 55%. The amount of oil added will be pro-rated based on EPA content in the EPA-rich triglyceride oil in a manner to assure that intake of EPA does not exceed 3 g/day. As an example, the maximum use-levels of the 55% oil are discussed herein.

On the basis of the composition of menhaden oil (total of approximately 20% DHA and EPA) and the EPA-rich triglyceride oil (maximum 55% EPA content), any limitation in the level of use of the EPA-rich triglyceride oil will have to be not more than 36% of the use-levels of menhaden oil to ascertain compliance with the safe intake level of 3.0 g/person/day for the sum of DHA and EPA. The individual intended food-uses and maximum use-levels for the EPA-rich triglyceride oil, which are consistent with the current GRAS uses of menhaden oil, are summarized in Table 2.

Table 2 Proposed Food-Uses and Maximum Use-Levels of EPA-rich triglyceride oil in the United States

Food Category[a]	Maximum Use-Level[b] (% as served)
Baked goods and baking mixes (1)[c]	1.8
Cereals (4)	1.44
Cheese products (5)	1.8
Chewing gum (6)	1.08
Condiments (8)	1.8
Confections and frostings (9)	1.8
Dairy product analogs (10)	1.8
Egg products (11)	1.8
Fats and oils (12)[d]	4.32
Fish products (13)	1.8
Frozen dairy desserts (20)	1.8
Gelatins and puddings (22)	0.36
Gravies and sauces (24)	1.8
Hard candy (25)	3.6
Jams and jellies (28)	2.52
Meat products (29)	1.8
Milk Products (31)	1.8
Nonalcoholic beverages (3)	0.18
Nut products (32)	1.8
Pastas (23)	0.72
Plant protein products (33)	1.8
Poultry products (34)	1.08
Processed fruit juices (35)	0.36
Processed vegetable juices (36)	0.36
Snack foods (37)	1.8
Soft candy (38)	1.44
Soup mixes (40)	1.08
Sugar substitutes (42)	3.6
Sweet sauces, toppings, and syrups (43)	1.8
White granulated sugar (41)	1.44

[a] Based on the final rule amending 21 CFR §184.1472 (FDA, 2005)
[b] Maximum use-levels (%) for EPA-rich triglyceride oil are based on a maximum EPA content of 55%.
[c] The number in parenthesis following each food category refers to the paragraph listing of the particular food category in 21 CFR 170.3(n).
[d] Not including infant formula.

Based on a maximum EPA content of 35-55% in EPA-rich triglyceride oil and a maximum intake of 3.0 g/person/day of EPA through the intended food-uses, the maximum intake of EPA-rich triglyceride oil would be approximately 5.45-8.57 g/person/day. For a 60 kg adult, this intake estimate would correspond to approximately 0.09-014 g/kg body weight/day of EPA-rich triglyceride oil.

c. Physiology of Long-Chain Polyunsaturated Fatty Acids

Fatty acids are building blocks for new membranes and provide an energy-rich fuel source for cellular metabolism. In adipose cells, for example, free fatty acids produced by lipoprotein lipase are converted to triglycerides and stored for later use. When fat is needed for fuel, adipose tissue triacylglycerol undergoes lipolysis and free fatty acids are released into the circulation bound to albumin. Fatty acids then undergo β-oxidation to produce 2-carbon substrates from the Kreb's cycle. The Kreb's metabolic cycle produces CO_2 and NADPH. NADPH is then oxidized in mitochondria to produce ATP, releasing two electrons added to O_2 to make H_2O. The ATP produced in this way provides energy for all cellular functions (IOM, 2005).

Long chain polyunsaturated fatty acids (LCPUFAs) are fatty acids of 20 carbons or more in length with two or more methylene-interrupted double bonds in the cis position. PUFAs can be grouped into two main families, omega-6 (or n-6) or omega-3 (or n-3), depending on the position of the first double bond proximate to the methyl end of the fatty acid. PUFAs are important structural components, modulating membrane fluidity and permeability and serving as precursors for a number of biologically active molecules such as eicosanoids, growth regulators, and hormones (Uauy et al., 1996; Jump, 2002; La Guardia et al., 2006; Berra et al.,2009).

The biosynthesis of long-chain polyunsaturated fatty acids (PUFAs) such as EPA from essential 18-carbon fatty acid precursors (α-linolenic acid and linolenic acid) obtained from the diet involves the sequential addition of 2-carbon units and desaturations catalyzed by Δ5- and Δ6-desaturases. The ability to convert α-linolenic acid to EPA and DHA and the pathways utilized in this process differ among mammalian species (IOM, 2005). The first three steps in the production of EPA involve a Δ6-desaturation of α-linolenic acid to SDA, a chain elongation to form omega-3-eicosatrienoic acid (ETA), and a Δ5-desaturation to form EPA. Alternate pathways have been identified in other organisms (e.g., some protists, including *Euglena gracilis)* and in some mammalian tissues, namely testis of rats and humans. In this pathway, the essential 18-carbon fatty acids are elongated to 20-carbon chain lengths followed by consecutive Δ8- and Δ5-desaturations (Huang et al., 2004; Green, 2004; Hulanicka et al, 1964; Albert and Coniglio, 1977; Albert et al., 1979).

d. Safety of Omega-3 Fatty Acids

The safety of omega-3 fatty acids is supported by their long history of ingestion as a component of the human diet and a large number of clinical trials investigating their effects. Although adverse effects, namely elevated LDL-cholesterol levels, prolonged bleeding time, and effects on glycemic control have been reported in some subpopulations, the available data indicate that intakes of up to 3 g of DHA + EPA/person/day pose no significant risk on these parameters. In its 1997 menhaden oil decision, the FDA concluded that combined consumption of up to 3 g of DHA + EPA/person/day poses no significant risk for bleeding time, produces no clinically significant effect on glycemic control, and is safe with respect to the effect on LDL cholesterol (FDA, 1997). In its final rule affirming menhaden oil as GRAS, the FDA (2005) published maximum use-levels to ensure that the total daily intake of combined DHA and EPA does not exceed 3.0 g/person. A discussion of clinical studies related to these parameters is provided in Appendix 3.

e. Safety of the Microorganism Utilized in the Production of EPA-rich Triglyceride Oil

Yarrowia lipolytica is a dimorphic ascomycetous yeast that is ubiquitous in nature. It is not considered to be pathogenic, nor have specific virulence factors suggestive of pathogenicity been identified. The available data suggest a low degree of toxicity and there have been no reports of *Y. lipolytica* producing toxins against humans or animals. DuPont enabled the synthesis of EPA (C20:5n-3) by integrating into the *Y. lipolytica* genome single or multiple copies of a variety of well-characterized genes involved in efficient fatty acid synthesis and specifically in omega-3 fatty acid synthesis. No antibiotic resistance marker has been used in the construction of the production organism. In addition, DNA and protein from the production organism is removed from the oil during the purification process. Additional information regarding the safety of *Yarrowia lipolytica* and the genetic modifications made to this organism are provided in Appendix 4.

f. Preclinical Studies Pertaining to the Safe Consumption of EPA-rich triglyceride oil

Non-clinical studies conducted by DuPont supporting the safety of EPA-rich triglyceride oil include a 28-day and a 90-day repeated dose toxicity study and a developmental toxicity study in rats and various genotoxicity studies such as, a reverse mutation assay, chromosomal aberration test, and mouse bone marrow micronucleus test. These studies are discussed in the following sections.

1 Subchronic Toxicity Studies

a. 28- Day Study

DuPont evaluated the potential repeated-dose toxicity of EPA-rich triglyceride oil produced from genetically modified *Yarrowia lipolytica* yeast, when administered by oral gavage to rats for approximately 28 days (DuPont, 2009a). This study was conducted in compliance with the U.S. FDA good laboratory practices and Redbook 2000 test guideline IV.C.3.a. Short-Term Toxicity studies with Rodents. Fifteen Crl:CD(SD) rats per sex were administered EPA-rich triglyceride oil (neat or in olive oil) by intragastric intubation at a dose volume of 3 mL/kg body weight for at least 28 days in dosages of 940, 1880, and 2820 mg/kg b.w./day (325, 650, and 976 mg EPA/kg b.w./day). Control animals received 3 mL/kg olive oil vehicle while comparison oil rats were treated with 3 mL/kg food-grade fish (sardine and anchovy) oil (nominally equivalent to 513 mg EPA/kg/day). The first 10 rats per group were designated for evaluation of repeated-dose toxicity and the remaining 5 rats per group were designated for evaluation of fatty acid profile in plasma and selected tissues.

No test substance-related deaths occurred, and no clinical or ophthalmological observations were attributed to exposure to the test substance. No test substance-related effects on body weight, body weight gain, food consumption, or food efficiency were observed. There were no treatment-related changes in hematology. Total serum cholesterol was significantly decreased in all EPA oil dose groups and in the fish oil group in males and females, primarily due to decreases in the non-HDL fraction. However, this is an expected response to EPA exposure, and the decrease is comparable to that observed with other polyunsaturated fatty acids (Sugiyama et al., 2008). No other test substance-related effects were observed on any other clinical chemistry parameters. Likewise, there were no changes in coagulation or urinalysis parameters.

There were no test substance-related gross observations at necropsy. A test substance-related increase in liver weights was observed in the male and female High EPA and fish oil groups, and in males in the Medium EPA oil group. The increased liver weights were considered to be a physiological adaptive response, and they were not correlated with clinical or microscopic pathology evidence of liver toxicity. No other test substance-related effects on organ weights were observed.

Male rats in the High EPA oil group had a slightly greater incidence of thyroid follicular cell hypertrophy than the study control or historical control incidences. Thyroid follicular cell hypertrophy was not observed in any female rats on study. Follicular cell hypertrophy is consistent with several different mechanisms of altered thyroid gland homeostasis, but the specific cause and the biological significance of the hypertrophic response in this study is not clear. However, thyroid follicular cell hypertrophy was also observed at a similar incidence in the male rats exposed to fish oil. This thyroid effect may represent an adaptive response to exposure to high levels of polyunsaturated fatty acids.

Under the conditions of this study, exposure to DuPont EPA-rich triglyceride oil, dosed up to 2820 mg/kg/day (976 mg EPA/kg/day), produced no adverse effects on in-life or clinical pathology parameters. All concentrations of EPA-rich triglyceride oil lowered cholesterol. Exposure to the high- or mid-dose (males only) EPA-rich triglyceride oil produced non-adverse increases in liver weights with no correlative clinical or microscopic pathology. Exposure to high-dose EPA-rich triglyceride oil produced minimal to mild thyroid follicular cell hypertrophy in male rats, which was also observed at similar incidence in the male rats exposed to fish oil, a substance considered to be GRAS by the U.S. FDA (USFDA CFSAN Office of Food Additive Safety. Agency Response Letter. GRAS Notice No. GRN 000138, April 20, 2004).

b. 90- Day Study

DuPont also evaluated the potential repeated-dose toxicity of EPA-rich triglyceride oil produced from genetically modified *Yarrowia lipolytica* yeast, when administered by oral gavage to rats for approximately 90 days (DuPont, 2009b). This study was conducted in compliance with the U.S. FDA good laboratory practices and Redbook 2000 test guideline IV.C.4.a Subchronic Toxicity Studies with Rodents. Twenty Crl:CD(SD) rats per sex were administered EPA-rich triglyceride oil (neat or in olive oil) by intragastric intubation for at least 28 days in dosages of 282, 1410, and 2820 mg/kg b.w./day (98, 488, and 976 mg EPA/kg b.w./day). These dose levels were chosen based on non-adverse effects observed at all dose levels in the 28-day toxicity study. Control animals received 3 mL/kg olive oil vehicle while comparison oil rats were treated with 3 mL/kg food-grade fish (sardine and anchovy) oil (513 mg/kg/day). The first 10 rats per group were designated for evaluation of neurobehavioral and clinical pathology and an additional 5 rats per group were designated for evaluation of fatty acid profile.

No adverse effects were observed on mean body weight, mean body weight gains, nutritional parameters, mortality, clinical signs, neurobehavioral or ophthalmological observations in male or female rats in any group. There were no treatment-related changes in hematology, coagulation, or urinalysis parameters. Minimal to mild decreases in total serum cholesterol were observed in the mid to high EPA oil dose groups and in the fish oil group in males and females primarily due to decreases in the non-HDL fraction. However, this is an expected response to EPA exposure, and the effect in the high-dose EPA group was of a magnitude comparable to that observed in the fish oil group. No other adverse, test substance-related effects were observed on any other clinical chemistry parameter.

There were no test substance-related deaths. A slight increase in pale livers was observed at necropsy in male high-dose EPA and fish oil groups, and liver weights were increased in male high-dose EPA and female mid- and high-dose EPA groups. These observations were not considered to be adverse as they were not associated with any clinical or microscopic pathology evidence of liver toxicity. No other test substance-related effects on organ weights were observed.

Oily material believed to be test substance was observed in the caudal nasal cavity of many rats, and was associated with secondary lesions (minimal to mild mucopurulent exudate and focal epithelial degeneration/regeneration) resulting from the foreign material. One or more of these lesions was observed in all groups except those dosed with deionized water; however, the incidence was highest in the mid- and high-dose EPA and the fish oil groups. These lesions were considered to be a reaction to test substance in the nose and not evidence of systemic target organ toxicity.

The follicular cell hypertrophy observed in the 28-day study was not observed in the 90-day study, suggesting that this adaptive response to PUFA may be transient and further supports a non-adverse response.

Exposure to the EPA-rich triglyceride oil produced from yeast for approximately 90 days did not produce adverse effects at daily doses up to 2820 mg/kg/day (976 mg EPA/kg/day), the NOAEL for the study. Based on all endpoints from the 90-day study, the overall safety profile of the EPA-rich triglyceride oil was comparable to that of GRAS fish oil (USFDA CFSAN Office of Food Additive Safety. Agency Response Letter. GRAS Notice No. GRN 000138, April 20, 2004).

2 Developmental Toxicity Study

The potential maternal and developmental toxicity of EPA-rich triglyceride oil produced from genetically modified *Yarrowia lipolytica* yeast was evaluated. Groups of 22 time-mated Crl:CD(SD) rats were dosed by gavage with EPA-rich triglyceride oil (neat or diluted with olive oil) at doses of 282, 1410, and 2820 mg/kg b.w./day (98, 488, and 976 mg EPA/kg/day) (DuPont, 2009c). This study was conducted in compliance with the U.S. FDA good laboratory practices and Redbook 2000 test guideline IV.C.9.b. Guidelines for Developmental Toxicity Studies. The control group was dosed with olive oil (vehicle control) on gestation days (GD) 6-20. The dose volume was 3 mL/kg for all groups. Maternal clinical observations, body weights, and food consumption data were collected. On GD 21, all dams were euthanized and a gross external and visceral examination was performed. The uterus of each pregnant female was removed and the uterine contents were examined and described; all fetuses were removed and individually identified, weighed, sexed, and examined for external alterations. Approximately one-half of the fetuses were examined for soft tissue alterations; all fetuses were examined for skeletal alterations.

