Arachidonic Acid Single-Cell Oil (ARA)

Handling/Processing

Identification of Petitioned Substance

Chemical Names:
Arachidonic acid single-cell oil

Other Name:
ARASCO
ARASCO oil
AA oil
ARA Single-cell Oil
ARA-rich oil
Arachidonic acid oil
Arachidonic acid-rich oil
Arachidonic acid-rich single-cell oil
Arachidonic acid-rich fungal oil
Arachidonate
Mortierella alpina oil
Arachidonsaeure
(NLM, 2011a,b; Hogan & Hartson L.L.P., 2000; FDA, 2011)

Trade Names:
ARASCO® – Martek Biosciences (Martek, 2010a)
Life'sARA™ – Martek Biosciences (Martek, 2010a)
SUN-TGA40S – Suntory Ltd. (EFSA, 2008)
RAO – Cargill, Inc (Casterton et al., 2009)

CAS Numbers:
None (Arachidonic acid single-cell oil)
506-32-1 (Arachidonic acid)

Other Codes:
IUPAC Name: icosa-5,8,11,14-tetraenoic acid (Arachidonic acid)

Characterization of Petitioned Substance

<u>Composition of the Substance</u>:

Arachidonic Acid (ARA) is a long-chain, polyunsaturated fatty acid (PUFA) of the omega-6 class (5,8,11,14-eicosatetraenoic acid). Omega-6 PUFA are synthesized within animals and humans through desaturation and elongation of dietary linoleic acid (Jump et al., 2009; NLM, 2011a). ARA can be found in the blood, fat, liver, brain, and glandular organs of humans and animals, where it serves as a structural agent and precursor to number of biosynthesized compounds, including prostaglandins, thromboxanes, and leukotrines. The class of compounds derived from ARA are collectively referred to as eicosanoids (Kyle, 1997). The molecular formula of ARA is $C_{20}H_{32}O_2$; the chemical structure for ARA is presented in Figure 1.

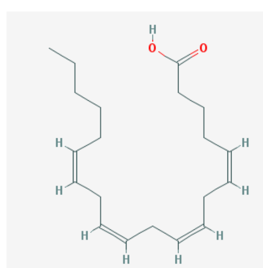

Figure 1. Chemical Structure of Arachidonic Acid (NLM, 2011b)

Arachidonic acid single-cell oil (ARA Single-cell Oil) is a triglyceride oil extracted from a soil fungus and enriched with a commercial grade high oleic sunflower oil to approximately 40% ARA by weight (Hogan & Hartson L.L.P., 2000; Wyeth-Ayerst, 1998; EFSA, 2008). The petitioned use of ARA Single-cell Oil is as a nutritional food ingredient added to infant formulas. ARA Single-cell Oil is added to infant formula to increase free ARA levels in formula to those comparable to ARA levels in human breast milk. ARA Single-cell Oil comprises approximately 95–99% triglyceride, 0.5–3.5% diglyceride, and 0.1–1.5% unsaponified material composed primarily of sterols (Hogan & Hartson L.L.P., 2000; EFSA, 2008). The trans fatty acid content of the mixture has been reported as not detected (Hogan and Hartson L.L.P., 2000) and as less than 2% of total fats (EFSA, 2008). Other fatty acids present in ARA Single-cell oil include oleic acid (~16–23%), palmitic acid (~7–10%), stearic acid (~7–10%), linoleic acid (~6–8%), gamma-linoleic acid (~3%), dihomo-gamma-linoleic acid (~1–3%), behenic acid (~2%), and a number of other fatty acids at levels less than one percent (Hogan & Hartson L.L.P, 2000). The primary sterol identified in ARA Single-cell Oil is desmosterol, which is a natural component of the human metabolic pathway for cholesterol biosynthesis and is purportedly found in a variety of animal and plant food sources and human milk (Hogan and Hartson L.L.P., 2000; EFSA, 2008). As shown in Figure 2, ARA in triglyceride oil (Martek trade name: ARASCO®) can be positioned in one of three places on the triglyceride molecule: sn-1 (outside position), sn-1 (inside position; as found in human breast milk), or sn-3 (outside position), each of which affects the form in which the ARA will be received in the body. The Petitioner reports that in most cases the triglyceride will be associated with only one ARA molecule, but some triglycerides will be associated with two ARA molecules (Figure 2).

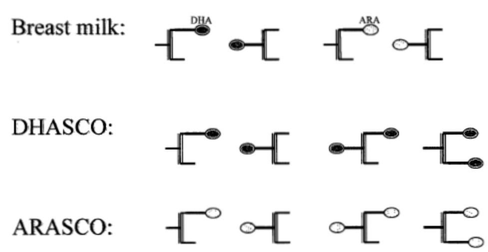

Figure 2. Triglyceride Structures in Human Breast Milk, Docosahexaenoic Acid Algal Oil (Trade Name: DHASCO®), and Arachidonic Acid Single-cell Oil (Trade Name: ARASCO®) Prior to Cleavage in the Gut of an Infant by Lipases (provided to U.S. FDA by Hogan & Hartson, L.L.P [2000] on behalf of Martek Biosciences Corporation)

<u>Properties of the Substance</u>:

The physical and chemical properties of ARA Single-cell Oil are presented in Table 1. The properties presented in Table 1 describe either a trademarked ARA Single-cell Oil product or free ARA, as indicated.

<u>Specific Uses of the Substance</u>:

ARA Single-cell Oil is presently used as a nutritional additive providing a source of ARA in term and preterm infant formula in amounts resulting in a range of 16–34 mg ARA/100 calories (Jump et al., 2009). ARA is considered an accessory nutrient by the U.S. Department of Agriculture (USDA). The term "accessory nutrient" has not been written into law, but the term has been used to refer loosely to substances that are not specifically classified as vitamins or minerals but are found to promote optimal health (NOSB, 2011). Accessory nutrients can be contrasted with the essential nutrients such as the fatty acids linoleic acid and alpha-linoleic acid, which cannot be synthesized by the body (Jump, 2009).

Although infants are capable of producing some ARA from essential fatty acids consumed in the diet, infants consuming breast milk generally have higher blood levels of ARA than those consuming formula (FDA, 2009a). In general, the rate of ARA synthesis from essential linoleic acid precursors seems to be insufficient to maintain stable levels of ARA in infants, resulting in an overall decline in ARA levels in infants fed unsupplemented formula as compared to those fed human milk (which naturally contains pre-formed ARA). As a result, there is an interest in adding oils containing pre-formed ARA to infant formula to replace the ARA not obtained from breast feeding and to supplement those essential oils (e.g., linoleic acid) in the infant formula that allow infants to produce their own ARA.

Table 1. Physical and Chemical Properties of ARA Single-cell Oil

Chemical or Physical Property	Value
Color	Yellow (ARASCO® - Hogan & Hartson L.L.P., 2000); Clear yellow (SUN-TGA40S - EFSA, 2008)
Physical State	Liquid (oil) (ARASCO® - Hogan & Hartson L.L.P., 2000)
Odor	Characteristic (ARASCO® - Hogan & Hartson L.L.P., 2000); Musky (ARASCO® - Martek, 2010b)
Molecular Weight	204.46688 g/mol (Free ARA - NLM, 2011b)
Melting Point	-4.95E+01 °C (Free ARA - NLM, 2011a)
Boiling Point	170 °C (Free ARA - Sciencelab.com, Inc., 2005)
Solubility	0.031 mg/L – insoluble (Free ARA - NLM, 2011a)
Stability	Stable under normal conditions; susceptible to heat (ARASCO® - Martek, 2010b) and oxidation (ARA Single-cell Oil - Bartee et al., 2007)
Reactivity	Not reactive (ARASCO® - Martek, 2010b); Reactive with oxidizing agents, reducing agents, alkalis. Slightly reactive to reactive with moisture (Free ARA – Sciencelab.com, Inc., 2005)
Oxidizing or Reduction Action	Susceptible to oxidation due to four *cis* double bonds (ARA Single-cell Oil - Bartee et al., 2007; Free ARA - Brash, 2001). Oxidation results in odor and off-flavor.
Flammability/Flame Extension	Flashpoint: >232 °C (ARASCO® - Martek, 2010b)
Explodability	MSDS contains warning that "porous material wetted with this product may undergo spontaneous combustion" (ARASCO® - Martek, 2010b)

While the only current use for ARA listed in the USDA National Nutrient Database for Standard Reference is in infant formula (USDA, 2010a), ARA Single-cell Oil has been proposed (but not petitioned or used) as an ingredient in infant foods and foods for pregnant women and nursing mothers, as an ingredient in functional foods, and as a supplement in capsule, granule, drink, or eneric feeding form (Higashiyama et al., 2000). ARA Single-cell Oil has also been proposed for use in animal feed intended for cattle, sheep, poultry, and other farm animals, or for farmed marine organisms such as fish and shellfish (Streekstra & Brocken, 2008). Manufacturers of ARA Single-cell Oil also variably describe its uses as a "beauty product[1]," food-grade "nutrition enhancer[2]," food additive[3], and "antipyretic analgesic and NSAID [non-steroid anti-inflammatory drug][4]."

ARA oil is also manufactured and sold as a dietary supplement in combination with "performance-enhancing" proprietary nutrient and vitamin blends. The ARA-containing supplements are marketed as anabolic nutrients that promote muscle growth in highly active adults.[5] The source of the ARA oil blend in dietary supplements is generally not provided (i.e., proprietary). Dietary supplements such as ARA do not need specific approval from FDA before they are marketed and sold, but the manufacturer is required to determine that the supplement is "safe" before the supplement is marketed (FDA, 2009b).

Approved Legal Uses of the Substance:

ARA Single-cell Oil is considered by FDA as GRAS in infant formula when used in combination with docosahexaenoic acid (DHA) (FDA, 2011a). ARA is not currently used in foods other than infant formulas, and no other U.S. government regulations currently exist for ARA Single-cell Oil.

Action of the Substance:

ARA Single-cell Oil is meant to be consumed directly as a food ingredient or nutrient supplement. Once in the body, absorption of ARA will be determined by the position of the ARA on the triglyceride molecule. ARA triglyceride oils are generally hydrolyzed by pancreatic enzymes to form free ARA if the ARA molecule is on the sn-1 or sn-3 position, as described previously in "Composition of the substance" and as shown in Figure 2. If, however, the ARA molecule is on the sn-2 position of the triglyceride, the ARA can

[1] http://cabio.en.alibaba.com/product/219633295-210026682/health_food_ARA_Oil.html
[2] http://cabio.en.alibaba.com/product/333634597-210026682/ARACHIDONIC_ACID_ARA_.html
[3] http://cabio.en.alibaba.com/product/200718417-210026682/Food_additive_ARA_Oil.html
[4] http://cabio.en.alibaba.com/product/222511702-210026682/ARACHIDONIC_ACID.html
[5] http://www.gnc.com/product/index.jsp?productId=3715948

be fully absorbed as the sn-2 monoglyceride (Hogan and Hartson LLP, 2000). After absorption in the small intestine, omega-6 fatty acids like ARA are either incorporated into tissue lipids, utilized as substrates for eicosanoid synthesis, or oxidized to carbon dioxide and water (Institute of Medicine, 2005).

The primary role of ARA in the body is a as a structural component of cell membranes. ARA incorporated into phospholipids of certain tissues can affect the fluidity, flexibility, and permeability of cell membranes and the activity of membrane-bound enzymes (Jump et al., 2009). Phospholipids in the brain and eyes, for example, contain high levels of DHA and ARA, suggesting they are important to central nervous system and retinal function (Friesen & Innis, 2009).

Endogenous synthesis of ARA also occurs through multiple desaturation (carbon double bond addition) and elongation (two-carbon addition) reactions of linoleic acid (Jump, 2009). The conversion rate of linoleic acid to ARA in infants, however, is not sufficient to maintain stable ARA levels in the body (FDA, 2009a). To supply the amount of ARA necessary to support the rapid organ development that takes place during the first year of life, breast milk provides additional pre-formed ARA to infants. ARA Single-cell Oil in infant formula has been suggested as a reasonable substitution for pre-formed ARA in breast milk (Koletzko et al., 2008).

In addition to its primary role as a structural lipid, ARA acts as the direct precursor for a number of circulating eicosanoids, or chemical messengers involved in immune and inflammatory response. During inflammation, ARA in cell membranes can be metabolized to form the cell-signaling molecules known as prostaglandins and leukotrienes (Jump et al., 2008). The omega-3 eicosapentaenoic acid (EPA) in cell membranes can also form eicosanoids, but the physiological reaction to eicosanoids from ARA and EPA differ. For example, eicosanoids derived from EPA are generally less potent inducers of inflammation, blood vessel constriction, and coagulation than eicosanoids derived from ARA. In the right proportion, however, ARA eicosanoids exhibit beneficial regulatory effects on lipoprotein metabolism, blood theology, vascular tone, leukocyte function and platelet activation (Kyle, 1997).

Combinations of the Substance:

ARA and ARA Single-cell Oil are not currently on the National List of Allowed and Prohibited Substances (hereafter referred to as the National List); nor are they precursors to or components of any substance identified on the National List. Research suggests that a balance of ARA and DHA are necessary to the normal growth and development of infants (Innis et al., 2002). FDA and some international regulatory bodies have approved ARA Single-cell Oil for use as a nutrient additive to infant formula when used in combination with added DHA Algal Oil, which is also not on the National List (FDA, 2009a). DHA is a large component of fish oil. Fish oil (Fatty acid CAS #'s: 10417–94–4, and 25167–62–8) is on the National List as a nonorganically produced agricultural product allowed as an ingredient in or on processed products labeled as "organic." (7 CFR 205.606(f)). DHA is usually found in fish oil with EPA, another long-chain PUFA. EPA and ARA compete for the same enzymatic pathways leading to the formation of eicosanoids (as described in "Action of the Substance"); thus, incorporation of high intake of EPA can depress ARA and ARA eicosanoid production in infants (Kyle et al., 1997).

A preservative (e.g., tocopherols, ascorbyl palmitate) will generally need to be added to ARA Single-cell Oil to prevent oxidation and related adverse effects on the nutritional quality, odor, and flavor of the oil (Bartee et al., 2007). High oleic sunflower oil is generally added to ARA Single-cell Oil in varying amounts (usually less than 10% dilution) to achieve consistent ARA potency across batches and products (Hogan & Hartson L.L.P., 2000; EFSA, 2008; Martek, 2010b).

ARA Single-cell Oil is proposed for addition to infant formula, which contains a number of nutrients included on the National List by inference to FDA requirements for nutrient vitamins and minerals, in accordance with 21 CFR 104.20, Nutritional Quality Guidelines For Foods (7 CFR 205.605). Furthermore, a mixture of food ingredients comprising carbohydrates, proteins, fats, and stabilizers are expected to be included in infant formula, but these ingredients will vary with the manufacturer.