There were no early deaths; all animals on study survived until scheduled sacrifice. There were no test substance-related clinical observations at any dose level tested. With the exception of two females (one each from the mid- and high-dose EPA oil groups) with red vaginal discharge observed on GD 15, all animals appeared normal throughout the study. The red vaginal discharge was not considered test substance related because it was transient, affected only one animal per each affected dose group, and did not precede any adverse reproductive outcome.

There were no test substance-related effects on any reproductive outcome or litter data endpoint. Group mean data for the numbers of corpora lutea, implantations, resorptions, live and dead fetuses, sex ratio, and fetal weight were comparable across all groups tested. One female from the high-dose EPA oil group was noted with total litter loss. This female was determined pregnant by ammonium sulfide staining, which revealed nine very early resorptions. Since this finding was observed in a single animal, it was considered spurious and unrelated to the test substance. Under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for both maternal and developmental toxicity was 2820 mg EPA oil/kg/day (976 mg EPA/kg/day) dose, the highest level tested.

3 Mutagenicity/Genotoxicity Studies

a. Bacterial Reverse Mutation Assay

EPA-rich triglyceride oil was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9 (DuPont, 2009d). Dimethyl sulfoxide (DMSO) was selected as the solvent. This study was conducted in compliance with the U.S. FDA good laboratory practices and Redbook 2000 test guideline IV.C.1.a Bacterial Reverse Mutation Test. The assay was performed in two phases, using the plate incorporation method. The first phase, the initial toxicity-mutation assay, was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. The second phase, the confirmatory mutagenicity assay, was used to evaluate and confirm the mutagenic potential of the test article. The positive control substance used for all strains with S9 activation was 2-aminoanthracene. In the absence of S9 activation, positive controls were 2-nitrofluorene (for strain TA98), sodium azide (for strains TA100 and TA1535), 9-aminoacridine (for strain TA1537), and methyl methanesulfonate (for WP2 *uvr*A).

Results are shown in Appendix 5. No positive mutagenic response was observed in either the dose-range finding assay or the confirmatory mutagenicity assay at doses up to 5000 µg per plate. These results indicate that, under the conditions of this study, EPA-rich triglyceride oil did not exhibit any mutagenic responses in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test article was concluded to be negative in this assay.

b. *In Vitro* Mammalian Cell Gene Mutation Test

EPA-rich triglyceride oil was also tested in the L5178Y/TK+/- Mouse Lymphoma Mutagenesis Assay in the absence and presence of Aroclor-induced rat liver S9 (DuPont, 2009e). The preliminary toxicity assay was used to establish the concentration range for the mutagenesis assay. The mutagenesis assay was used to evaluate the mutagenic potential of the test article. This study was conducted in compliance with the U.S. FDA good laboratory practices and Redbook 2000 test guideline IV.C.1.c. Mouse Lymphoma Thymidine Kinase Gene Mutation Assay.

Based on the results of the preliminary toxicity assay, the concentrations tested in the initial mutagenesis assay ranged from 5.0 to 750 µg/mL for both the non-activated and S9-activated cultures with a 4-hour exposure. Visible precipitate was present at concentrations ≥500 µg/mL in treatment medium. Data are shown in Appendix 5. The concentrations chosen for cloning were 50, 100, 200, 350, and 500 µg/mL with and without S9 activation. No cloned cultures exhibited mutant frequencies ≥90 mutants per 106 clonable cells over that of the solvent control. There was no concentration-related increase in mutant frequency.

Based on the results of the preliminary toxicity and initial mutation assays, the concentrations tested in the extended treatment assay ranged from 5.0 to 750 µg/mL for non-activated cultures with a 24-hour exposure. Data are shown in Appendix 5. Visible precipitate was present at concentrations ≥500 µg/mL in treatment medium. The concentrations chosen for cloning were 50, 100, 200, 350, and 500 µg/mL. No cloned cultures exhibited mutant frequencies ≥90 mutants per 106 clonable cells over that of the solvent control. There was no concentration-related increase in mutant frequency.

All criteria for a valid test were met. Under the conditions of this study, EPA-rich triglyceride oil was concluded to be negative in the presence and absence of S9 metabolic activation in the L5178Y/TK+/- Mouse Lymphoma Mutagenesis Assay.

c. Mouse Bone Marrow Erythrocyte Micronucleus Test

The potential of EPA-rich triglyceride oil to increase the incidence of micronucleated polychromatic erythrocytes in bone marrow of male and female ICR mice was tested in the mouse micronucleus assay (DuPont, 2009f). The definitive micronucleus study consisted of four groups, each containing 10 male and 10 female mice. Mice in these groups were treated either with the vehicle control or with EPA-rich triglyceride oil at 25%, 50%, or 100%. Cyclophosphamide monohydrate (CP), at a dose of 50 mg/kg, was used as the positive control substance. Test or control substances were administered at a dose volume of 20 mL/kg body weight by a single oral gavage.

Five mice/sex/treatment were euthanized 24 hours after dosing and the remaining five mice/sex/treatment were euthanized 48 hours after dosing. An additional group (group 5) of 5 male and 5 female mice was treated with CP, and these animals were euthanized along with 24 hour treatment groups. At the time of euthanasia, femoral bone marrow was collected and bone marrow smears (slides) were prepared and stained with May-Gruenwald-Giemsa stain. Bone marrow cells [polychromatic erythrocytes (PCEs)] all 24-hour groups and the vehicle and high dose 48-hour groups were examined microscopically for the presence of micronuclei (micronucleated PCEs; MPCEs). A statistical analysis of data was performed using the Kastenbaum-Bowman Tables (binomial distribution, $p \leq 0.05$). The incidence of micronucleated PCEs and the ratio of PCEs to total erythrocytes (PCEs/ECs ratio) served as indication of test article clastogenicity and cytotoxicity, respectively.

No mortality was observed in any treatment group. All animals dosed with the control substances (vehicle or positive) and all female mice dosed with EPA-rich triglyceride oil in all treatment groups appeared normal. All male mice dosed with EPA-rich triglyceride oil had diarrhea.

As shown in Appendix 5, no appreciable reductions in the ratio of polychromatic erythrocytes to total erythrocytes in the test article-treated groups relative to the respective vehicle control groups. No statistically significant increase in the incidence of micronucleated polychromatic erythrocytes in test article-treated groups relative to the respective vehicle control groups was observed in male or female mice at 24 or 48 hours after dose administration. CP, the positive control, induced a statistically significant increase in the incidence of micronucleated PCEs ($p \leq 0.05$) in both male and female mice. The number of micronucleated PCEs in the vehicle control groups did not exceed the historical vehicle control range. Based upon this, all criteria for a valid test were met as specified in the protocol.

A single oral administration of EPA-rich triglyceride oil at doses up to and including 100% (neat test article) did not induce a significant increase in the incidence of micronucleated polychromatic erythrocytes in bone marrow of male or female ICR mice. Therefore, the EPA-rich triglyceride oil was concluded to be negative in the mouse micronucleus assay.

G. Clinical Study Pertaining to the Safety of EPA-rich triglyceride oil

The effects of EPA-rich triglyceride oil and DHA oil on adverse events, safety parameters, and cardiovascular disease risk factors were compared to olive oil control (600 mg/day) in a randomized, blinded, placebo-controlled study. Healthy volunteers (n = 110; ages 21-70 years) off lipid-altering medications or supplements, received EPA-rich triglyceride oil (equivalent to 600 or 1800 mg EPA/day) or DHA oil (equivalent to 600 mg DHA/day) or olive oil control (600 mg/day) for 6 weeks.

The safety tests included a comprehensive metabolic profile (measured by Quest Diagnostics, Cambridge, MA) which included fasting values of glucose, blood urea nitrogen, creatinine, sodium, potassium, chloride, carbon dioxide, calcium, total protein, albumin, globulin, bilirubin, alkaline phosphatase, AST, and ALT. Researchers also measured prothrombin time, thyroid function (T3, T4), and a complete blood count (hemoglobin, hematocrit, while blood cell count, differential, red blood cell count). Data are presented in Appendix 6.

There were no major adverse events. Treatments were well tolerated, except for complaints of occasional fishy odors. No significant alterations in safety tests versus baseline values were noted. No significant effects on body weight, systolic blood pressure, pulse, fasting glucose, or fasting insulin levels were noted versus baseline in any group. Likewise, there were no significant effects on lipids, apolipoproteins, or inflammatory markers observed versus baseline for the low EPA group.

In the high-EPA group, there was a significant ($p<0.05$) reduction of 5.8% for lipoprotein-associated phospholipase A_2 ($LpPLA_2$) (a marker of inflammation in the arterial wall). In the DHA group, there were significant increases in low density lipoprotein (LDL) cholesterol in both the fasting and fed state of 14.2% and 16.3%, respectively. There were significant reductions in fed triglyceride levels of 9.5% in the DHA group. In contrast, the olive group had a significant increase in high density lipoprotein cholesterol of 7.1% and a significant decrease in LDL cholesterol of 6.0%.

No significant effects on the clotting parameters prothrombin time and fibrinogen, inflammatory markers adiponectin, C reactive protein, interleukin-6, and tumor necrosis factor alpha, or the adhesion molecules ICAM1 and VCAM, were noted for any intervention. In addition there were no significant effects on small density LDL cholesterol, remnant lipoprotein cholesterol, apolipoprotein (apo) B, apoB-48, apoA-I, Lp(a), HDL particles, or free fatty acids.

Based on these data, researchers concluded that this human study indicates that the EPA-rich triglyceride oil given to human subjects at 600 mg or 1800 mg of EPA/day is well tolerated and safe at least over a 6 week period.

H. Summary and Basis for GRAS Conclusion

DuPont's GRAS determination for EPA-rich triglyceride oil from a genetically modified strain of *Yarrowia lipolytica* for its intended use as a direct food ingredient in the food categories listed in 21 CFR 184.1472(a)(3) (not to be used in infant formula) and not to be combined or augmented with any other food ingredient containing EPA and/or DHA at levels which would exceed a maximum intake of 3.0 g/day would not be harmful under the intended conditions is based on scientific procedures and includes the evaluation of data from safety studies conducted on DuPont's product, as well as other related sources of EPA.

The safety of the EPA-rich triglyceride oil has been demonstrated in the absence of mutagenic or genotoxic activity *in vitro* and *in vivo*. The subchronic toxicity potential of EPA-rich triglyceride oil has also been evaluated in rats. Exposure to the EPA-rich triglyceride oil, dosed up to 2820 mg/kg/day (976 mg EPA/kg/day) for up to 90 days, produced no adverse effects on in-life or clinical pathology parameters. A developmental study conducted with EPA-rich triglyceride oil showed no developmental toxicity. In addition, no adverse effects on any parameters, including clotting time and blood glucose levels, were seen in a 6-week clinical trial at doses up to 1800 mg/day EPA. The overall safety profile of the EPA-rich triglyceride oil is also comparable to that of GRAS fish oil.

In its final rule affirming menhaden oil as GRAS, the FDA (2005) published maximum use-levels to ensure that the total daily intake of combined DHA and EPA does not exceed 3.0 g/person. Given that the EPA level in the EPA-rich oil may range up to 2.75-fold higher than the combined levels of DHA and EPA in menhaden oil, the proposed use-levels for EPA-rich triglyceride oil have been established at levels which are not more than 36% of those GRAS-affirmed for

menhaden oil and would ensure that the intake of EPA is within the limits considered safe by the FDA. The maximum intake of EPA-rich triglyceride oil resulting from these intended food uses would be approximately 5.45-8.57 g/person/day (for 55 or 35% EPA-rich triglyceride oil). For a 60 kg adult, this intake estimate would correspond to approximately 0.09-0.14 g/kg body weight/day EPA-rich triglyceride oil, respectively. As discussed in section 4.B use levels of EPA-rich triglyceride oil containing lesser amounts of EPA would be prorated on this basis.

DuPont has concluded the EPA-rich triglyceride oil is GRAS under the intended conditions of use on the basis of scientific procedures. This determination is further supported by an Expert Panel evaluation of the safety of EPA-rich triglyceride oil under the intended conditions of use.

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APPENDICES

EPA-RICH TRIGLYCERIDE OIL GRAS NOTIFICATION

Submitted: August, 2010

Notifier: E.I. du Pont de Nemours and Company

Applied BioSciences
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Wilmington, DE 19803

APPENDIX 1

EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF EPA-RICH TRIGLYCERIDE OIL

Expert Panel Report Concerning the Generally Recognized As Safe (GRAS) Status of EPA-Rich Triglyceride Oil for Use As a Food Ingredient

DuPont convened a panel of independent biomedical scientists (hereinafter referred to as the Expert Panel), qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, to independently and critically evaluate the available information on (EPA)-rich triglyceride oil and to determine if the intended uses of (EPA)-rich triglyceride oil are safe and would be Generally Recognized As Safe (GRAS) based on scientific procedures. (EPA)-rich triglyceride oil is intended to provide omega-3 polyunsaturated fatty acids, and specifically, EPA, in a range of 35 to 55%, for use as a direct food ingredient in the food categories listed in 21 CFR 184.1472(a)(3) and is not to be combined or augmented with any other food ingredient containing EPA and another omega-3 fatty acid, docosahexaenoic acid (DHA) at levels which would exceed a maximum intake of 3.0 g/day. The Expert Panel consisted of Professors Joseph F. Borzelleca, Ph.D. (VCU School of Medicine), Michael W. Pariza, Ph.D. (University of Wisconsin, Madison) and Ernst J. Schaeffer, M.D. (Tufts University School of Medicine) and David Bechtel, Ph.D. (CANTOX U.S., Inc.).

The Expert Panel independently and collectively critically evaluated information contained in a comprehensive package of scientific information and data compiled from the literature and other sources through January 2010 by Cantox. The Expert Panel convened by teleconference on 03 May 2010 and unanimously concluded that the intended uses of (EPA)-rich triglyceride oil produced consistent with cGMP and meeting the food grade specifications presented in the dossier are Generally Recognized As Safe (GRAS) based on scientific procedures.