Status

Historic Use:

The patent for production of ARA Single-cell Oil from fungal species *Pythium insidiosum* and *M. alpina* was filed in 1995 and registered in 1997 (Kyle et al., 1997). As cited in the patent application, no commercial infant formulas were known to contain ARA in triglyceride form in 1995. Another patent filed in 1995 by Clandinin and Chappell (1997) suggested that infant nutritional requirements for ARA be met using a supplement blend of egg yolk, fish oil or red blood cell phospholipids and vegetable oils as the fat component of a proposed infant formula. Kyle et al. (1997) claim, however, that fish oil contains high quantities of eicosapentaneoic acid (EPA), which is known to depress ARA synthesis in infants. Furthermore, they claim that egg yolks contain a relatively low concentration of ARA, such that the mixture proposed by Clandinin and Chappell is "not economically viable." The patent by Kyle et al. (1997) describes the production of an "unmodified" edible fungal oil containing predominantly ARA as the end product. The intent of the manufacturer of the oil was to include the oil only in infant formulas (Kyle et al., 1997).

While a study of DHA infant formula supplementation reports that both DHA and ARA have been added to U.S. infant formulas since 2002 (Birch et al., 2010), Martek claims to have begun adding DHA and ARA oils to infant formula as early as 1994 (Martek, 2010a). The use of ARA Single-cell Oil as a nutritional food ingredient has thus far been limited to infant formula.

OFPA, USDA Final Rule:

Neither ARA nor ARA Single-cell Oil are listed in the Organic Foods Production Act of 1990 or the National Organic Program Final Rule.

A 2006 letter ruling from the USDA National Organic Program (NOP) determined that ARA was allowed in organic food and baby formula and is in compliance with 7 CFR 205.605(b) of the National List because "[n]utrients allowed under section 205.605(b) are not limited to the nutrients listed in section 104.20(d)(3), because section 104.20(f) provided that nutrients may be added to foods as permitted or required by applicable regulations established elsewhere by FDA. Thus, for example, ARA and DHA are covered under section 205.605(b) of the National List because the FDA permits their use as nutrients that are GRAS" (USDA, 2010b). In April 2010, the NOP concluded that its interpretation of Section 104.20 was incorrect and requested that NOSB re-evaluate their recommendation for nutrient vitamins and minerals. Companies or interest groups were invited to petition to add accessory nutrients to the National List (USDA, 2010b).

International

Some international organizations allow the use of ARA Single-cell Oil as an ingredient in infant formulas, but currently no international organizations have allowed the use of ARA Single-cell Oil in organic handling or processing.

The June 2011 (Amended) version of the Organic Production Systems Permitted Substances Lists produced by the Canadian General Standards Board does not include ARA or ARA Single-cell Oil as allowable substances in any category. Substances produced through similar processes (e.g., algal products, vegetable oil) are allowed for certain production and processing uses, but only if a nonsynthetic solvent is used for extraction (CGSB, 2011). Health Canada began allowing addition of ARASCO® (trade name for Martek's ARA Single-cell Oil) to non-organic infant formula in 2003, after assessing the toxicology, chemistry, microbiology, and nutrition of ARASCO® as a food ingredient (Health Canada, 2003). Allowable levels of ARASCO® in baby formula are determined by Health Canada on a case-by-case basis during the premarket notification process for infant formula.

Other counties that have approved ARA Single-cell Oil as a novel food ingredient or food additive for infant formula are Australia, New Zealand, China, France, and the Netherlands (Martek, 2010a). The

European Union similarly allows "ARA Single-cell Oil from *M. alpina*" in infant formula (European Commission, 2008).

No standard for ARA Single-cell Oil as a food ingredient has been determined by the CODEX Alimentarius Commission (Codex Alimentarius Commission, 2010). Edible fats and oils not covered under individual standards, however, are allowed by the CODEX Alimentarius Commission as "foodstuffs" provided they do not contain substances prohibited under the standard (e.g., added colors, concentrations of heavy metals and pesticide residues above maximum limits) (Codex Alimentarius Commission, 1981).

Evaluation Questions for Substances to be used in Organic Handling

<u>Evaluation Question #1:</u> Describe the most prevalent processes used to manufacture or formulate the petitioned substance. Further, describe any chemical change that may occur during manufacture or formulation of the petitioned substance when this substance is extracted from naturally occurring plant, animal, or mineral sources (7 U.S.C. § 6502 (21)).

ARA Single-cell Oil is synthesized primarily by the non-genetically-modified soil fungus *Mortierella alpina* (Hogan & Hartson L.L.P., 2000), which is not believed to cause disease in humans and biota (EFSA, 2008). Other microorganisms, such as other Mortierella species, other fungi, ciliated protozoa, amoeba, and algae also produce ARA (Bajpai et al., 1991), but research has focused on the Mortierella fungi due to high relative ARA production capacity and purported similarity to chemical structures in breast milk (Kyle, 1997). In a study comparing the ARA production of 33 Mortierella species, *M. alpina* produced the highest percentage of ARA (Eroshin et al., 1996). The process described here is for the production of single-cell oil by fungal species; however, the process is similar to that used to process single-cell oil from algal species.

Synthesis begins with aerobic fermentation of the fungus in shake flasks containing a growth medium. The fermentation broth typically includes mixtures of nitrogen and carbon sources such as glucose, molasses, high-fructose corn syrup, soy flour, hydrolyzed starch, and yeast extract, among others (Kyle, 1997; EFSA, 2008). The fermentation broth can also include a number of bulk nutrients, trace minerals, starch, and saccharifying enzymes (Ono et al., 2011). The fermentation process is finished in stirred tank fermenters, in which temperature, pH, air flow, pressure, agitation, dextrose concentration, and dissolved oxygen are monitored and controlled (Hogan & Hartson, 2000). The pH of the fermentation broth can particularly affect microbial growth and the amount and profile of the oils produced; as a result, pH profiling is conducted through addition of food acids and bases to maintain pH at a desirable level (Kyle, 1997).

The microbial biomass is then harvested from the fermentation broth using filtration, centrifugation, or spray drying (Kyle, 1997). Oil can be extracted directly from the crumbled wet mycelial cake (i.e., the harvested biomass) using polar solvents such as ethanol or isopropyl alcohol in a reaction kettle. Supercritical fluid extraction can also be used on the wet cake by employing carbon dioxide (CO_2)or nitric oxide (NO) solvents in a manner similar to that used in decaffeination of coffee beans.

Alternatively, the mycelial cake can be dried after harvest via vacuum drying, fluid drying, spray drying, or lyophilization, after which the oil is extracted using a nonpolar solvent and wet grinding (Bajpai et al., 1991; Kyle, 1997; Ono et al., 2011). Although the preferred solvent is hexane, other solvents that can be used for this process include ether, methanol, ethanol, chloroform, dichloromethane, and petroleum ether (Ono et al., 2011). The extraction process results in an oil clouded with suspended fine solids that can interfere with the refinement of the crude oil. Cloudy crude oil can be clarified through addition of a more polar solvent such as acetone. The mixture is then desolventized through treatment by heat and vacuum, and the solvent is recovered and re-used (Kyle, 1997; Martek, 2010b). The Petitioner reports that no detectable hexane residues remain in the oil mixture after desolventation (Hogan & Hartson, L.L.P, 2000). A Swiss study that examined 41 vegetable oils for hexane residues, however, did detect hexane residues in 12% of oils tested using a detection limit of 0.01 mg/kg, indicating that residual hexane from processing of food-grade oils can occur, albeit at levels below accepted tolerances (Kantonales Laboratorium, 2004). Oil can also be extracted from the dry biomass through counter-current extraction in commercially available extraction units designed to extract dirt and soil. Extraction efficiencies using this process are not as high those processes that include grinding of the biomass, the but the result is a clearer oil.

Although the crude oil can be used in products, further steps to purify and deodorize the oil for use in food and consumer products are often conducted. Kyle (1997) reports that these processes "do not chemically or covalently modify the ARA-containing lipids or the ARA itself." This is in contrast to a method reported by Shinmen et al. (1995) where isolated methyl esters of ARA-containing lipids are hydrolyzed (i.e., broken down by chemical reaction with water) with alkali before extraction with an organic solvent. ARA crude oil can be purified by adjusting the pH to neutralize fatty acids and remove "undesirable" residues first through lowering the pH by addition of an acid (e.g., citric acid) followed by raising the pH through addition of sodium hydroxide. The pH adjustment results in the formation of gums, soaps, and water that can be removed through heating and centrifugation. Any remaining polar compounds, trace metals, and oxidation products can then be removed physically through filtration or addition of adsorbents and chelaters (e.g., citric acid, silica, clay) (Martek, 2010b). A deodorizer can be added to the oil under heated and vacuum conditions to remove peroxides and remaining low-molecular weight compounds that might contribute to off-flavors and odors. To prevent oxidation, food-grade antioxidants (e.g., tocopherols, ascorbyl palmitate) can be added to oil. Finally, high oleic sunflower oil can be added in varying amounts (usually less than 10% dilution) to achieve consistent ARA potency across batches and products (Hogan & Hartson L.L.P., 2000; EFSA, 2008; Martek, 2010b).

<u>Evaluation</u> <u>Question #2:</u> Is the substance synthetic? Discuss whether the petitioned substance is formulated or manufactured by a chemical process, or created by naturally occurring biological processes (7 U.S.C. § 6502 (21)).

ARA Single-cell Oil is produced naturally via fermentation of *M. alpina* and some other single-celled organisms. However, to extract the ARA Single-cell Oil from the fungus, a nonpolar solvent (usually hexane) is used. A U.S. patent describing the production process of ARASCO® on behalf of Martek Biosciences Corporation claims that the extracted oil is "unmodified," stating that "As used herein 'unmodified' means that the chemical properties of the fatty acids, or the oils themselves, have not been covalently altered" (Kyle, 1997). The crude oil extracted using the nonpolar solvent is often further purified to clarify and deodorize the oil for use as a food ingredient. The same patent states that "Processes such as those used in the preparation of lecithin from vegetable products, and known to those of skill in the art, can be used in this additional purification step. Such processes do not chemically or covalently modify the ARA-containing lipids or the ARA itself" (Kyle, 1997). Processes are employed to remove any extraction and purification solvents from the oil, after which the solvents can be recycled and reused. No residual hexane from the extraction process has been detected in samples of ARA Single-cell Oil using methods with detection limits of 0.3 ppm (Hogan and Hartson L.L.P., 2000) or 2 mg/kg (EFSA, 2008).

In its April 2010 guidance to the NOP, the NOSB Joint Materials and Handling Committee sought to clarify the definition of synthetic with the following statement: "extraction with a synthetic not on the National List would not result in a material being classified as synthetic unless either the extraction resulted in chemical change or the synthetic remained in the final material at a significant level"(NOSB, 2010). Hexane is not currently on the National List.

Some stabilizers added to the ARA Single-cell Oil to prevent oxidation (e.g., tocopherols) are included on the National List as synthetics allowed on 7 CFR 205.605(b), while others (e.g., ascorbyl palmitate) are not on the National List. The petitioner claims that the concentration of synthetic food-grade antioxidants are <0.1% by weight in ARA Single-cell Oil (Martek, 2010a).

Given that (1) the synthetic solvents used during processing do not appear to alter the chemical identity of the naturally produced ARA Single-cell Oil; (2) that these solvents are removed from the oil, leaving no detectible concentrations; and (3) synthetic food-grade ingredients are present at what could be considered a "less than significant level," ARA Single-cell Oil does not appear to be a synthetic substance.

<u>Evaluation Question #3:</u> Provide a list of non-synthetic or natural source(s) of the petitioned substance (7 CFR § 205.600 (b) (1)).

ARA Single-cell Oil can be produced in the cells of ciliated protozoa, amoebae, algae, and other microorganisms, but Mortierella fungi produce the greatest amount of ARA Single-cell Oil by weight (Bajpai et al., 1991). No effective, solvent-free, alternative process has been reported for extraction of ARA Single-cell Oil from microorganisms. Pre-formed ARA can also be found in meat, dairy products, and farm-raised fish, but the yield is relatively small (approximately 0.2% by weight) compared to that of ARA Single-cell Oil produced by *M. alpina* (reported yields up to 95%; Shimada et al. 1998).

The only natural pre-formed ARA alternative to ARA Single-cell Oil that is used in infant formula is ARA-rich egg phospholipid. Research has shown that by varying the diet of chickens, eggs with virtually any desired ratio of DHA to ARA can be produced (Carlson, 1997). The biomass of single-cell organisms is often used to supplement the chicken feed to produce the desired level of ARA in the egg. Nature's One® produces a DHA/ARA infant formula supplement powder.[6] The manufacturer claims that "Baby's Only Essentials® DHA & ARA Fatty Acid is naturally derived from the goodness of egg phospholipids using an aqueous (water) process. This differs from C. cohnii oil (algae) & M. alpina oil (fungus) used in all other organic and conventional infant formulas, which are treated with hexane solvent, acid, and bleach" (Nature's One, 2011). Using egg phospholipid as a commercial source of ARA, however, is considered by some as not economically feasible and wasteful of resources because ARA contents in egg phospholipids are relatively low (1.5–2.8%; Clandinin and Chappell, 1997) and most of the egg is often discarded after phospholipid extraction (Ahn et al., 2006). The lipid fraction of an egg yolk is about 31%, of which about 29% is phospholipids (Ahn et al., 2006).

An aqueous process (as reported for Nature's One® Baby's Only Essentials® DHA & ARA Fatty Acid) is described in a patent for "The Aqueous Extraction Process To Selectively Remove Phospholipid From Egg Yolks" (Merkle & Ball, 2001). In general, an aqueous method is utilized to separate the majority of proteins from the egg yolk using ionic strength, pH, and gravitational centrifuge forces. First, the egg yolks are separated from the albumen (i.e., egg white) by hand or using mechanical methods, and the egg whites are generally discarded but can be used for other purposes. Egg yolks are then diluted with water, and the pH of the diluted egg yolk material is adjusted by the addition of food-grade acids, bases, or salts. The adjusted and mixed egg yolk material is then exposed to gravitational separation via centrifugation, and a viscous precipitate is removed, leaving the supernatant fraction containing most of the egg phospholipids. The precipitate can be discarded or reused for other purposes. Viscosity agents such as algin or carboxy methylcellulose are then added to the supernatant fraction and again exposed to gravitational separation forces for separation into a cream fraction and an aqueous subnatant fraction. The cream and subnatant of the algin separation contain approximately 35.5% and 1.3% fat, respectively, with the cream layer accounting for approximately 13% of the total volume (Merkle & Ball, 2001). Other manufacturing methods are described that use ethanol, a food-grade solvent, to initiate the separation of the lipid and protein fractions of the egg yolk (Nielson, 2007; Schneider, 2010).

The claim by Nature's One® that egg phospholipids in the Baby's Only Essentials DHA & ARA supplement are extracted using an aqueous process indicates that the aqueous process described in the previous paragraph (or a similar process) has been used for ARA products currently on the market. It is not clear whether ethanol-extracted egg phospholipids have been used in infant formulas.