DuPont's EPA-rich triglyceride oil is produced from a fermentation process using a genetically engineered strain of *Yarrowia lipolytica*, a dimorphic ascomycetous yeast that is ubiquitous in nature. *Y. lipolytica* is non-toxigenic and non-pathogenic and specific virulence factors suggestive of pathogenicity have not been identified. DuPont enabled the synthesis of EPA (C20:5n-3) by integrating into the Y. lipolytica genome single or multiple copies of a variety of well-characterized genes involved in efficient fatty acid synthesis and specifically in omega-3 fatty acid synthesis. DuPont's EPA-rich triglyceride oil may be produced with an EPA content ranging from 35 to 55% by using strains with different copy numbers and promoters. No antibiotic resistance marker was used in the construction of the production organism. In addition, any DNA from the production organism is removed from the oil during the purification process.

The process used to produce EPA-rich triglyceride oil involves fermentation, dewatering the *Yarrowia* yeast biomass and then drying it. The dried yeast biomass is disrupted to release the oil, which is then refined using processes commonly practiced in the seed oil and fish oil industries. The process is conducted in a nitrogen-rich environment, and natural antioxidants (*e.g.*, tocopherols, ascorbyl palmitate, rosemary extract, and ascorbic acid) are added at various steps of the process to ensure the oxidative stability of the EPA-rich triglyceride oil. Analyses of non-consecutive batches demonstrate that the manufacturing process results in product that consistently meets product specifications. Additional analyses demonstrate that DuPont EPA-rich

triglyceride oil is free of organochlorine pesticides, general pesticides, heavy metals, PCBs, microbial contaminants, solvents, and aflatoxins.

Based on an EPA content of 35 to 55% in EPA-rich triglyceride oil and a maximum intake of 3.0 g/person/day of EPA through the intended food-uses, the maximum intake of EPA-rich triglyceride oil would be approximately 5.45 g/person/day. For a 60 kg adult, this intake estimate would correspond to approximately 0.09 g/kg body weight/day of EPA-rich triglyceride oil.

Omega-3 fatty acids have a long history of safe ingestion as a component of the human diet. A number of clinical trials investigating the physiological and clinical effects and safety of omega-3 fatty acids have been reported. Adverse effects including elevated LDL-cholesterol levels, prolonged bleeding time, and effects on glycemic control were reported in some subpopulations. However, the available data indicate that intakes of up to 3 g of DHA + EPA/person/day do not pose a significant risk on these parameters and the USFDA has confirmed this.

The sub chronic toxicity potential of DuPont's EPA-rich triglyceride oil was evaluated in Sprague-Dawley rats that received gavage daily doses of DuPont's EPA-rich triglyceride oil of 98, 488, and 976 mg/kg bw for up to 90 days. The NOAEL was 976 mg/kg bw/day, the highest dose tested. In a developmental study, Sprague-Dawley rats received gavage doses of DuPont's EPA-rich triglyceride oil of 98, 488, and 976 mg/kg bw on gestation days 6-20. The NOAEL for maternal and developmental toxicity was 976 mg/kg bw/day, the highest dose tested. DuPont's EPA-rich triglyceride oil was not mutagenic or genotoxic in the Ames test, mouse lymphome assay, and the mouse micronucleus test.

In a six-week clinical trial with DuPont's EPA-rich , no adverse effects on any parameters, including clotting time and blood glucose levels, were reported at doses up to 1800 mg/day EPA.

The safety profile of DuPont's EPA-rich triglyceride oil is comparable to that of menhaden fish oil, which is GRAS.

Conclusion

We, the Expert Panel, have independently and collectively critically evaluated the information summarized above and unanimously conclude that there is reasonable certainty that no harm will result from the intended uses of DuPont's EPA-rich triglyceride oil.

We further conclude that the intended uses of DuPont's EPA-rich triglyceride oil, manufactured consistent with cGMP and meeting the food grade specifications presented in the dossier, are Generally Recognized As Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

(b) (6)

29 June 2010

Date 29 June 2010

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APPENDIX 2

PRODUCT ANALYSIS

Product Specifications and Analysis

Test	Specification	Lot F12	Lot F14	Lot F17	Lot DS09434 A	Lot DS09021 A	Lot DS09026 A
		35% EPA			50% EPA		
Fatty Acids							
Eicosapentaenoic acid (EPA)	350 - 550 mg FA/g oil	357.9	352.1	357.1	507.3	513.2	518.5
Docosahexaenoic acid (DHA)	< 10 mg FA/g oil	1.0	1.1	1.3	0.0	0.4	0.0
Peroxide Value	<3 meq/kg	<0.10	<0.10	<0.10	<0.10	0.8	1.36
P-Anisidine Value	<12	2.14	2.17	1.52	4.27	5.96	7.09
Totox	<18	2.14	2.17	1.52	4.27	7.56	9.81
% Max Unsaponifiables	<2%	1.22	1.31	1.06	0.92	0.85	0.86
Color	Clear, slight yellow	Clear, slight yellow	Clear, slight yellow	Clear, slight yellow	Clear, slight yellow	Clear, slight yellow	Clear, slight yellow
Acid Value	< 6 mg KOH/g	4.8	4.7	3.5	0.98	0.30	0.32
Total Plate Count	< 2000 CFU/mL	<2000	<10	<10	<5	<5	<5
E. coli	< 5 CFU/mL	Negative	Negative	Negative	<5	<5	<5
Salmonella	Negative (Absent)	Negative	Negative	Negative	Negative	Negative	Negative
Yeast and molds	< 100 CFU/mL	<10	<10	<10	<5	<5	<5
S. aureus	< 5 CFU/mL	Negative	Negative	Negative	<5	<5	<5
Coliforms	< 5 CFU/mL	Negative	Negative	Negative	<5	<5	<5

Sterols

Sterols	mg/100g		
	F12	F14	F17
Total sterols (mostly ergosterol)	404	467	277

Method of Analysis: JAOCS vol. 60, no.8 (August 1983)

Proximate Analysis

Proximate Analysis	Method of Analysis	F12	F14	F17
Total Fat (%)	AOAC 950.46	98.80	99.60	99.70
Total Nitrogen (%)	AOCS Ba 4e-93	0.32	0.30	0.28
Total Amino Acids (µg/ml)	Agilent Amino Acid Quant System	17	22	11
Carbohydrates (by difference; %)	Calculation	0.90	0.10	0.00
Ash (%)	AOAC 920.155c	<0.1	<0.04	<0.03
Dietary Fiber (%)	AOAC 920.155c	<0.1	<0.1	<0.1

Organochlorine Pesticide Analysis

Organochlorine Pesticides	Det. Limit in parentheses (ppb)		
	F12	F14	F17
a-BHC	ND(3)	ND(3)	ND(3)
HXCB	ND(2)	ND(2)	ND(2)
b-BHC	ND(6)	ND(6)	ND(6)
g-BHC (LINDANE)	ND(3)	ND(3)	ND(3)
d-BHC	ND(4)	ND(4)	ND(4)
HEPTACHLOR	ND(3)	ND(3)	ND(3)
OXYCHLORDANE	ND(3)	ND(3)	ND(3)
HEPTACHLOR EPOXIDE	ND(3)	ND(3)	ND(3)
o,p' DDE	ND(4)	ND(4)	ND(4)
g-CHLORDANE	ND(4)	ND(4)	ND(4)
a-CHLORDANE	ND(3)	ND(3)	ND(3)
t-NONACHLOR	ND(3)	ND(3)	ND(3)
p,p' DDE	ND(3)	ND(3)	ND(3)
o,p' DDD	ND(7)	ND(7)	ND(7)
p,p' DDD	ND(6)	ND(6)	ND(6)
o,p' DDT	ND(4)	ND(4)	ND(4)
c-NONACHLOR	ND(4)	ND(4)	ND(4)
p,p' DDT	ND(4)	ND(4)	ND(4)
MIREX	ND(4)	ND(4)	ND(4)

Method of Analysis: OC Pesticides Marine Oil - USEPA 505

General Pesticide Analysis

General Pesticides	Result (ppm)		
	F12	F14	F17
Alachlor	ND	ND	ND
Aldrin + Dieldrin	ND	ND	ND
Azinphos-methyl	ND	ND	ND
Bromopropylate	ND	ND	ND
Chlordane	ND	ND	ND
Chlorfenvinphos	ND	ND	ND
Chlorpyrifos	ND	ND	ND
Chlorpyrifos-methyl	ND	ND	ND
Cypermethrin	ND	ND	ND
DDT+isomers	ND	ND	ND
Deltamethrin	ND	ND	ND
Diazinon	ND	ND	ND
Dichhlorvos	ND	ND	ND
Dithiocarbamates	ND	ND	ND
Endusulfan	ND	ND	ND
Endrin	ND	ND	ND
Ethion	ND	ND	ND
Fenitrothion	ND	ND	ND
Fenvalerate	ND	ND	ND
Fonofos	ND	ND	ND
Heptachlor	ND	ND	ND
HCB	ND	ND	ND
Hexachlorocyclohexane isomers	ND	ND	ND
Lindane	ND	ND	ND
Malathion	ND	ND	ND
Methidithion	ND	ND	ND
Parathion	ND	ND	ND
Parathion methyl	ND	ND	ND
Permethrin	ND	ND	ND
Phosalone	ND	ND	ND
Piperonyl butoxide	ND	ND	ND
Primiphos-methyl	ND	ND	ND
Pyrethrine	ND	ND	ND
Quintozene	ND	ND	ND

Method of Analysis: USP<561>
ND: <0.02

Heavy Metals Analysis

Heavy Metals	Result (ppm)		
	F12	F14	F17
Arsenic	<0.1	<0.1	<0.1
Cadmium	<0.01	<0.01	<0.01
Lead	<0.01	<0.01	<0.01
Mercury	<0.01	<0.01	<0.01

Methods of Analysis: USEPA 305.1, 200.7, 200.8, US EPA 245.6

PCBs Analysis

PCBs	Result (ppt)		
	F12	F14	F17
Mono-chloro	ND	ND	ND
Di-chloro	ND	ND	ND
Tri-chloro	ND	85.1	67.4
Tetra-chloro	15.9	27.1	37.2
Penta-chloro	21.6	47.6	25.7
Hexa-chloro	17.1	47.4	38.9
Hepta-chloro	16.3	14.8	18.8
Octa-chloro	ND	ND	ND
Nona-chloro	ND	ND	ND
Deca-chloro	ND	ND	ND
Total PCBs	71	222	188
CRN/GOED Monograph totals	71	75	88
WHO TEQs (ND=0)	0	0	0
WHO TEQs (ND=DL)	0.16	0.04	0.04

Methods of Analysis: PCB - USEPA 1614; Dioxin Like PCB's - Prop 65

Residual Solvents Analysis

Solvents	Result (ppm)		
	F12	F14	F17
Isohexane	<1	<1	<1
Acetone	<1	<1	<1

Method of Analysis: AOCS Ca 2c-25

Mycotoxin Analysis

Mycotoxins	F12	F14	F17
Total Aflatoxins (ng/g)	<5	<5	<5
Total Vomitoxins (µg/g)	<0.5	<0.5	<0.5

Methods of Analysis: MAFLA-01 and MVOM-01

Analysis of Recombinant DNA Content and Total Protein

A procedure was developed for the detection of DNA in EPA-rich triglyceride oil produced from genetically modified *Yarrowia lipolytica*. The procedure consists of DNA extraction and purification steps followed by polymerase chain reaction (PCR)-based detection. Using the procedure, ≥ 100 pg of genetically modified *Y. lipolytica* DNA was detectable when spiked into 1 ml aliquots of EPA-rich triglyceride oil derived from the same strain. No DNA was detected in non-spiked 1 ml aliquots. This indicates that the EPA-rich triglyceride oil contained < 100 pg of *Y. lipolytica* DNA per ml of oil. This procedure can be used for EPA-rich triglyceride oil generated by other production strains as long as the PCR primer binding sites are not modified in these strains.

To assess the potential presence of unintended protein in EPA-rich triglyceride oil, the concentration of total amino acids was determined (see Table 2-3). Total amino acid analysis was determined to be in the range of 11-22 µg/mL, or 0.0011 – 0.0022% (w/w) in the three batches F12, 14 and 17. This result would be a sum of proteins, peptides and free amino acids. The result of extremely low presence of protein in this highly refined oil is consistent with the findings from others, including the Food Allergen Labeling and Consumer Protection Act of 2004, which exempts highly refined oils from food allergen labeling for consumer protection.

APPENDIX 3

SAFETY OF OMEGA-3 FATTY ACIDS

The safety of omega-3 fatty acids is supported by their long history of ingestion as a component of the human diet. Although the high LCPUFA doses used in animal studies (>1g/kg body weight/day) may produce adaptive non-specific effects in liver metabolism and histomorphology, these effects are related to the extra metabolic workload and are not relevant to exposure at much lower doses.

Adverse effects associated with omega-3 polyunsaturated fat consumption have been reported in a few special subpopulations (IOM, 2005). It has been suggested that individuals exhibiting glucose intolerance or diabetic conditions use caution with omega-3 fatty acids as some have required increased doses of hypoglycemic agents (Glauber *et al.*, 1988; Kasim *et al.*, 1988; Friday *et al.*, 1989; Stacpoole *et al*, 1989; Zambon *et al.*, 1992). Clarke *et al.* (1990) reported increased episodes of nosebleeds in individuals with familial hypercholesterolemia during fish oil supplementation. High levels of eicosapentaenoic acid (EPA) content may reduce growth in infants (Carlson *et al.*, 1993).

The FDA critically evaluated available information on the safety on the omega-3 fatty acids, EPA and DHA, and identified three potential safety issues, namely prolongation of bleeding time, modification of glycemic control, and elevation of LDL cholesterol (US FDA, 1993). Similarly, the Societa Italiana di Nutrizone Umana (1996) and Hughes and Pinder (1997) reported that the intake of excessive omega-3 fatty acids may result in toxic effects, including increased bleeding rate, alteration of immune function, and formation of toxic lipoperoxides (Societa Italiana di Nutrizone Umana, 1996; Hughes and Pinder, 1997). It has been noted that the intake of omega-3 fatty acids should also include adequate quantity of vitamin E or tocopherols to limit oxidation of PUFAs by free radicals (Witting and Lee, 1975; Brude *et al.,* 1997; La Guardia *et al.,* 2006).

The results of numerous clinical studies published since the FDA review in 1993 indicate the DHA provided in fish or marine-derived oils at levels up to 6 g DHA/person/day would not be expected to produce adverse effects on these parameters (Kroes *et al.*, 2003). These results are consistent with the FDA conclusion in its 1997 menhaden oil decision that the combined consumption of up to 3 g of DHA + EPA/person/day poses no significant risk for bleeding time, produces no clinically significant effect on glycemic control, and is safe with respect to the effect on LDL cholesterol (FDA, 1997).

The US Department of Health and Human Services Agency for Healthcare Research and Quality (2004) identified 148 studies on omega-3 fatty acids that evaluated over 20,000 subjects for adverse effects. The most common side effects were gastrointestinal complaint, reported among 6.6% of patients taking omega-3s versus 4.3% in placebo groups. An increased incidence of bleeding was not observed, and only 1 of the 148 studies reported such an association in patients randomized to 6 g/day of omega-3. There were no reported deaths or life-threatening illnesses as a consequence of omega-3 consumption. No adverse effects were reported in 77 of the 148 studies. Based on this review, the agency concluded that adverse effects related to consumption of fish oil or α-linolenic supplements appear to be minor.

Wang *et al.* (2006) recently reported the results of an evidence-based, systematic review of the literature on the effects of omega-3 fatty acids consumed as fish, or fish oils rich in EPA and DHA, or as alpha-linolenic acid, on cardiovascular disease outcomes and adverse effects associated with their consumption. Adverse effects were generally considered to be minor. Gastrointestinal side effects were the most often reported, but only 4% of complaints occurred at dosages of less than 3 g/day. GI symptoms increased to approximately 20% at a dose of 4 g/day. Nine studies included in the review addressed the risk of clinically significant bleeding episodes. Four of these studies reported no bleeding in either arm, while in the remaining five, no consistent association found between the omega-3 fatty acid dose and the risk of bleeding, even in those patients taking aspirin or warfarin.

Similarly, in their evidence-based systematic review of the data related to the use of omega-3 fatty acids, Balk *et al.* (2007) found that although only a limited number of the reviewed articles provided information on adverse events, and the reporting of adverse events in these studies was generally inadequate and incomplete (*e.g.*, rates of adverse events were not systematically or consistently monitored and reported), no evidence of substantial adverse events related to omega-3 fatty acid consumption beyond mild gastrointestinal symptoms was found.

Effects on Bleeding Time

A link between omega-3 polyunsaturated fatty acid intake and reduced risk of coronary heart disease has been suggested, with reduced platelet aggregation proposed as a possible mechanism. This effect may also result in prolonged bleeding time, although intervention studies examining the effects of omega-3 fatty acids on bleeding time have produced equivocal results (IOM, 2005).

Thorngren and Gustafson (1981) reported that ingestion of omega-3 fatty acids by individuals taking anticoagulants such as aspirin, warfarin, and coumadin may result in prolonged bleeding times. Collective analysis of numerous clinical studies with EPA/DHA supplements, ranging in dose levels from 2 to 15 g/day, has shown no dose response for EPA and DHA on bleeding times. None of the studies indicated excessive bleeding times, bleeding episodes, or bruising (IOM, 2005). Some studies have indicated an increased risk of hemorrhagic stroke among Greenland Eskimos with high EPA and DHA intakes (Kromann and Green, 1980; Kristensen, 1983). While a prospective study in the U.S. showed no association between intake of omega 3 fatty acids and risk of hemorrhagic stroke, the median intakes ranged from only 0.077 to 0.481 g/day, which were considered to be relatively low levels of intake in the U.S. (Iso *et al.*, 2001; IOM, 2002). Increased lipid peroxidation and oxidative damage of erythrocytes, liver, and kidney membranes and bone marrow DNA have been reported in feeding studies with DHA in laboratory animals (Ando *et al.*, 1998; Yasuda *et al.*, 1999; Song and Miyazawa, 2001; Umegaki *et al.*, 2001); however, consumption of vitamin E reportedly reduced or prevented the oxidative damage (Leibovitz *et al.*, 1990; Ando *et al.*, 1998; Yasuda *et al.*, 1999). Bays (2007) reported that the clinical trials have shown high-dose fish oil omega-3 fatty acid consumption to be safe, even when concurrently administered with other agents that may increase bleeding, such as

aspirin and warfarin. He noted that in certain clinical settings, it could be argued that the reported antiatherothrombotic effects of fish oils rich in omega-3 fatty acids are a benefit that outweighs the unproved bleeding risks, especially in select patients at high risk for thrombosis, which may include patients with acute atherosclerotic CAD.

Likewise, a recent comprehensive report by Harris (2007) concluded that virtually no increased risk for clinically remarkable bleeding has been seen in patients with doses of omega-3 fatty acids up to 7 g/d of DHA and EPA.

Emsley *et al.* (2008) evaluated the effects of EPA treatment on body mass, glucose metabolism, lipid profiles, prolactin secretion, bleeding time, hematology and liver functions in subjects with schizophrenia. Eighty four subjects participated in a 12 weeks, in a randomized, controlled trial in which they were treated with either EPA 2 g/day or placebo in addition to their antipsychotic medication. Forty-seven of these subjects entered a 40-week open-label extension phase of EPA 2 g/day. Seventy-four patients were included in the analysis. The incidence of adverse events was similar for the two groups. There were no significant between group differences. In the open-label extension, there was again a modest increase in BMI. There were no striking changes in any of the safety measures, and no significant between-group differences were recorded. However, there were several significant changes from baseline values in the EPA group in both the blinded and open-label phase, while there were none in the placebo group. In the blinded phase the EPA treated patients showed significant increases in BMI and bleeding time. Compared to the placebo group, the increase in bleeding time approached significance (P=0.06). However, values remained within the normal range and were not associated with clinically significant events. Furthermore, there were no concomitant changes in other hematological measures. There was likewise no effect for EPA on prolactin levels or liver function tests. Based on these data, the authors concluded that 2 g EPA/day is generally well tolerated.

Tanaka *et al.* (2008) reported no increase in bleeding tendency in patients with nonalcoholic steatohepatitis (NASH) with advanced fibrosis following treatment with 2700 mg EPA/day for 12 months. Likewise, body weight, blood glucose, insulin, and adiponectin concentrations remained unchanged.

As reviewed by Harris (2009), substudies conducted as part of the JELIS trial demonstrated that EPA treatment was not associated with an increased risk for clinically significant bleeding.

Effects on LDL cholesterol

Prospective cohort studies and a trial of secondary prevention of ischemic heart disease suggested that intakes of greater than 1 g/day of omega-3 LCPUFAs offer protection from fatal ischemic heart disease (Hu *et al.*, 2002; Albert *et al.*, 1998, Anonymous, 1999). Likewise, a mixture of EPA and DHA has been shown to lower serum triacylglycerol concentrations, though a slight and, concurrent increase in LDL cholesterol concentrations may occur, particularly in

hypertriacylglycerolemic subjects (Harris, 1989; 2003; Davidson *et al.*, 1997; Anonymous, 1999); Mori *et al.*, 2000; Montori *et al.*, 2000; Theobald *et al.* 2004) while favorable effects on lipoprotein particle distributions may occur (Li *et al.*, 2004). Increased LDL levels are associated with increased risk of cardiovascular disease, thus increases in LDL could counteract other beneficial cardiovascular effects associated with omega-3 oils.

Studies using purified EPA (Harris, 1997) or DuPont EPA-rich triglyceride oil generally have not reported increases in LDL cholesterol. DuPont EPA-rich triglyceride oil, rich in EPA but containing low levels of DHA compared to fish oils, would thus be anticipated to offer the beneficial health effects associated with omega-3 fatty acids without the potential LDL-increasing effects attributed to DHA.

A decrease in total VLDL particle concentrations has previously been reported after consumption of EPA and DHA-rich fish oil (Li *et al.*, 2004). In the 2005 study by Goyens and Mensink, however, the proportion of EPA in plasma phospholipids was significantly increased with the consumption of both experimental diets, whereas DHA was not affected. This suggested to authors that EPA may be responsible for the changes reported in VLDL concentrations after consumption of fish oil.

Emsley *et al.* (2008) reported significant decreases in total cholesterol and HDL levels following treatment with 2 g/day EPA in a 12 week randomized, controlled trial followed by a 40-week open-label extension phase.

Tanaka *et al.* (2008) reported no increase in bleeding tendency in patients with nonalcoholic steatohepatitis (NASH) with advanced fibrosis following treatment with 2700 mg EPA/day for 12 months. Likewise, body weight, blood glucose, insulin, and adiponectin concentrations remained unchanged.

The dose–response effects of EPA oil, administered in combination with α-tocopherol, in young and older male subjects on blood pressure, plasma lipid, α-tocopherol and soluble adhesion molecule concentrations, the susceptibility of lipoproteins to copper-induced peroxidation, and indices of oxidative stress were evaluated by Cazzola *et al.* (2008). Young (18 to 42 years) and older males (53 to 70 years) received EPA at doses of 0, 1.35, 2.7, or 4.05 g/day for 12 weeks. No effects blood pressure or on plasma total, LDL or HDL cholesterol were observed. EPA lowered plasma triacylglycerols, with the maximal effect at the lowest dose. Plasma lipoperoxides were decreased in all groups. EPA also decreased the lag time of copper-induced lipoprotein peroxidation and the ratio of reduced to total glutathione in the older subjects. The highest dose of EPA increased soluble E-selectin in young subjects, while increasing EPA tended to decrease soluble intercellular adhesion molecule 1 in young and older subjects. Young and older males will gain cardiovascular benefit from increased intake of EPA. Young males are unlikely to suffer adverse consequences from high EPA intake, whereas in older males there may be an increased risk of lipoprotein peroxidation. These authors attributed this risk to diminished antioxidant defenses compared to the younger subjects, a greater in plasma

triacylglycerols than in lipid hydroperoxides, which altered the ratio between the oxidized and native lipids, and due to the added "EPA load".

Effects on Glycemic Control

Montori *et al.* (2000) reviewed the data from eighteen trials including 823 type 2 diabetic subjects dosed with 3 to 18 g of fish oil per day for 12 weeks. Researchers found that fish oil supplementation in type 2 diabetics lowered triglycerides, raised LDL cholesterol, and had no statistically significant effect on fasting glucose or glycemic control.

Emsley *et al.* (2008) reported a trend towards increased fasting blood glucose (P=0.06) following treatment with 2 g/day EPA in a 12 week randomized, controlled trial followed by a 40-week open-label extension phase.

Tanaka *et al.* (2008) reported no increase in bleeding tendency in patients with nonalcoholic steatohepatitis (NASH) with advanced fibrosis following treatment with 2700 mg EPA/day for 12 months. Likewise, body weight, blood glucose, insulin, and adiponectin concentrations remained unchanged.

Alteration of Immune Function

Suppression of various aspects of human immune function has been reported *in vitro* or *ex vivo* in peripheral blood mononuclear cells, in isolated neutrophils or monocytes, or in subjects provided omega-3 polyunsaturated fatty acids as a supplement or in experimental diets. The minimum dose observed for such an effect was 0.9 g/day EPA and 0.6 g/day DHA given as fish oil for 6 to 8 weeks to healthy adults (Cooper *et al.*, 1993). Single treatment studies suggest that intakes of EPA and DHA at levels 7 to 15 times the typical U.S. intake diminish the potential of the immune system to attack pathogens (Lee *et al.*, 1985; Schmidt *et al.*, 1989; Kelley *et al.*, 1998, 1999). These doses have also reportedly been associated with suppression of inflammatory responses. However, Blok *et al.* (1997) administered fish oil supplements (0, 3, 6, or 9 g/day fish oil supplement resulting in EPA intakes of 0, 0.81, 1.62, or 2.43 g/day and DHA intakes of 0, 0.16, 0.33, or 0.49 g/day) for 1 year to 58 healthy men. *Ex vivo* endotoxin stimulated production of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, or IL-1Ra did not differ among treatment groups up to 6 months after supplementation was stopped, supporting a lack of long-term adverse effects of fish oil supplementation on cytokine activity.

Hawkes *et al.* (2002) examined the effects of supplementation with DHA-rich tuna oil on the human milk cytokines interleukin 1β, interleukin 6, and tumour necrosis factor α. DHA levels in maternal plasma, human peripheral blood mononuclear cells (PBMCs), milk, and human milk cells (HMCs) were increased in a double-blind, prospective, randomized trial in which mothers of healthy full-term infants were asked to consume a nutritional supplement containing either 300 mg DHA + 70 mg EPA or 600 mg DHA + 140 mg EPA for 4 weeks. DHA concentrations reportedly increased in a linear manner. Despite the increased n-PUFA concentrations in

relevant tissues, no perturbations in cytokine concentrations were observed. Side effects were reported by 7 to 15% of the subjects and included gas (n=4), fishy burps (n=4), unsettled babies (n=1), and rash (n=1).

The effects of dietary fatty acid supplementation from various sources on endothelial function and tone in healthy subjects were examined by Khan *et al.* (2003). Volunteers took oil supplements containing oleic acid rich sunflower oil, evening primrose oil, soybean oil, tuna fish oil (6% EPA acid and 27% DHA), or tuna/evening primrose oil mix for 8 months. Endothelium-dependent and endothelium-independent responses to iontophoresis of acetylcholine and sodium nitroprusside, respectively, were measured in the forearm skin using laser Doppler imaging. Acetylcholine responses were significantly improved after tuna oil supplementation while significant positive correlations were also seen between acetylcholine responses and omega-3 fatty acid levels in the plasma and erythrocyte membrane phospholipids in subjects consuming the tuna oil supplements. No significant differences were observed with any of the other oils. Total cholesterol, red and white blood cell counts, hemoglobin, and hematocrit levels were not significantly affected by tuna oil supplementation. The authors concluded that modification of the diet involving a 6% increase in EPA acid and 27% DHA might have significant beneficial effects on cardiovascular function and health.

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APPENDIX 4

SAFETY OF THE MICROORGANISM UTLIZED IN THE PRODUCTION OF EPA-RICH TRIGLYCERIDE OIL

Yarrowia lipolytica is a dimorphic ascomycetous yeast that is readily isolated from various food products. It was originally classified as a *Candida lipolytica* until the perfect form (sexual stage) was identified, and then it was reclassified first as *Endomycopsis lipolytica*, then as *Saccharomycopsis lipolytica* and finally as *Y. lipolytica*. All strains are heterothallic (mating types MATA and MATB) and both haploid and diploid cells are vegetatively stable. Mating frequencies are very low and dependent on genetic background, growth medium, cell density, growth phase and temperature. *Yarrowia lipolytica* is not considered pathogenic (Holzschu *et al.,* 1979).

Y. lipolytica is a strict aerobe with a maximum growth temperature of approximately 32 °C, and is able to utilize aliphatic carbon sources, including fatty acids and alkanes (Kerscher *et al.*, 2002; Madzak *et al.*, 2004). It has been used for numerous biotechnological applications. Traditional roles include processing of cheese and other dairy products, but it has also been exploited for the production of single cell protein for animal feeds, for the production of organic compounds like citric acid and α-ketoglutaric acid, and for the bioconversions of aliphatic compounds (Arzumanov *et al.,* 2000; Chernyavskaya *et al.*, 2000 ; Suzzi *et al.*, 2001; Vannini *et al.*, 2001).