<u>Evaluation Question #4:</u> Specify whether the petitioned substance is categorized as generally recognized as safe (GRAS) when used according to FDA's good manufacturing practices (7 CFR § 205.600 (b)(5)). If not categorized as GRAS, describe the regulatory status. What is the technical function of the substance?

ARA Single-cell Oil is characterized as GRAS under three different names submitted by four different applicants: Martek Biosciences (GRN No. 41), Mead Johnson Nutritionals (GRN No. 80), Abbott Laboratories (GRN No. 94), and Cargill, Inc. (GRN No. 326) when used in term and preterm infant formula

[6] Nature's One, Inc., 8754 Cotter St., Lewis Center, OH 4303. Toll Free (US Only): (888) 227-7122; Customer Service: (614) 898-9758; Corporate Office: (740) 548-0135

along with GRAS concentrations of DHA (FDA, 2011a). FDA originally categorized ARASCO® as GRAS as a source of ARA when added to infant formulas intended for consumption by healthy term infants at a level up to 1.25 percent total dietary fat and at a ratio of DHA to ARA of 1:1–1:2 (FDA, 2001a). In its GRAS approval letter, FDA expressed an expectation that infant formula manufacturers monitor infants consuming formulas to which ARASCO® has been added through scientific studies and post-market surveillance. The designated GRAS level of ARASCO® was later elevated to 1.88 percent of total dietary fat following another GRAS submission (FDA, 2001b).

ARA-rich fungal oil from *M. alpina* is also categorized as GRAS as a source of ARA in preterm infant formula intended for consumption by hospitalized preterm infants, post-discharge premature infants, and term infants at a mean level of 0.4 percent of total fatty acids in the formula (FDA, 2006a). And most recently, ARA-rich oil from *M. alpina* strain I_{49}-N_{18} has been designated GRAS as a source of ARA for term infants at a mean concentration of 0.75 g/100 g total fat and for pre-term infants at a mean concentration of 0.40 g/100 g total fat when used in combination with DHA from tuna at ARA:DHA ratios of 1:1–2:1 (FDA, 2011b).

<u>Evaluation Question #5:</u> Describe whether the primary function/purpose of the petitioned substance is a preservative. If so, provide a detailed description of its mechanism as a preservative (7 CFR § 205.600 (b)(4)).

The primary function of ARA Single-cell Oil in infant formula is to impart nutritional qualities on the food, not to act as a preservative. The oil is relatively susceptible to oxidation due to the high number of cis double bonds in ARA, the primary constituent of ARA Single-cell Oil. Without the addition of a preservative, ARA Single-cell Oil will oxidize, resulting in an adverse effect on the nutritional quality, odor, and taste of the oil (Bartee et al., 2007). Because ARA Single-cell Oil requires the use of a preservative to maintain its stability, ARA Single-cell Oil cannot itself be considered a preservative.

<u>Evaluation Question #6:</u> Describe whether the petitioned substance will be used primarily to recreate or improve flavors, colors, textures, or nutritive values lost in processing (except when required by law) and how the substance recreates or improves any of these food/feed characteristics (7 CFR § 205.600 (b)(4)).

The primary function of ARA Single-cell Oil is as a nutritional GRAS ingredient in infant formula. ARA Single-cell Oil is not added to replace nutritive value lost in processing, but to augment the essential fatty acids necessary to create ARA with pre-formed ARA for consumption by infants.

<u>Evaluation Question #7</u>*:* Describe any effect or potential effect on the nutritional quality of the food or feed when the petitioned substance is used (7 CFR § 205.600 (b)(3)).

ARA Single-cell Oil is added to infant formula to deliberately increase the level of ARA to levels comparable to those in human milk (Spherix Consulting, Inc., 2010). In a recent FDA GRAS notification, Spherix Consulting, Inc. (2010) on behalf of Cargill, Inc. compared the fatty acid profiles of an infant formula with no added ARA, an infant formula supplemented with SUNTGA40S (trade name for Suntory, Ltd. ARA Single-cell Oil), and an infant formula supplemented with RAO (trade name for Cargill, Inc. ARA Single-cell Oil). The authors reported “virtually no effect on the final formula fatty acid composition, with the exception of the intentional increase in levels of ARA”. No information was identified that discussed the effect of ARA on the bioavailability of other nutrients in the enriched foods.

<u>Evaluation Question #8:</u> List any reported residues of heavy metals or other contaminants in excess of FDA tolerances that are present or have been reported in the petitioned substance (7 CFR § 205.600 (b)(5)).

No residues of heavy metals or other contaminants have been reported in ARA Single-cell Oils at levels higher than FDA tolerances (Martek, 2010b; EFSA, 2008). Contaminants that were tested for and not detected by the Petitioner in ARASCO® (Martek Biosciences) include arsenic, cadmium, chromium, copper, iron, lead, manganese, mercury, molybdenum, nickel, and phosphorous. The detection limit for lead (0.1 mg/kg) in ARA Single-cell Oil products, however, is higher than the maximum level (0.020

mg/kg wet weight) provided in the European Union Annex, Section 3 of Commission Regulation (EC) 1181/2006 for lead in infant formula (EFSA, 2008). Concentrations of pesticides or PAHs have not been provided for ARA Single-cell Oil products.

As discussed in Evaluation Question #1, the Petitioner reports that no detectable hexane residues remain in the oil mixture after desolventation (Hogan & Hartson, L.L.P, 2000). A Swiss study that examined 41 vegetable oils for hexane residues, however, did detect hexane residues in 12% of oils tested using a detection limit of 0.01 mg/kg, indicating that residual hexane from processing of food-grade oils can occur, albeit at levels below accepted tolerances (Kantonales Laboratorium, 2004).

<u>Evaluation Question #9:</u> Discuss and summarize findings on whether the manufacture and use of the petitioned substance may be harmful to the environment or biodiversity (7 U.S.C. § 6517 (c) (1) (A) (i) and 7 U.S.C. § 6517 (c) (2) (A) (i)).

No information was found on the effect of ARA Single-cell Oil on the environment or biodiversity, but limited information is available on the behavior of ARA in the environment. The Environment Canada Domestic Substances List characterizes the toxicity of ARA to aquatic organisms as "uncertain," but the Substances List does not characterize ARA are persistent or bioaccumulative in the environment (Environment Canada, 2011). A producer of ARA notes in a material safety data sheet (MSDS) that "[p]ossibly hazardous short term degradation products are not likely. However, long term degradation products may arise. The product itself and its products of degradation are not toxic" (Sciencelab.com, Inc., 2005). A MSDS for Martek's ARA Single-cell Oil product ARASCO® notes, however, that "porous material wetted with this product may undergo spontaneous combustion," which might have implications on the environmental effects of this substance (Martek, 2010b).

<u>Evaluation Question #10:</u> Describe and summarize any reported effects upon human health from use of the petitioned substance (7 U.S.C. § 6517 (c) (1) (A) (i), 7 U.S.C. § 6517 (c) (2) (A) (i)) and 7 U.S.C. § 6518 (m) (4)).

Humans are not capable of synthesizing the omega-3 and omega-6 families of PUFA; instead, these PUFA must be derived from shorter-chain PUFA that are present in the diet. As a result, the parent omega-3 and omega-6 PUFA for DHA and ARA (i.e., alpha-linoleic acid and linoleic acid, respectively) are considered essential to the human diet (Koletzko et al., 2008). Both ARA and DHA are considered to be important structural components of brain and retinal tissues of humans. These LCPUFA begin to accumulate rapidly in the fetal brain during the mid to last trimester of pregnancy when the fetal brain undergoes a dramatic growth spurt, and accumulation of LCPUFA continues well into the second year of infancy (Birch et al., 2007). The ARA eicosanoids are also key components in the growth and maturation of multiple organs within the immune system and gastrointestinal tract in a developing fetus (Friesen & Innis, 2009). Some of the ARA eicosanoids promote blood clotting, induce pain, and cause smooth muscle contraction, while others are powerful inflammatory agents (DC Nutrition, 2006).