Y. lipolytica differs considerably from other ascomycetous yeasts on the genetic level. Its secretory apparatus (importance of the co-translational pathway, high efficiency, low overglycosylation) makes it closer to mammalian cells than to many other yeasts in some regards, and thus makes it a useful host, especially for the production of complex proteins. Vectors and recipient strains are available and easy to handle, and amplification systems and the potential for scaled up production has been studied (Madzak *et al.*, 2004).

Oleaginous strains can accumulate up to 35 to 40% dcw as lipid when starved for nitrogen in the presence of excess glucose or alkanes. The composition of the lipid depends on the carbon source. Lipid from glucose-grown cells is comprised mostly of triglycerides and a small amount of sterols. Oleic acid is the prominent fatty acid. Lipids from oleic acid-grown cells contain more sterols and oleic acid than glucose-grown cells.

The *Y. lipolytica* genome has been sequenced. It is comprised of 21Mb DNA containing 6650 ORFs arranged on 6 chromosomes. It is considered to be amenable for genetic development and to be a reliable and versatile host for heterologous production as it combines performance and regularity (Ogrydziak, 1988; Muller *et al.*, 1998; Kerscher *et al.,* 2002; Madzak *et al*, 2004). As of 2004, forty-two heterologous proteins have been produced using *Y. lipolytica* (Madzak *et al.*, 2004). Genetic transformation occurs by integration of linear DNA into the genome by homologous or non-homologous recombination. Transformants are selected by complementation of auxotrophic mutations.

Y. lipolytica is GRAS for the commercial production of food grade citric acid (21 CFR 173.165). It has also been produced commercially as a source of single cell protein for animal feeds

(Toprina) and to produce γ-decalactone as a flavoring agent. Other applications include production of wax esters, 2-ketoglutaric, 2-hydroxyglutaric and isopropylmalic acids and secretion of heterologous proteins, including several food enzymes. *Y. lipolytica* secretes lipases and proteases that contribute to the ripening of dairy products. Its lipase may be also useful for the synthesis of 2,4-dimethylglutaric acid monoesters and transesterification of meso-cyclopentane diols.

Pathogenicity and Virulence

Y. lipolytica is not considered to be pathogenic (Holzschu *et al.*, 1979), but has been isolated in association with disease. In three separate studies (Holzschu *et al.*, 1979; Walsh *et al.*, 1989; Yoshida and Hashimoto, 1986) *Y. lipolytica* at high cell concentrations (10^7-10^8 CFU) was injected intravenously (i.v.) into mice with little indication that this organism could invade, colonize, and produce disease. Although the relevance of these studies for occupational health is limited, these studies do provide potency information relative to other pathogenic and nonpathogenic yeasts. For example in the Holzschu paper, *Candida albicans* was clearly pathogenic, while *Saccharomyces cereveisiae* (bakers yeast) was clearly not pathogenic. *Y. lipolytica* acted similarly to bakers yeast in the same test. Similarly, the pathogenicity of *Y. lipolytica* was studied relative to other yeast species by administering 10^7 CFU intravenously to mice and observing them for clinical signs and mortality for 14 days. Liver, kidney, and brain were collected and cultured for *Y. lipolytica*. Out of 42 yeast species *Y. lipolytica* species ranked 25 and 34, indicating a lower potential for pathogenicity than many of the other yeasts tested (Yoshida and Hashimoto, 1986). In another study, groups of 2 male CD-1 mice received 0.1 mL of 10-fold dilutions (highest concentration was 10^8 CFU) *via* the tail vein. Clinical signs and gross pathology was recorded after 14 days. All mice survived, and no *Y. lipolytica* was found in organs (except for 2 kidney abscesses in one mouse) (Walsh *et al.*, 1989).

Cases where *Y. lipolytica* has been isolated associated with disease include nail infection (Segal *et al.*, 1996), an infection associated with an extrinsically contaminated intravenous anesthetic agent (McNeil *et al.*, 1999), an infection associated with a cholescytectomy (Hazen, 1995), five cases of fungemia reported in a 9-week period in a pediatric ward (Shin *et al.*, 2000), fungemia associated with a leukemia patient (Chang *et al.*, 2001), three cases of traumatic ocular infection, one case of catheter-associated thrombophlebitis, and one case of polymicrobial sinusitis (Walsh *et al.*, 1989). In most if not all cases, the isolation of *Y. lipolytica* was associated with medically compromised individuals or other pathogenic microorganisms.

Y. lipolytica is adaptable to a wide range of physical and chemical conditions (Zvyagilskaya *et al.*, 2001). It can antagonize the growth of pathogenic species (Lanciotti *et al.*, 2005). *Y. lipolytica* has a high hydrophobicity and adherence to surfaces among yeast species (Zvyagilskaya *et al.*, 2001). *Y. lipolytica* is osmotolerant and halotolerant, and is able to thrive at a pH range of 4.5 to 7.5. And finally, *Y. lipolytica* is resistant to many antibiotics (Romanos *et al.*, 1992). All of these factors contribute to the hardy ubiquitous nature of this organism.

In spite of the range of environments colonized by *Y. lipolytica,* specific virulence factors suggestive of pathogenicity were not identified in the literature. Two virulence factors associated with the ability of yeast to cause disease are phospholipase A and lysophospholipase (enzymes associated with adherence) and the ability to impair the host immune response. *Y. lipolytica* was among a panel of 20 yeast strains tested for their ability to adhere to human gastrointestinal cells and stimulate the production of IL-8 (proinflammatory cytokine). *Y. lipolytica* did not adhere nor elicit cytokine production, while known pathogenic yeast did exhibit these capabilities (Kumura, 2004). The i.v. studies in mice and the low incidence of isolation in diseased patients already medically compromised, support a low virulence potential. *Y. lipolytica* can survive in human tissues and fluids, but does not function as a virulent pathogen.

Toxicity

Two lines of evidence suggest a low degree of toxicity for *Y. lipolytica*. First, the results from the group of i.v. mouse studies described above did not suggest toxicity. The second is based on feeding studies of single-cell protein derived from *Y. lipolytica*. In the latter case, viable microorganisms were not considered, only the remaining cell debris from the fermentation process.

In the 1960's, an animal feed protein-containing product from the dried cell mass of *Y. lipolytica* (Toprina) was tested in animals (*e.g.*, finishing pigs, beagle dogs) for health effects (Bizzi *et al.*, 1980; Sedgman *et al.*,1985; Davies *et al.*, 1977; Jackson and Kirkpatrick, 1978; Hanssen and Farstad, 1980; Newberne and Young, 1975). Although these studies were limited with respect to full toxicological assessment; clinical signs, weight gains, and feed consumptions suggest a low toxicity to relatively high concentrations of these proteins in feed.

Toxin Production

There have been no reports of *Y. lipolytica* producing toxins in humans or animals. The genetic modifications made to this organism are designed for increased oil production and are not related to toxin production.

Extracellular Products

Y. lipolytica produces an abundance of extracellular proteolytic and lipolytic enzymes (*e.g.,* alkaline protease, RNase, lipase, and acid proteases). These enzymes are important in the cheese ripening process (Suzzi et al., 2001).

Hazard/Risk Information for Similar Microorganisms

Two species of yeasts (*Saccharomyces cerevisiae* and *Saccharomyces uvarum*) are listed in BioTSCA as exempt based on the EPA final risk assessments indicating a low level of risk when these yeasts are present in or associated with food processes. Their ubiquitous nature, lack of pathogenicity, lack of toxins produced against humans and animals, and low degree of virulence support their exempt BioTSCA status (US EPA, 1997a,b).

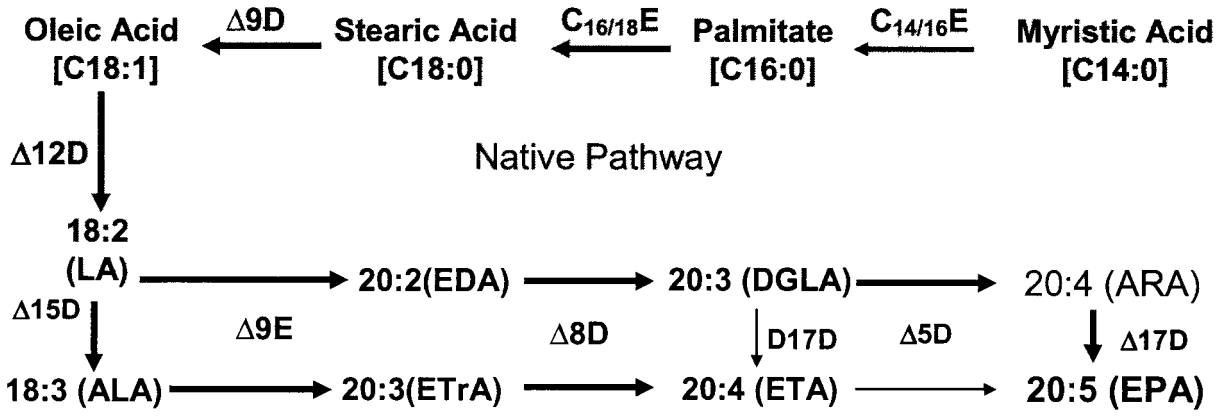
EPA-Rich Triglyceride Oil Production Organism

Yarrowia lipolytica can accumulate oil to at least 30% dcw as an energy reserve when starved for nitrogen in the presence of excess glucose, but the only polyunsaturated fatty acid it synthesizes is linoleic acid (C18:2n-6). DuPont enabled the synthesis of EPA (C20:5n-3) by integrating into the *Y. lipolytica* genome single or multiple copies of a variety of well-characterized genes involved in efficient fatty acid synthesis and specifically in omega-3 fatty acid synthesis. All genes were synthesized *in vitro* according to respective sequences from source microorganisms and have been sequenced and, in several cases, codon-optimized for expression in *Yarrowia.* The EPA-rich triglyceride oil may be produced with an EPA content ranging from 35 to 55% by using strains with a different copy numbers and promoters. Genetic transformation occured by integration of linear DNA into the genome by homologous or non-homologous recombination and using selectable markers that complemented auxotrophic mutations. A combination of targeted and random insertion was used to incorporate all of the DNA modifications. The genetic elements used to control gene expression (e.g., promoters and terminators) are derived from *Y. lipolytica* wild-type. Information about the structural genes is presented in below:

Table 1: Selection of Structural genes used in the production strain

Enzyme	Substrate	Product	Source of Gene
Δ5-desaturase	C20:3n-6 C20:4n-3	C20:4n-6 C20:5n-3	*Euglena gracilis* *Eugalena anabaena* *Peridinium* sp *CCMP626*
Δ8-desaturase	C20:2n-6 C20:3n-3	C20:3n-6 C20:4n-3	*Euglena gracilis* *Eugalena anabaena*
Δ9-desaturase	C18:0	C18:1n-9	*Yarrowia lipolytica*
Δ12-desaturase	C18:1n-9	C18:2n-6	*Fusarium moniliforme*
Δ17-desaturase	C20:3n-6 C20:4n-6	C20:4n-3 C20:5n-3	*Pythium aphanidermatum* *Phytophthora ramorum*
Δ9-elongase	C18:2n-6 C18:3n-3	C20:2n-6 C20:3n-3	*Euglena gracilis* *Eugalena anabaena* *Eutreptiella sp.* CCMP389
$C_{16/18}$-elongase	C16:0	C18:0	*Mortierella alpina*
Malonyl-CoA Synthetase	Malonate and CoA	Malonyl-CoA	*Rhizobium leguminosarum bv. viciae 3841*
Diacylglycerol cholinephospho Transferase (CPT)	CDPcholine, 1,2 diacyl-glycerols	Phosphatidyl-choline	*Yarrowia lipolytica*
Cholinephosphate cytidylyltransferase (PCT)	CTP and choline phosphate	CDP-choline	*Yarrowia lipolytica*
Acyl-CoA:1-acylglycerol-3-phosphate acyltransferase (LPAAT)	Acyl-CoAs Lysophosphatidic acid or phospholipids	Altered composition of acyl-CoAs and phospholipids	*Mortierella alpina*

Metabolic pathway introduced into the production strain



The safety of the production organism as well as the EPA-rich triglyceride oil was assessed using the decision tree approach by Pariza and Johnson (2001), developed for the evaluation of microbial enzyme preparation safety used in food processing as shown below. As a result of this assessment, the test article (EPA-rich triglyceride oil) is acceptable for use in foods. It is noteworthy that the EPA-rich triglyceride oil is highly refined and absent of recombinant DNA and proteins, a characteristic that differentiates the oil from often crude enzyme/protein preparations used in food processing.

The decision tree was developed to evaluate the safety of microbial enzyme preparations. Some of the questions in the decision tree were modified to make them applicable for safety evaluation of the EPA-rich triglyceride oil ("The oil"). Though the production organism produces recombinant enzymes that enable oil accumulation, the decision tree was not applied for recombinant enzymes, since proteins are removed from the oil during the purification process. Modified questions are marked as such below.

EPA-Rich Triglyceride Oil and Production Organism Safety Evaluation Using the Pariza and Johnson (2001) Decision Tree

1. Is the production strain genetically modified?
   Answer: **Yes** (If yes, go to 2.)

2. Is the production strain modified using rDNA techniques?
   Answer: **Yes** (If yes, go to 3.)

3. Issues related to the introduced DNA
   a. Does the oil have a history of safe use in food? (modified)
      Answer: **No**. All the oil components, including the various fatty acids and EPA as primary component have a history of safe use in food. However, since the composition of the EPA-rich triglyceride oil is novel, as compared to incumbent oils, the answer to this question is "no". (If no, go to 3b)

   b. Is the NOAEL for the oil in appropriate short-term oral studies sufficiently high to ensure safety? (modified)
      Answer: **Yes**. Exposure of rats to the EPA-rich triglyceride oil, dosed up to 100% (approximately 976 mg/kg/day of EPA) for up to 90 days, produced no adverse effects on in-life or clinical pathology parameters. The maximum intake of EPA-rich triglyceride oil resulting from these intended food uses would be approximately 5.45-8.57 g/person/day (for 55 or 35% EPA-rich triglyceride oil. For a 60 kg adult, this intake estimate would correspond to approximately 0.09-0.14 g/kg body weight/day EPA-rich triglyceride oil, respectively. (If yes, go to 3c)

   c. Is the oil free of transferable antibiotic resistance gene DNA? (modified)
      Answer: **Yes.** No antibiotic resistance marker has been used in the construction of the production organism. In addition, any DNA from the production organism is removed from the oil during the purification process. (If yes, go to 3e)

   d. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?
      Answer: **Yes**. To eliminate the possibility of introducing adventitious sequences into the production organism, all genes introduced into the strain were chemically

synthesized rather than being isolated. Structural genes are listed in Appendix 4. In all cases the DNA's sequences, structures and functions are fully known and well characterized. Therefore, the inserted DNA meets the "well characterized" criteria. (If yes, go to 4)

4. Is the introduced DNA randomly integrated into the chromosome?
   Answer: **Yes**. Some of the cassettes (Promoter – Structural gene(s) – Terminator) are randomly integrated into the chromosome. (If yes, go to 5)

5. Is the production strain sufficiently well characterized so that one may reasonable conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification that was employed?
   Answer: **Yes.** Several observations support the answer of "yes". The production strain is based upon an organism that is not known to produce toxins or other unsafe metabolites. All cassettes integrated into the chromosome contain a terminator genetic element to ensure transcription termination of intended structural genes. The stability of the genetic modifications of the production strain has been demonstrated over hundreds of generations. (If yes, go to 6)

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?
   Answer: **No**. Although the production strain is derived from a safe lineage, the answer is no, since we are not aware if this evaluation procedure has been applied before to the *Yarrowia lipolytica*. (If no, go to 7)

7. Is the organism nonpathogenic?
   Answer: **Yes.** (If yes, go to 8)

8. Is the test article free of antibiotics?
   Answer: **Yes.** No antibiotics are used in the manufacturing of the EPA-rich triglyceride oil. (If yes, go to 9)

9. Is the test article free of oral toxins known to be produced by other members of the same species?
   Answer: **Yes.** No reports of have been identified that indicate oral toxin production of *Y. lipolytica*. Repeat dose studies in rats with EPA-rich triglyceride oil have shown no adverse effects. (If yes, go to 11)

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to demonstrate safety?

    Answer: **Yes.** The preclinical safety studies conducted with the EPA-rich triglyceride oil established a NOAEL of 976 mg/kg/day of EPA (highest dose tested). Based on a maximum intake of 3.0 g/person/day of EPA through the intended food-uses, the maximum intake for a 60 kg adult would correspond to approximately 50 mg/kg body weight/day of EPA, a margin of safety of about 20-fold.

    If yes, the test article is ACCEPTED.

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APPENDIX 5

PRECLINICAL TRIAL DATA

Results of the Bacterial Mutagenicity Assay (Initial Toxicity Mutation Assay)

Test Article Id : H-28297
Study Number : AC14XM.503.BTL
Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Activation Condition : None

Dose (µg per plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	16 ± 1	93 ± 1	10 ± 2	9 ± 1	22 ± 0
1.5	15 ± 1	103 ± 1	10 ± 4	12 ± 2	26 ± 10
5.0	16 ± 1	119 ± 5	9 ± 4	12 ± 1	24 ± 3
15	11 ± 4	106 ± 13	12 ± 0	14 ± 4	28 ± 1
50	13 ± 3	107 ± 13	17 ± 2	12 ± 4	21 ± 1
150	15 ± 2	109 ± 2	7 ± 0	14 ± 1	32 ± 1
500	21 ± 6	101 ± 1	11 ± 1	14 ± 0	22 ± 6
1500	20 ± 6	112 ± 17	14 ± 2	15 ± 5	24 ± 2
5000	14 ± 13	107 ± 6	13 ± 3	10 ± 0	24 ± 3
Positive	221 ± 13	346 ± 15	267 ± 17	2047 ± 40	331 ± 13

Activation Condition : Rat Liver S9

Dose (µg per plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	21 ± 11	119 ± 13	10 ± 3	7 ± 4	40 ± 9
1.5	23 ± 1	135 ± 19	10 ± 7	9 ± 7	34 ± 8
5.0	26 ± 0	143 ± 7	11 ± 1	8 ± 3	40 ± 5
15	18 ± 1	167 ± 23	10 ± 4	8 ± 4	24 ± 4
50	19 ± 1	155 ± 1	10 ± 1	7 ± 2	37 ± 2
150	22 ± 3	144 ± 11	18 ± 6	9 ± 1	33 ± 6
500	20 ± 3	184 ± 34	14 ± 4	8 ± 2	32 ± 1
1500	21 ± 5	194 ± 31	17 ± 4	9 ± 2	24 ± 9
5000	24 ± 3	172 ± 50	15 ± 0	12 ± 4	23 ± 5
Positive	422 ± 37	412 ± 68	113 ± 9	68 ± 11	319 ± 6

Vehicle = Vehicle Control
Positive = Positive Control (50 µL plating aliquot)
Plating aliquot = 50 µL

Results of the Bacterial Mutagenicity Assay (Confirmatory Mutagenicity Assay)

Test Article Id : H-28297
Study Number : AC14XM.503.BTL
Experiment No : B2 and B3
Activation Condition : None

Dose (µg per plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	18 ± 3	142 ± 19	11 ± 3	14 ± 4	22 ± 1
50	16 ± 3	144 ± 12	10 ± 5	10 ± 2	22 ± 3
150	16 ± 3	134 ± 7	13 ± 2	13 ± 5	23 ± 4
500	18 ± 1	149 ± 7	15 ± 3	10 ± 2	22 ± 1
1500	23 ± 3	137 ± 10	15 ± 1	10 ± 3	25 ± 3
5000	17 ± 4	138 ± 28	16 ± 5	11 ± 4	21 ± 3
Positive	261 ± 65	593 ± 59	455 ± 28	1176 ± 71	207 ± 15

Activation Condition : Rat Liver S9

Dose (µg per plate)	TA98	TA100[a]	TA1535	TA1537	WP2 uvrA
Vehicle	19 ± 9	109 ± 16	13 ± 3	10 ± 3	29 ± 0
50	31 ± 2	118 ± 9	14 ± 4	9 ± 6	23 ± 4
150	22 ± 5	120 ± 6	12 ± 1	10 ± 3	31 ± 2
500	23 ± 7	111 ± 2	11 ± 2	9 ± 3	26 ± 6
1500	26 ± 7	107 ± 25	12 ± 1	8 ± 6	23 ± 6
5000	26 ± 3	120 ± 20	11 ± 3	12 ± 3	25 ± 1
Positive	700 ± 533	261 ± 7	98 ± 25	54 ± 20	242 ± 38
Positive[b]		438 ± 13			

Vehicle = Vehicle Control
Positive = Positive Control (50 µL plating aliquot)
Plating aliquot = 50 µL
[a] Data from Experiment B3
[b] 2-Aminoanthracene at 2.0µg per plate

Data Summary for the Initial Assay (-S9, 4-hr exposure)

DOSE LEVEL (µg/mL)	PRECIP.	TOTAL SUSP. GROWTH	% SUSP. GROWTH	TFT COLONIES PLATE COUNTS 1	2	3	MEAN	VC COLONIES PLATE COUNTS 1	2	3	MEAN	TOTAL MUTANT FREQUENCY (PER 10^6 CELLS)	INDUCED MUTANT FREQUENCY (PER 10^6 CELLS)	% TOTAL GROWTH
SOLVENT 1		20.9	100	26	26	26	**26**	202	246	186	**211**	25	N/A	100
SOLVENT 2		20.7		*	31	19	**25**	140	140	140	**140**	36		
50 A		17.9	86	39	20	34	**31**	*	145	136	**141**	44	**14**	69
50 B		20.8	100	43	24	27	**31**	123	139	151	**138**	46	**15**	78
100 A		19.5	93	31	18	16	**22**	172	159	155	**162**	27	**-3**	86
100 B		19.3	93	22	26	*	**24**	182	155	162	**166**	29	**-1**	88
200 A		18.6	90	26	27	13	**22**	153	159	137	**150**	29	**-1**	76
200 B		19.3	92	15	19	18	**17**	131	152	182	**155**	22	**-8**	82
350 A		18 9	91	22	22	26	**23**	152	133	143	**143**	33	**3**	74
350 B		19 7	95	34	26	36	**32**	98	136	163	**132**	48	**18**	71
500 A	P	20.4	98	30	26	30	**29**	136	151	137	**141**	41	**10**	79
500 B	P	18.1	87	23	30	34	**29**	148	149	156	**151**	38	**8**	75
POSITIVE CONTROL:		Methyl methanesulfonate (MMS)								(µg/mL)				
20		8.8	42	35	42	22	**33**	26	7	13	**15**	430	**400**	4
15		12.2	58	73	79	78	**77**	35	42	40	**39**	393	**363**	13

MEAN SOLVENT TOTAL SUSPENSION GROWTH: 20.8
MEAN SOLVENT CLONING EFFICIENCY: 88%
MEAN SOLVENT MUTANT FREQUENCY: 30 *(PER 10^6 CELLS)*

Solvent = DMSO A and B or 1 and 2 are duplicate cultures

* - Plate lost to contamination P = precipitating concentration

Data Summary for the Initial Assay (+S9, 4-hr exposure)

DOSE LEVEL (µg/mL)	PRECIP.	TOTAL SUSP. GROWTH	% SUSP GROWTH	TFT COLONIES PLATE COUNTS 1	2	3	MEAN	VC COLONIES PLATE COUNTS 1	2	3	MEAN	TOTAL MUTANT FREQUENCY (PER 10^6 CELLS)	INDUCED MUTANT FREQUENCY (PER 10^6 CELLS)	% TOTAL GROWTH
SOLVENT 1		16.8	100	20	28	18	**22**	167	147	118	**144**	31	N/A	100
SOLVENT 2		17.9		34	22	22	**26**	118	105	174	**132**	39		
50 A		15 3	88	30	27	31	**29**	131	133	118	**127**	46	**11**	81
50 B		18.6	107	30	22	30	**27**	131	105	133	**123**	44	**10**	96
100 A		16 9	98	16	23	13	**17**	141	160	162	**154**	22	**-12**	109
100 B		16 9	97	9	28	27	**21**	92	135	151	**126**	34	**-1**	89
200 A		15.3	88	19	39	23	**27**	136	135	132	**134**	40	**5**	86
200 B		16.9	98	16	27	34	**26**	132	143	164	**146**	35	**0**	103
350 A		16.8	97	20	23	23	**22**	132	145	141	**139**	32	**-3**	98
350 B		16.0	92	16	15	22	**18**	106	135	137	**126**	28	**-7**	84
500 A	P	12 3	71	23	19	15	**19**	166	144	160	**157**	24	**-11**	80
500 B	P	12.3	71	19	27	15	**20**	156	145	149	**150**	27	**-8**	77
POSITIVE CONTROL:		7,12-dimethylbenz(a)anthracene (DMBA)								(µg/mL)				
1.25		1 4	10	82	129	129	**113**	31	35	34	**33**	680	**645**	2
1		3.1	18	98	123	144	**122**	63	57	48	**56**	435	**400**	7

MEAN SOLVENT TOTAL SUSPENSION GROWTH: 17.3
MEAN SOLVENT CLONING EFFICIENCY: 69%
MEAN SOLVENT MUTANT FREQUENCY: 35 *(PER 10^6 CELLS)*

Solvent = DMSO A and B or 1 and 2 are duplicate cultures P = precipitating concentration

Data Summary for the Extended Treatment Assay (-S9, 24-hr exposure)

DOSE LEVEL (µg/mL)	PRECIP.	TOTAL SUSP. GROWTH	% SUSP. GROWTH	TFT COLONIES				VC COLONIES				TOTAL MUTANT FREQUENCY (PER 10^6 CELLS)	INDUCED MUTANT FREQUENCY (PER 10^6 CELLS)	% TOTAL GROWTH
				PLATE COUNTS				PLATE COUNTS						
				1	2	3	MEAN	1	2	3	MEAN			
SOLVENT 1		40.0	100	13	33	35	27	114	106	139	120	45	N/A	100
SOLVENT 2		39 6		24	26	*	25	139	151	****	145	34		
50 A		34 0	85	29	29	40	33	133	109	128	123	53	13	79
50 B		33 9	85	20	49	47	39	93	97	128	106	73	33	68
100 A		39.6	99	35	43	52	43	95	106	87	96	90	50	72
100 B		37 8	95	55	45	37	46	81	120	88	96	95	55	69
200 A		37 1	93	18	32	20	23	130	123	118	124	38	-2	87
200 B		34.6	87	18	49	49	39	115	97	139	117	66	26	77
350 A		35 0	88	22	13	27	21	104	123	144	124	33	-6	82
350 B		36.7	92	32	36	31	33	*	*	*				
500 A	P	39.1	98	27	14	37	26	124	120	86	110	47	7	81
500 B	P	39.3	99	19	40	23	27	109	144	*	127	43	3	94
POSITIVE CONTROL:				Methyl methanesulfonate (MMS)				(µg/mL)						
7.5		17 3	43	55	43	54	51	18	23	19	20	507	467	7
5		25 4	64	112	87	60	86	109	33	75	72	239	199	35
MEAN SOLVENT TOTAL SUSPENSION GROWTH: 39.8														
MEAN SOLVENT CLONING EFFICIENCY: 66%														
MEAN SOLVENT MUTANT FREQUENCY: 40 (PER 10^6 CELLS)														

Solvent = DMSO A and B or 1 and 2 are duplicate cultures * - plate lost to contamination

P = precipitating concentration ****-Actual value cannot be determined based on hard evidence

Summary of bone Marrow Micronucleus Analysis Following a Single Oral Administration of EPA-rich triglyceride oil in ICR Mice

Treatment (20 mL/kg)	Sex	Time (hr)	Number of Animals	PCE/Total Erythrocytes (Mean +/- SD)	Change from Control (%)	Number of MPCE/1000 PCE (Mean +/- SD)	Number of MPCE/PCE Scored
Olive Oil	M	24	5	0.448 ± 0.04	---	0.8 ± 0.45	8 / 10000
	F	24	5	0.478 ± 0.04	---	0.7 ± 0.27	7 / 10000
H-28297							
25%	M	24	5	0.470 ± 0.05	5	0.8 ± 0.57	8 / 10000
	F	24	5	0.474 ± 0.03	-1	0.8 ± 0.27	8 / 10000
50%	M	24	5	0.463 ± 0.04	3	0.7 ± 0.76	7 / 10000
	F	24	5	0.465 ± 0.04	-3	0.9 ± 0.22	9 / 10000
100%	M	24	5	0.481 ± 0.03	7	0.7 ± 0.27	7 / 10000
	F	24	5	0.478 ± 0.03	0	0.7 ± 0.57	7 / 10000
Cyclophosphamide							
50 mg/kg	M	24	5	0.461 ± 0.02	3	26.5 ± 4.51	*265 / 10000
	F	24	5	0.465 ± 0.03	-3	25.5 ± 2.78	*255 / 10000
Olive Oil	M	48	5	0.518 ± 0.01	---	0.7 ± 0.57	7 / 10000
	F	48	5	0.506 ± 0.01	---	0.6 ± 0.22	6 / 10000
H-28297							
100%	M	48	5	0.499 ± 0.02	-4	0.0 ± 0.00	0 / 10000
	F	48	5	0.513 ± 0.02	1	0.5 ± 0.50	5 / 10000

*Statistically significant increase, $p \leq 0.05$ (Kastenbaum-Bowman Tables)

Induction of Micronucleated Polychromatic Erythrocytes in Bone Marrow Collected 24 Hours Following a Single Oral Administration of EPA-rich triglyceride oil in ICR Mice.