The relationship between accretion of LCPUFA and rapid brain growth has led to a number of studies investigating the potential benefits and adverse effects on neural (and visual) function associated with DHA/ARA supplementation of infant formula. While many randomized trial studies have reported statistically significant improvements to retinal maturation, visual acuity, and cognitive function during infancy following supplementation of infant formula with DHA/ARA, other studies have reported no benefit (as summarized in Birch et al., 2007). Similarly, studies investigating the effects of supplementation of infant formula with DHA/ARA on growth of pre-term infants have reported mixed results, with some studies reporting statistically significant increases in growth of pre-term infants fed supplemented formula compared to those fed non-supplemented formula (Innis et al., 2002) and others reporting no effect (O'Connor et al., 2001; Foreman-van Drongelen et al., 1996; Vanderhoof et al., 1999). The same pattern of mixed results (i.e., studies reporting benefits and studies reporting no effect) has been reported for intestinal effects (e.g., reduced necrotizing enterocolitis, inflammation) in pre-term infants (as summarized in Calder et al., 2007). Despite mixed results on many of the purported benefits of ARA supplementation in infant formula, adverse effects in infants fed formulas enriched with ARA/DHA have not been observed in randomized trials for up to one year (Jump et al., 2009; Spherix Consulting, Inc., 2010).

In its review of the Martek Biosciences Corporation notification for its ARA Single-cell Oil, ARASCO®, FDA discussed some adverse effects that were observed in studies and panel reports that evaluated infant consumption of DHA and ARA from sources such as fish oil and egg phospholipid. Some studies of infants that consumed formula containing long-chain PUFAs showed unexpected deaths, but these were were attributed to necrotizing colitis, sepsis, or Sudden Infant Death Syndrome (SIDS). Other studies have reported increased flatulence, diarrhea, apnea, and jaundice in infants that were fed formulas with long-chain PUFAs (FDA, 2001a).

One study has reported statistically significantly lower mean blood pressure and diastolic blood pressure in children six years of age that had been fed ARA/DHA supplemented formula for the first four months of life compared to children given unsupplemented infant formula (Forsyth et al., 2003). The study authors claim that because blood pressure in childhood is often predictive of blood pressure as an adult, early exposure to dietary ARA/DHA might have lasting beneficial effects of reduced blood pressure and reduced cardiovascular risk.

Safety assessments of ARA Single-cell Oil have been conducted, and the results are comparable among the different formulations of commercially available ARA infant formula supplements (Spherix Consulting, Inc., 2010). The most recent safety assessment of ARA Single-cell Oil was conducted by Cargill, Inc. for its ARA Single-cell Oil product RAO, which was evaluated for reverse mutation, chromosome aberration and gene mutation, and in a 90-day Wistar rat feeding study with in utero exposure to 0.5%, 1.5% and 5% RAO. All results of the genotoxicity assays were negative. Although some statistically significant effects were observed for selected histopathology, clinical chemistry, and organ weight endpoints, only one endpoint (increased absolute and relative monocytes in both sexes of high-dose rats) was deemed relevant to treatment with ARA Single-cell Oil. No adverse effects attributed to consumption of the ARA Single-cell Oil were observed even at the highest dose of RAO (~ 3,000 mg/kg body weight/day), which is 29-times higher than the anticipated intake of 42 mg ARA/kg body weight/day (104 mg of RAO/kg body weight/day) for term infants and 45-times higher than the intake of 27 mg ARA/kg body weight/day (67 mg of RAO/kg body weight/day) for preterm infants (Spherix Consulting, Inc, 2010).

Food Standards Australia New Zealand (FSANZ) reviewed the toxicological database for ARA Single-cell Oil and determined that ARA Single-cell oil did not induce any histopathological, biochemical, or hematological changes that would be indicative of toxicity at doses up to 2500 mg /kg body weight/day (FSANZ, 2003). FSANZ determined that the observed changes (e.g., increased liver weights, decreased serum cholesterol and triglycerides) in the toxicology database were consistent with the physiological changes observed in response to the administration of high levels of LCPUFA, irrespective of source, and were not concluded to be a manifestation of toxicity specific to the administration of ARA Single-cell Oils.

<u>Evaluation Information #11:</u> Provide a list of organic agricultural products that could be alternatives for the petitioned substance (7 CFR § 205.600 (b)(1)).

Three main sources of ARA are used for supplementing infant formula: ARA Single-cell Oil, fish oil, and egg phospholipids (FSANZ, 2003). As discussed in Evaluation Question #3, the fatty acid profile of eggs can be manipulated by feeding chickens the biomass of ARA-producing fungus (Carlson, 1997). As a result, egg phospholipids could be organic alternatives for the petitioned substance, ARA Single-cell Oil, if the eggs are produced in adherence with organic practices.

Before the large-scale production of ARA Single-cell Oil and DHA Algal Oil for fatty acid supplementation of infant formula, fish oil was the primary source of fatty acids to formula-fed infants. Though fish oil is not an organic agricultural product per se, fish oil is on the National List as a non-organically produced agricultural product allowed for use as an ingredient in or on processed products labeled as "organic" (7 CFR § 205.606(f)). Fish oil does not contain high levels of pre-formed ARA, however, so fish oil used today is often supplemented with another source of ARA (e.g., egg phospholipid or ARA Single-cell Oil) to achieve a fatty acid profile for optimal nutrition. Furthermore, fish oil contains high levels of EPA, which can result in adverse effects on growth of pre-term infants even at low concentrations (Carlson et al., 1999).

Additional Questions Specific to ARA Single-cell Oil

The following additional questions were posed by the NOSB Handling Committee to aid the National List review for ARA Single-cell Oil use in handling (USDA, 2011).

Additional Question #1: Describe the FDA approval process for the use of ARA single-cell oil in foods and infant formula.

Infant formula is considered a food by FDA; therefore, infant formula and other foods are subject to the same regulatory provisions governing the use of ARA Single-cell Oil as a food ingredient. Under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act (FFDCA), "any substance that is intentionally added to food is a food additive, that is subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excluded from the definition of a food additive" (FDA, 2004). Infant formula is subject to additional statutory and regulatory requirements provided in 21 CFR 106 and 107 to ensure the nutritional quality and safety of what is considered the "sole source of nutrition by a vulnerable population during a critical period of growth and development" (FDA, 2009a).

Because ARA Single-cell Oil is generally recognized as safe for human consumption, even in vulnerable infant populations, the FFDCA does not require FDA premarket review and approval before using ARA Single-cell Oil in infant formula and other foods. Manufacturers of a food ingredient intended for specific use (e.g., ARA Single-cell Oil as an ingredient in infant formula) may submit a GRAS notice to FDA that includes a "GRAS exemption claim" comprising a short description of the substance, the applicable conditions of use, and the statutory basis for the GRAS determination (e.g., through scientific procedures or through experience based on common use in food) (FDA, 2004). A GRAS notice also includes information about the identity and properties of the notified substance and a discussion of the notifier's reasons for concluding that the substance is GRAS for its intended use. However, the GRAS notification program is voluntary; if a food ingredient for a specified use is designated GRAS by the manufacturer, FDA has no regulatory authority over that ingredient in that use. Only if an ingredient is determined to be injurious to human health does FDA have authority (under the adulteration provision (section 402(a)(1)) of the FFDCA) to remove products containing that ingredient from the market.