Treatment (20 mL/kg)	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE scored)
Olive Oil	M	1	0.474	3 / 2000
		2	0.425	1 / 2000
		3	0.487	2 / 2000
		4	0.398	1 / 2000
		5	0.458	1 / 2000
	F	6	0.523	2 / 2000
		7	0.439	2 / 2000
		8	0.473	1 / 2000
		9	0.516	1 / 2000
		10	0.438	1 / 2000
H-28297 25%	M	11	0.495	2 / 2000
		12	0.508	1 / 2000
		13	0.513	2 / 2000
		14	0.408	3 / 2000
		15	0.426	0 / 2000
	F	16	0.476	1 / 2000
		17	0.423	2 / 2000
		18	0.493	1 / 2000
		19	0.465	2 / 2000
		20	0.513	2 / 2000
50%	M	21	0.521	3 / 2000
		22	0.458	0 / 2000
		23	0.436	0 / 2000
		24	0.411	1 / 2000
		25	0.488	3 / 2000
	F	26	0.404	2 / 2000
		27	0.488	2 / 2000
		28	0.515	1 / 2000
		29	0.483	2 / 2000
		30	0.437	2 / 2000
100%	M	31	0.470	2 / 2000
		32	0.492	1 / 2000
		33	0.523	1 / 2000
		34	0.443	1 / 2000
		35	0.478	2 / 2000
	F	36	0.434	3 / 2000
		37	0.492	0 / 2000
		38	0.457	1 / 2000
		39	0.497	2 / 2000
		40	0.508	1 / 2000
Cyclophosphamide 50 mg/kg	M	41	0.441	58 / 2000
		42	0.455	54 / 2000
		43	0.479	63 / 2000
		44	0.493	51 / 2000
		45	0.439	39 / 2000
	F	46	0.477	58 / 2000
		47	0.499	56 / 2000
		48	0.425	47 / 2000
		49	0.460	46 / 2000
		50	0.466	48 / 2000

Induction of Micronucleated Polychromatic Erythrocytes in Bone Marrow Collected 48 Hours Following a Single Oral Administration of EPA-rich triglyceride oil in ICR Mice.

Treatment (20 mL/kg)	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE scored)
Olive Oil	M	51	0.523	2 / 2000
		52	0.524	1 / 2000
		53	0.515	3 / 2000
		54	0.509	0 / 2000
		55	0.519	1 / 2000
	F	56	0.519	1 / 2000
		57	0.483	1 / 2000
		58	0.507	1 / 2000
		59	0.516	1 / 2000
		60	0.507	2 / 2000
H-28297 100%	M	81	0.511	0 / 2000
		82	0.462	0 / 2000
		83	0.494	0 / 2000
		84	0.510	0 / 2000
		85	0.518	0 / 2000
	F	86	0.532	1 / 2000
		87	0.528	2 / 2000
		88	0.482	0 / 2000
		89	0.504	2 / 2000
		90	0.521	0 / 2000

Summary of EPA-Rich Triglyceride Oil Preclinical Safety Studies

Lab Report	Cell Type/Species	Treatment	Results
Subchronic Toxicity			
DuPont, 2009a (28-day oral toxicity)	Rat (Crl:CD(SD))	0, 940, 1880, 2820 mg EPA-rich triglyceride oil/kg/day (0, 325, 650, and 976 mg EPA/kg b.w./day) or 513 mg EPA/kg/day oin fish oil	NOAEL: 976 mg EPA/kg/day
DuPont, 2009b (90-day oral toxicity)	Rat (Crl:CD(SD))	0, 282, 1410, and 2820 mg EPA-rich triglyceride oil/kg/day (0, 98, 488, and 976 mg EPA/kg b.w./day) or 513 mg EPA/kg/day in fish oil	NOAEL: 976 mg EPA/kg/day
Developmental Toxicity			
DuPont, 2009c (oral toxicity)	Rat (Crl:CD(SD))	0 , 98, 488, and 976 mg EPA/kg b.w./day on GD 6-20	NOAEL for both maternal and developmental toxicity was considered to be 976 mg EPA/kg/day
Genotoxicity			
Reverse Mutation Assay			
DuPont, 2009d	*Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvr*A	1.5 to 5000 µg/plate EPA-rich oil with or without S9 metabolic activation	Negative
Chromosome Aberration Test			
DuPont, 2009e	L5178Y/TK$^{+/-}$ mouse lymphoma cells	**4-Hour Exposure** 50 to 500 µg/mL EPA-rich oil with and without S9 activation. **24-Hour Exposure** 50 to 500 µg/mL EPA-rich oil without S9 activation.	Negative
In Vivo Micronucleus Test			
DuPont 2009f	Mouse (ICR)	EPA-rich triglyceride oil at 5%, 25%, 50%, 75% or 100% (neat test article).	Negative

APPENDIX 6

CLINICAL TRIAL DATA

Olive Oil Placebo (n=26)

Parameter	Baseline	Final	% Change	P Value
Comprehensive Metabolic Panel				
Glucose (mg/dL)	93.5 ± 15.8	92.4 ± 14.1	-1.6	0.60
Urea Nitrogen (mg/dL)	15.4 ± 4.6	15.8 ± 4.8	1.3	0.43
Creatinine (mg/dL)	0.9 ± 0.2	0.9 ± 0.2	-0.8	0.96
Sodium (mmol/L)	140.2 ± 2.2	139.9 ± 1.8	-0.2	0.47
Potassium (mmol/L)	4.2 ± 0.3	4.2 ± 0.3	-0.5	0.90
Chloride (mmol/L)	105.0 ± 1.9	104.1 ± 3.0	-0.9	0.09
Carbon Dioxide (mmol/L)	24.7 ± 2.4	26.2 ± 2.6	5.1	0.005
Calcium (mg/dL)	9.3 ± 0.3	9.2 ± 0.4	-0.5	0.55
Total Protein (g/dL)	7.3 ± 0.4	7.2 ± 0.5	-0.6	0.78
Albumin (g/dL)	4.5 ± 0.2	4.5 ± 0.2	-0.2	1.00
Globulin (g/dL)	2.8 ± 0.5	2.8 ± 0.5	-1.4	0.64
Bilirubin (mg/dL)	0.8 ± 0.3	0.9 ± 0.5	-7.8	0.51
Alkaline Phosphate (U/L)	66.8 ± 13.6	68.3 ± 12.8	2.2	0.22
Aspartate Aminotransferase (U/L)	26.5 ± 19.3	27.6 ± 21.9	-0.9	0.74
Alanine Aminotransferase (U/L)	28.5 ± 28.2	32.5 ± 39.0	3.1	0.31
Complete Blood Count				
White Blood Cells (k/μL)	5.4 ± 1.5	5.3 ± 1.5	-2.5	0.4
Red Blood Cells (M/μL)	4.6 ± 0.4	4.5 ± 0.4	-1.9	0.11
Hemoglobin (g/dL)	14.2 ± 1.5	14.1 ± 1.4	-0.7	0.62
Hematocrit (%)	42.3 ± 4.5	41.4 ± 4.2	-2.3	0.046
Mean Cell Volume (fL)	92.8 ± 4.9	92.5 ± 5.0	-0.4	0.08
Platelets (k/μL)	210.7 ± 47.6	211.6 ± 53.9	-1.2	0.83
Absolute Neutrophils (cells/μL)	3181.0 ± 1021.2	3114.2 ± 1075.7	-36.1	0.63
Absolute Lymphocytes (cells/μL)	1641.3 ± 746.7	1521.3 ± 795.4	-61.6	0.11
Absolute Monocytes (cells/μL)	428.1 ± 163.8	392.7 ± 132.5	-10.3	0.063
Absolute Eosinophils (cells/μL)	132.7 ± 81.7	138.6 ± 92.8	-6.0	0.65
Absolute Basophils (cells/μL)	28.9 ± 13.0	29.0 ± 15.2	-2329.4	0.98
Neutrophils (%)	59.0 ± 9.2	60.3 ± 8.2	2.3	0.12
Lymphocytes (%)	30.2 ± 8.7	29.2 ± 8.4	-4.9	0.22
Monocytes (%)	7.9 ± 2.0	7.4 ± 1.9	-9.0	0.09
Eosinophils (%)	2.4 ± 1.2	2.6 ± 1.4	-5.0	0.43
Basophils (%)	0.5 ± 0.2	0.6 ± 0.3	-97.7	0.82
Thyroid Function Tests and Prothrombin Time				
Triiodothyronine (%)	31.9 ± 2.9	31.7 ± 2.8	-0.8	0.78
Thyroxine (mcg/dL)	7.2 ± 1.0	7.3 ± 1.2	0.40	0.48
Free Thyroxine (%)	2.3 ± 0.3	2.3 ± 0.4	0.30	0.43
Prothrombin Time (sec)	10.3 ± 0.4	10.3 ± 0.4	-0.10	0.94

Group means ± standard deviations and mean percent change of 26 individuals who consumed a daily olive oil placebo. P values are based on 2-tailed independent t-tests of the absolute changes from baseline to final over a 6 week period and are not corrected for multiple comparisons.

000070

EPA 600 mg (n=27)

Parameter	Baseline	Final	% Change	P Value
Comprehensive Metabolic Panel				
Glucose (mg/dL)	90 ± 11.0	92.1 ± 11.0	1.3	0.47
Urea Nitrogen (mg/dL)	16.3 ± 4.3	16.0 ± 4.8	-4.4	0.67
Creatinine (mg/dL)	1.0 ± 0.2	0.9 ± 0.2	-3.8	0.13
Sodium (mmol/L)	140.0 ± 1.8	139.9 ± 1.6	0	0.93
Potassium (mmol/L)	4.2 ± 0.2	4.2 ± 0.3	1.1	0.26
Chloride (mmol/L)	105.9 ± 2.1	105.3 ± 1.8	-0.5	0.21
Carbon Dioxide (mmol/L)	24.3 ± 2.4	25.1 ± 2.4	2.9	0.074
Calcium (mg/dL)	9.4 ± 0.3	9.4 ± 0.5	-0.3	0.93
Total Protein (g/dL)	7.2 ± 0.4	7.2 ± 0.4	0.5	0.55
Albumin (g/dL)	4.5 ± 0.2	4.5 ± 0.3	0.5	0.46
Globulin (g/dL)	2.7 ± 0.4	2.7 ± 0.3	-0.2	1
Bilirubin (mg/dL)	0.7 ± 0.2	0.6 ± 0.2	-12.3	0.09
Alkaline Phosphate (U/L)	71.4 ± 19.0	73.4 ± 18.4	2.3	0.30
Aspartate Aminotransferase (U/L)	20.7 ± 8.5	22.3 ± 10.7	3.2	0.08
Alanine Aminotransferase (U/L)	23.1 ± 17.4	25.7 ± 22.7	-2.2	0.16
Complete Blood Count				
White Blood Cells (k/μL)	5.3 ± 1.4	5.5 ± 1.3	3.3	0.21
Red Blood Cells (M/μL)	4.5 ± 0.4	4.4 ± 0.4	-0.9	0.22
Hemoglobin (g/dL)	14.1 ± 0.9	14.2 ± 0.9	0.1	0.77
Hematocrit (%)	42 ± 2.9	41.7 ± 2.7	-0.8	0.35
Mean Cell Volume (fL)	94.0 ± 5.6	94.2 ± 5.4	0.2	0.55
Platelets (k/μL)	215.1 ± 41.3	222.9 ± 42.8	2.8	0.11
Absolute Neutrophils (cells/μL)	3240.3 ± 948.2	3315.4 ± 929.0	0.2	0.55
Absolute Lymphocytes (cells/μL)	1500.3 ± 551.6	1567.4 ± 440.5	4.0	0.31
Absolute Monocytes (cells/μL)	446.4 ± 154.7	471.8 ± 154.9	3.1	0.14
Absolute Eosinophils (cells/μL)	126.6 ± 88.1	144.1 ± 104.9	6.6	0.09
Absolute Basophils (cells/μL)	27.4 ± 11.8	31.1 ± 15.1	-1.6	0.21
Neutrophils (%)	60.4 ± 7.5	59.6 ± 7.1	-1.9	0.44
Lymphocytes (%)	28.2 ± 6.4	28.4 ± 7.3	-2.2	0.84
Monocytes (%)	8.4 ± 1.8	8.5 ± 1.9	-0.9	0.68
Eosinophils (%)	2.4 ± 1.7	2.6 ± 1.7	3.7	0.32
Basophils (%)	0.5 ± 0.2	0.6 ± 0.3	-4.6	0.39
Thyroid Function Tests and Prothrombin Time				
Triiodothyronine (%)	31.5 ± 3.4	31.3 ± 3.1	-0.7	0.74
Thyroxine (mcg/dL)	7.3 ± 1.2	7.6 ± 1.6	1.9	0.20
Free Thyroxine (%)	2.3 ± 0.4	2.4 ± 0.5	1.7	0.24
Prothrombin Time (sec)	10.3 ± 0.3	10.3 ± 0.5	0.1	0.77

Group means ± standard deviations and mean percent change of 27 individuals who consumed 600 mg of EPA daily. P values are based on 2-tailed independent t-tests of the absolute changes from baseline to final over a 6 week period and are not corrected for multiple comparisons.