Pre-market requirements do exist for addition of macroingredients to infant formula. Within FDA, the Center for Food Safety and Applied Nutrition (CFSAN) is responsible for regulating infant formula in the United States. Manufacturers that wish to market a new or reformulated infant formula are required to register with FDA, submit a notification 90 days before marketing the formula, and submit a statement that summarizes the test results that verify that the product complies with the FFDCA (FDA, 2009a). The notification for a new infant formula must include (1) the quantitative formulation of the infant formula, (2) a description of any reformulation of the formula or change in processing of the infant formula, (3) assurances that the infant formula will not be marketed unless it meets the quality factors and the nutrient requirements of the FFDCA, and (4) assurances that the processing of the infant formula complies with good manufacturing practices, including quality control procedures.

The CFSAN Office of Nutritional Products, Labeling, and Dietary Supplements (ONPLDS) evaluates whether the manufacturer of the formula has met the requirements in section 412 of the FFDCA. The ONPLDS then consults with the Office of Food Additive Safety (OFAS) regarding the safety of the ingredients in the formula and the packaging materials for the formula. OFAS evaluates the safety of the ingredients in the formula according to sections 201(s) and 409 of FFDCA. The manufacturer can market a new infant formula without providing a pre-market notification to FDA, but the formula is then automatically defined as adulterated under section 412(a)(1) of the FFDCA, and FDA has the authority to take compliance action (FDA, 2009a). Compliance actions may range from sanctions to removal of products from the market.

<u>Additional Question #2:</u> Describe how the FDA approves ingredients to be considered essential, required, and/or allowed in foods and infant formula. Does FDA consider ARA to be essential, required, and/or allowed in foods and infant formula?

Guiding principles for appropriate addition of nutrients to foods is provided primarily by the FDA Fortification Policy of 1980 (21 CFR 104.20). Only essential nutrients are subject to the Fortification Policy (Schneeman, 2010). The Policy states that these essential nutrients "may appropriately be added to a food to correct a dietary insufficiency recognized by the scientific community to exist and known to result in nutritional deficiency disease" (21 CFR 104.20) if the addition will "correct a dietary insufficiency, restore nutrients to a certain level, maintain a balanced nutrient profile, improve the quality of a replacement food, or be added as permitted or required by another FDA regulation" (Schneeman, 2010). Any nutrients not codified in 21 CFR 109 (c)(8)(iv) are not considered "essential" nutrients and are therefore outside the scope of the guiding principles of the Fortification Policy. As a result, nutrients not codified in 21 CFR 109 (c)(8)(iv) would be categorized as food additives or GRAS substances and would be allowed in food products following premarket review and approval by FDA or determination of GRAS status, as required in sections 201(s) and 409 of the FFDCA (Schneeman, 2010).

The Infant Formula Act of 1980 was enacted after the Fortification Policy and the recommended daily values of essential nutrients in the Fortification Policy were established for children aged 4 years and above, not for younger children and infants (Schneeman, 2010). The nutrient requirements of infant formula are therefore considered to be outside of the scope of Fortification Policy. Minimum amounts for 29 specified nutrients are required in infant formulas, and maximum amounts are provided for 9 of those nutrients in 21 CFR Part 107. Any infant formula ingredient not specified in 21 CFR Part 107 is subject to the same regulations as a food ingredient and would be allowed in infant formula following premarket review and approval by FDA (if characterized as a food additive) or determination of GRAS status (if characterized as a GRAS substance), as required in sections 201(s) and 409 of the FFDCA (FDA, 2006b).

<u>Additional Question #3:</u> Describe how the FDA regulates the use of ARA in foods and infant formula. What is the maximum amount of ARA that is permitted? How does the FDA regulate what foods can be fortified with ARA single-cell oil?

As discussed in "Additional Question #1," infant formula is considered a food by FDA; therefore, infant formula and other foods that are enriched with ARA Single-cell Oil are subject to the same FDA approval process. Under sections 201(s) and 409 of the FFDCA "any substance that is intentionally added to food is a food additive that is subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excluded from the definition of a food additive" (FDA, 2004).

As discussed in "Additional Question #1," manufacturers of a food ingredient intended for specific use (e.g., ARA Single-cell Oil as an ingredient in infant formula) may submit a GRAS notice to FDA that includes a "GRAS exemption claim" comprising a short description of the substance, the applicable conditions of use, and the statutory basis for the GRAS determination (i.e., through scientific procedures or through experience based on common use in food) (FDA, 2004). A GRAS notice also includes information about the identity and properties of the notified substance and a discussion of the notifier's reasons for concluding that the substance is GRAS for its intended use. However, this program is voluntary for inclusion of GRAS substances as ingredient in most food items.

FDA does not set a maximum amount of ARA that can be added to food products. Instead, the manufacturer of the ARA must show through their food-additive petition that adding ARA to a given food item at the levels proposed would be safe or that adding ARA to the food item at the levels proposed is GRAS (FDA, 2004). ARA has not currently been petitioned for addition to food items other than infant formula.

Infant formula is subject to additional statutory and regulatory requirements provided in 21 CFR 106 and 107 to ensure the nutritional quality and safety of what is considered the "sole source of nutrition by a vulnerable population during a critical period of growth and development" (FDA, 2009a).

FDA is not required to approve infant formulas before they can be marketed and sold, but all formulas have to meet federal requirements for basic nutrients. As discussed in the response to "Additional Question #1," manufacturers of infant formulas also have to notify FDA 90 days before they market a new formula. Nutrient requirements for infant formula are stipulated in section 412(d) of FFDCA and in 21 CFR 107.100. The only exception to these rules are "exempt infant formulas" which are specially formulated for infants with "…an inborn error of metabolism or low birth weight, or who otherwise has an unusual medical or dietary problem." Substances that can be used in infant formulas are GRAS substances for use in infant formula and those substances used in accordance with FFDCA sections 201(s) and 409 (FDA, 2009a).

<u>Additional Question #4:</u> What is the recommended daily allowance of ARA for humans at various stages of growth and maturity?

<u>Infants</u>

The recommended daily intakes of ARA in international infant formulas range from 27 to 60 mg/kg body weight/day (up to 0.9% of total fatty acids) for preterm infants and from 18 to 40 mg/kg body weight/day (0.2–0.6% of total fatty acids) for term infants (as summarized in Martek, 2010b; Spherix Consulting, Inc., 2010). The WHO recommends that ARA should be supplied in the diets of infants aged 0–6 months within the range of 0.2–0.3% total energy based on human milk consumption (WHO, 2008). The Institute of Medicine established adequate intakes for total omega-6 PUFA of 4.4 g/day for infants aged 0–6 months and 4.6 g/day for infants aged 7–12 months (Institute of Medicine, 2005).

<u>Children 12-36 months</u>

The Superior Health Council of Belgium recently recommended ARA intake of 45-110 mg/day for children aged 12-36 months (Superior Health Council, 2009).

<u>Adults</u>

Dietary ARA intake is not considered necessary for a normal healthy adult consuming dietary linoleic acid greater than 2.5% total energy (WHO, 2008).

<u>Additional Question #5:</u> What are the effects on humans if more than the recommended amount of ARA is consumed at various stages of growth and maturity?