EPA 1800 mg (n=29)

Parameter	Baseline	Final	% Change	P Value
Comprehensive Metabolic Panel				
Glucose (mg/dL)	91.7 ± 8.9	91.9 ± 7.7	0.0	0.87
Urea Nitrogen (mg/dL)	15.4 ± 3.8	15.4 ± 3.8	-3.0	0.95
Creatinine (mg/dL)	1.0 ± 0.2	0.9 ± 0.2	-2.5	0.14
Sodium (mmol/L)	140.2 ± 2.5	139.8 ± 2.1	-0.3	0.29
Potassium (mmol/L)	4.3 ± 0.3	4.2 ± 0.3	-1.5	0.27
Chloride (mmol/L)	105.8 ± 2.5	105.3 ± 2.6	-0.5	0.22
Carbon Dioxide (mmol/L)	24.4 ± 2.5	25.1 ± 2.4	2.3	0.10
Calcium (mg/dL)	9.3 ± 0.4	9.3 ± 0.4	-0.7	0.27
Total Protein (g/dL)	7.1 ± 0.5	7.2 ± 0.5	0.1	0.84
Albumin (g/dL)	4.4 ± 0.2	4.4 ± 0.2	-0.4	0.69
Globulin (g/dL)	2.7 ± 0.4	2.7 ± 0.5	0.5	0.42
Bilirubin (mg/dL)	0.8 ± 0.3	0.7 ± 0.4	-6.3	0.92
Alkaline Phosphate (U/L)	64.1 ± 13.5	63.1 ± 12.4	-1.8	0.42
Aspartate Aminotransferase (U/L)	19.9 ± 5.0	21.2 ± 10.2	-1.5	0.52
Alanine Aminotransferase (U/L)	20.2 ± 8.8	21.5 ± 11.8	-0.9	0.46
Complete Blood Count				
White Blood Cells (k/μL)	5.1 ± 1.0	5.1 ± 1.2	-1.8	1
Red Blood Cells (M/μL)	4.5 ± 0.4	4.5 ± 0.4	-2.1	0.0497
Hemoglobin (g/dL)	14.0 ± 1.1	13.9 ± 1.3	-1.1	0.41
Hematocrit (%)	41.7 ± 2.5	41.1 ± 3.7	-1.8	0.13
Mean Cell Volume (fL)	91.9 ± 4.4	92.2 ± 4.7	0.3	0.39
Platelets (k/μL)	219.8 ± 41.1	220.2 ± 42.5	-1.4	0.74
Absolute Neutrophils (cells/μL)	2872.6 ± 782.8	2812.6 ± 827.7	-5.2	0.63
Absolute Lymphocytes (cells/μL)	1637.1 ± 430.6	1696.6 ± 497.9	1.7	0.30
Absolute Monocytes (cells/μL)	419.5 ± 132.4	395.0 ± 115.7	-7.7	0.15
Absolute Eosinophils (cells/μL)	144.7 ± 112.6	137.8 ± 80.5	-15.2	0.68
Absolute Basophils (cells/μL)	29.6 ± 11.6	27.2 ± 13.0	-22.6	0.20
Neutrophils (%)	55.8 ± 7.2	55.0 ± 7.7	-2.1	0.47
Lymphocytes (%)	32.4 ± 6.6	33.8 ± 7.3	2.6	0.16
Monocytes (%)	8.3 ± 1.9	7.9 ± 1.9	-5.7	0.13
Eosinophils (%)	2.9 ± 2.2	2.7 ± 1.6	-12.4	0.57
Basophils (%)	0.6 ± 0.2	0.5 ± 0.2	-21.5	0.10
Thyroid Function Tests and Prothrombin Time				
Triiodothyronine (%)	31.9 ± 2.6	31.9 ± 2.7	-0.1	1
Thyroxine (mcg/dL)	7.1 ± 1.4	6.8 ± 1.5	-7.6	0.25
Free Thyroxine (%)	2.2 ± 0.4	2.4 ± 1.2	1.2	0.38
Prothrombin Time (sec)	10.4 ± 0.4	10.4 ± 0.3	0.4	0.33

Group means ± standard deviations and mean percent change of 29 individuals who consumed 1800 mg of EPA daily. P values are based on 2-tailed independent t-tests of the absolute changes from baseline to final over a 6 week period and are not corrected for multiple comparisons.

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DHA 600 mg (n=28)

Parameter	Baseline	Final	% Change	P Value
Comprehensive Metabolic Panel				
Glucose (mg/dL)	94.8 ± 14.5	96.4 ± 19.1	0.7	0.42
Urea Nitrogen (mg/dL)	15.4 ± 4.9	15.9 ± 5.4	-0.1	0.50
Creatinine (mg/dL)	0.9 ± 0.2	0.9 ± 0.2	0.6	0.46
Sodium (mmol/L)	139.6 ± 1.6	139.8 ± 1.5	0.2	0.47
Potassium (mmol/L)	4.2 ± 0.2	4.2 ± 0.2	-0.8	0.57
Chloride (mmol/L)	104.7 ± 2.4	104.8 ± 2.2	0.1	0.78
Carbon Dioxide (mmol/L)	24.5 ± 2.3	24.8 ± 2.7	0.6	0.52
Calcium (mg/dL)	9.3 ± 0.4	9.3 ± 0.3	-0.1	0.95
Total Protein (g/dL)	7.3 ± 0.3	7.3 ± 0.4	-0.3	0.81
Albumin (g/dL)	4.5 ± 0.3	4.5 ± 0.2	0.8	0.34
Globulin (g/dL)	2.9 ± 0.4	2.8 ± 0.4	-2.0	0.17
Bilirubin (mg/dL)	0.7 ± 0.3	0.7 ± 0.3	-7.9	0.66
Alkaline Phosphate (U/L)	74.0 ± 20.7	74.3 ± 21.6	-0.4	0.87
Aspartate Aminotransferase (U/L)	21.8 ± 6.1	20.8 ± 5.5	-6.2	0.22
Alanine Aminotransferase (U/L)	20.9 ± 10.1	20.5 ± 8.8	-2.8	0.74
Complete Blood Count				
White Blood Cells (k/µL)	5.5 ± 1.6	5.4 ± 1.8	-5.1	0.37
Red Blood Cells (M/µL)	4.6 ± 0.5	4.6 ± 0.6	-0.9	0.48
Hemoglobin (g/dL)	14.0 ± 1.3	14.0 ± 1.4	-0.3	0.95
Hematocrit (%)	41.7 ± 3.6	41.4 ± 4.1	-0.9	0.45
Mean Cell Volume (fL)	90.3 ± 5.7	90.3 ± 5.5	0.0	0.98
Platelets (k/µL)	221.9 ± 57.7	231.3 ± 60.3	3.0	0.20
Absolute Neutrophils (cells/µL)	3397.5 ± 1292.3	3247.1 ± 1421.7	-9.6	0.35
Absolute Lymphocytes (cells/µL)	1526.3 ± 363.7	1526.1 ± 376.1	-1.3	1
Absolute Monocytes (cells/µL)	429.5 ± 177.2	432.1 ± 216.8	-4.6	0.89
Absolute Eosinophils (cells/µL)	135.6 ± 104.7	131.8 ± 88.9	-9.1	0.70
Absolute Basophils (cells/µL)	30.6 ± 15.2	30.9 ± 16.9	-7.8	0.91
Neutrophils (%)	60.3 ± 7.8	58.8 ± 8.4	-3.3	0.22
Lymphocytes (%)	28.9 ± 7.4	30.0 ± 7.5	2.1	0.28
Monocytes (%)	7.8 ± 1.9	8.2 ± 3.5	-0.2	0.33
Eosinophils (%)	2.5 ± 1.9	2.5 ± 1.3	-8.5	0.81
Basophils (%)	0.6 ± 0.2	0.6 ± 0.3	-3.1	0.67
Thyroid Function Tests and Prothrombin Time				
Triiodothyronine (%)	30.2 ± 3.8	30.1 ± 3.6	-0.5	0.80
Thyroxine (mcg/dL)	7.7 ± 1.3	7.9 ± 1.2	1.7	0.35
Free Thyroxine (%)	2.3 ± 0.3	2.4 ± 0.3	1.3	0.44
Prothrombin Time (sec)	10.2 ± 0.3	10.3 ± 0.3	0.6	0.24

Group means ± standard deviations and mean percent change of 28 individuals who consumed 600 mg of DHA daily. P values are based on 2-tailed independent t-tests of the absolute changes from baseline to final over a 6 week period and are not corrected for multiple comparisons.

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SUBMISSION END

AM

Ramos-Valle, Moraima

From: Matthias Liebergesell [Matthias.Liebergesell@usa.dupont.com]
Sent: Tuesday, November 09, 2010 10:54 AM
To: Ramos-Valle, Moraima
Subject: RE: GRAS Notice 355

Dear Moraima,

Please find below the proposed answers to the questions you have forwarded earlier. I feel confident that we addressed those questions appropriately and would like to receive your feedback on whether our interpretation of the questions matches the review teams intend.

Please suggest a date and time to discuss. I then plan to submit the answers to the questions as amendment to the DuPont GRAS notification to FDA.

Best regards,

Matthias

On page 46 of the notice, the notifier states that "*Y. lipolytica* is GRAS for the commercial production of food grade citric acid (21 CFR 173.165)." However, per 21 CFR 173.165, *Y. lipolytica* is a secondary direct food additive. While 21 CFR 173.165 indicates that the organism is a safe source for an enzyme as a secondary direct food additive, the regulation does not make a reference to GRAS status of any use. Please rephrase your reference to 21 CFR 173.165 to account for the regulatory status of *Y. lipolytica.*

DuPont redact the statement made on page 46 of the GRAS notification regarding *Y. lipolytica* having GRAS status. As indicated elsewhere in the notification, *Y. lipolytica* is FDA approved as secondary direct food additive in the production of citric acid according to 21 CFR 173.165.

2) Please provide the strain designation for the production strain and host strain.

The specific yeast strain used to develop the eicosopentaenoic acid (EPA)-rich oil producing *Y. lipolytica* has the designated American Type Culture Collection (ATCC) number 20362. The organism is identified in ATCC as *Yarrowia lipolytica* (Wickerham et al.) van der Walt et von Arx, teleomorph deposited as *Candida lipolytica* (Harrison) Diddens et Lodder, anamorph.

Various strains based on strain ATCC 20362 have been developed by DuPont, by using combinations and variation in copy number of genes as outlined in table 1 of appendix 4 of the GRAS notification (page 50). This variety of strains with a common basis of genetic make-up enables DuPont to manufacture EPA-rich triglyceride oil with various levels of eicosopentaenoic acid (EPA) in the oil. The strain used to manufacture the 35% EPA oil has the strain designation Y4305F1B1 and the strain used to manufacture the 50 % oil has the strain designation Y8672.

3) Please discuss whether the production strain and/or host strain has been deposited into

a culture collection. If not, please discuss how your strain is/was verified.

The variety of production strains developed by DuPont is all based upon strain ATCC 20362. None of the production strains have been deposited in a culture collection.

4) Please discuss how the host strain was obtained (i.e. where did it come from)?

The host strain (ATCC 20362) was obtained from the American Type Culture Collection (ATCC).

5) Please discuss any steps taken to ensure purity of the *Y. lipolytica* culture during fermentation.

The purity of *Y. lipolytica* during fermentation is ensured through growth in sterilized media and aseptic transfer of *Y. lipolytica* pure culture into the fermentor. The fermentor is cleaned and sterilized prior the fermentation with Cleaning in Place (CIP) and/or Sterilization in Place (SIP) practices commonly used in the food fermentation industry. Samples are taken at 4 to 8 h intervals during the fermentation and cell colonies are examined visually for contamination on agar plates (e.g., Trypticase soy agar) after incubation at various temperatures. At the end of the fermentation, samples are analyzed spefically for contamination according to the specifications as outlined in the GRAS notification "Product specifications and analysis".

6) Please provide examples of the types of foods in which *Y. lipolytica* is naturally found, and provide references.

Members of the *Y. lipolytica* species are widely distributed in nature and have been found as epiphytic microflora on surfaces of plants, dairy products, salads containing meat and shrimp, insects, and fish (Zvyagilskaya *et al.*, 2001). *Y. lipolytica* is part of the normal microflora of cheese and dairy products (Suzzi *et al.*, 2001). Its presence imparts a particular function in these foods such as flavor, aroma, appearance, texture, and ripening. *Y. lipolytica* is regarded as a good candidate for ripening cheese (Suzzi *et al.*, 2001; Lanciotti *et al.*, 2005b). *Y. lipolytica* is one of the predominant species in camembert and blue-veined cheeses and is frequently found at concentrations exceeding 106-107 CFU/gram in these foods (Fickers *et al.*, 2005).

Suzzi, G. *et al.* (2001) Proteolytic, lipolytic, and molecular characterization of *Yarrowia lipolytica* isolated from cheese. Intl. J. Food Microbiol. 69:69-77.

Zvyagilskaya, R. *et al.* (2001) Isolation and characterization of a novel leaf-inhabiting osmo-, sal-, and alkali-tolerant *Yarrowia lipolytica* yeast strain. J. Basic Microbiol., 41:289-303.

Lanciotti, R. *et al.* (2005b) Evaluation of the ability of *Yarrowia lipolytica* to impart strain-dependent characteristics to cheese when used as a ripening adjunct. Intl. J Dairy Technol., 58(2):89-99.

Fickers, P. *et al.* (2005) Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications. FEMS Yeast Res. 5:527-543.

7) Please expand the manufacturing section by discussing information regarding the storage of the oil and its stability?

The following is added to the section 2 of the GRAS notification ("Details on the Manufacturing

Process for EPA-rich triglyceride oil"):

d Oil Storage and Stability

Prior to shipping the oil to a customer, the oil is stored below 7 Degree Celsius under a nitrogen blanket in containers suitable to hold food. An accelerated stability study with oil containing 35% EPA indicated that the oil is stable for 18 months (1.5 years) under oxidative stress (i.e., opened container) at room tepmperature (i.e., 22 Degree Celsius). The parameters measured to investigate stability (i.e., compliance with specifications) were EPA content, peroxide valus, P-anisidine value, acid value, and color.

8) Are the ingredients used in the manufacturing of the ingredient suitable for food use (i.e. food-grade?)?

The EPA-rich triglyceride oil is manufactured under food cGMP according to 21 CFR 110. The ingredients used to manufacture the oil are suitable for processing into food.

9) We noted that throughout the notice the notifier describes information about the ingredient referring different percentages (35%, 50%, and 55%) of EPA content. Please clarify the lipid content for the ingredient.

The EPA-rich triglyceride oil is a highly refined oil and consists primarily of triglycerides and minor fractions of free fatty acids, phospholipids, and unsaponifiables. The lipid content is comparable to other edible vegetable or fish oils.

10) The specifications section provides the fatty acid profile of 3 batches of the 35% and 50% EPA-rich
triglyceride oil. Please provide the fatty acid profile of the 55% EPA-rich triglyceride oil?

The fermentation conditions used to manufacture EPA-rich triglyceride oil with 50% EPA content, though controlled to deliver a consistent product in terms of oil composition, has a certain variability that may lead to EPA content slightly higher than 50% in the oil (see Table 1; page 6 in the notification) and not more than 55% EPA. This variability therefore is addressed in the EPA-rich triglyceride oil specifications as EPA content of 350 - 550 mg FA/g oil (35 to 55% of total lipids). The fatty acid profiles of a 55% EPA oil and a 50% EPA oil are very comparable.

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