No upper limit for intake of omega-6 PUFA has been established because no defined intake level has been identified at which an adverse effect occurs (Institute of Medicine, 2005). Because of the pro-inflammatory, blood-clotting, and other potentially adverse activities of some of the ARA eicosanoids, concern has been expressed over what effect high dietary ARA might have on cardiovascular, immune, and respiratory health. One study examined the effect of increasing dietary ARA seven-fold in healthy volunteers in a 7-week controlled feeding study, and no effects on platelet aggregation, bleeding times, balance of vasoactive metabolites, serum lipid levels, or immune response were observed (as summarized in Harris et al., 2009). Furthermore, in a meta-analysis of 25 case-control studies (including 1998 cases and 6913 controls) evaluating blood/tissue omega-6 PUFA content and cardiovascular events, ARA was shown to be unrelated to coronary heart disease risk (Harris et al., 2007). No effects in humans at high ARA doses were identified.

One GRAS notification for ARA Single-cell Oil states that "five independent studies have shown that very high acute oral doses (up to 20 grams of DHASCO or ARASCO/kg body weight) did not have any major toxicological consequences in rats (Hogan and Hartson L.L.P., 2000). The only potentially adverse effect noted at a high dose in rats was an impaired concentrating ability of the kidneys at 4900 mg ARASCO/kg body weight/day alone or in combination with 3650 mg DHASCO/kg body weight/day in a subchronic study (FSANZ, 2003).

<u>Additional Question #6:</u> Where is added ARA listed on the nutrition panel for products?

Manufacturers of infant formulas and foods containing ARA Single-cell Oil are not permitted to list added ARA to the nutrition panel of food products (Institute of Medicine, 2005). However, most infant formula manufacturers do provide this information outside of the nutrition panel. The nutrient requirements of infant formula and related labeling specification are provided in 21 CFR Part 107; if a nutrient not listed in the nutrient requirements in 21 CFR 107.100 is added to the formula, that nutrient may only appear in specific locations in the list of vitamins and minerals if that nutrient "(i) has been identified as essential by the National Academy of Sciences through its development of a recommended dietary allowance or an estimated safe and adequate daily dietary intake range, or has been identified as essential by the Food and Drug Administration through a FEDERAL REGISTER publication or establishment of a U.S. Recommended Daily Allowance, and (ii) is provided at a level considered in these publications as having biological significance, when these levels are known" (21 CFR 107.10).

Only the nutrients listed by FDA as mandatory or voluntary in 21 CFR 101.9(c) may be listed in the nutrition panel for foods intended for adults and children over age four (FDA, 2009c). ARA may not be listed on the nutrient panel of infant formulas because neither the National Academy of Sciences nor the FDA have established recommended intake levels for ARA. Furthermore, ARA is not on FDA's list of mandatory or voluntary nutrients provided in the FDA nutrition regulations.

Certain statements about added ARA to infant formula and other foods may appear outside of the nutrition panel on the food label, but these statements must not be misleading in any way . While the amount or percent of the nutrient added may be presented on the food label outside of the nutrition panel, no qualifying statements (e.g., high in omega-6, low in EPA) may be made (FDA, 2009c).

<u>Additional Question #7:</u> What assumptions are made to determine the amount of ARA permitted for addition to products, such as fluid milk, infant formula, and cookies?

As discussed in the responses to "Additional Question #1" and "Additional Question #3", if the manufacturer determines that ARA added to a specific food item is GRAS based on FDA requirements, the onus is on the manufacturer to ensure safety of the product (FDA, 2004). FDA does not "permit" specific amounts of a GRAS substance in food products, but rather relies on the manufacturer to interpret the relevant scientific data and add a reasonable and safe amount of substance to the food product.

ARA is not presently used or petitioned for use in food products other than infant formula and growing-up milks. Sufficient ARA can be obtained from conversion of dietary linoleic acid and intake of ARA from common dietary sources such as eggs and meat once the child transitions from infant formula to a more diverse diet. For example, although the recommended ratio of omega-6 to omega-3 fatty acids in the diet ranges from 2:1–4:1, Americans typically consume 14–25 times more omega-6 fatty acids than omega-3 fatty acids (Ehrlich, 2009).

<u>Additional Question #8:</u> What foods naturally provide ARA to the human diet?

The National Cancer Institute (2010) compiled a list of the primary sources of ARA in the human diet ranked in order of descending contribution to intake of ARA. The list is based on data from the 2005-2006 National Health and Nutrition Examination Survey of dietary intakes for the U.S. population aged 2 years and older (Table 2). Chicken and eggs are the primary sources of ARA in the U.S. diet. The Australian diet was also examined for ARA content, and relative intake by food group was somewhat comparable to U.S. values (Table 3), with eggs providing the highest source of ARA by weight. The Australian diet also includes more organ meats, which are generally high in ARA (Mann et al., 1995).

Table 2. Food Sources of Arachidonic Acid Listed in Descending Order by Percentages of Contribution to Intake of ARA (National Cancer Institute, 2010)

Food Item	Percent Contribution to Total ARA Intake
Chicken and chicken mixed dishes	26.9
Eggs and egg mixed dishes	17.8
Beef and beef mixed dishes	7.3
Sausage, franks, bacon, and ribs	6.7
Other fish and fish mixed dishes	5.8
Burgers	4.6
Cold cuts	3.3
Pork and pork mixed dishes	3.1
Mexican mixed dishes	3.1
Pizza	2.8
Turkey and turkey mixed dishes	2.7
Pasta and pasta dishes	2.3
Grain-based desserts	2.0

Table 3. Concentration of Arachidonic Acid in Foods of the Australian Diet in Descending Order (Mann et al., 1995)

Product	ARA Concentration (mg/100 g edible food)	Standard Deviation
Duck egg yolk	891	172
Chicken egg yolk	390	72
Duck pate	311	25
Ox liver	294	64
Chicken pate, brand B	160	19
Lamb kidney	153	11
Chicken pate, brand A	142	19
Atlantic salmon (no skin)	100	92
Turkey composite (no skin)	75	24
Turkey composite (with skin)	63	13
Chicken legs (no skin)	56	3
Pork leg steak (lean)	56	8
Cold turkey loaf, brand A	50	17
Lamb fillet	49	7
Lamb leg steak	41	8
Cold turkey loaf, brand B	37	11
Rump steak (lean)	35	5
Chicken breast (no skin)	31	2
Beef sirloin (lean)	30	6
Barrumundi (fish)	26	6

<u>Additional Question #9:</u> Describe the commercial availability of naturally occurring sources of ARA.

The natural agricultural products highest in ARA (e.g., eggs, poultry, beef, some fish) are commonly available in supermarkets, grocery stores, and farmer's markets. These foods are common components of the U.S. diet, and are therefore highly available and easily accessible on the commercial market.

<u>Additional Question #10:</u> What is the trend in the marketplace for foods fortified with ARA?

ARA does not currently appear to be incorporated into any foods but infant formula, and little information was identified to suggest that ARA will be incorporated into other foods in the future. Patent applications regarding ARA-rich oil for potential use in foods are the only sources identified that suggest that ARA might someday be incorporated into foods other than infant formula. Because sufficient ARA can be obtained from a normal diet and from conversion of dietary linoleic acid in children and adults (Ehrlich, 2009), there appears to be no need to supplement foods with ARA, and substantial near-term market growth is not anticipated for food other than infant formula.

An overall market trend for ARA in infant formula was not identified, but information was available regarding the market growth for Martek's ARA Single-cell Oil product, ARASCO®, which is currently the most widely used ARA infant formula supplement on the market (Seeking Alpha, 2011).

Following the 2001 FDA GRAS determination for the use of the Martek Biosciences Corporation ARASCO® nutritional oil product in specified amounts and ratios in infant formulas, ARASCO® was almost immediately incorporated into several common infant formula brands including Mead Johnson Nutritionals (Enfamil®LIPIL®), Abbott Nutrition (Similac® ADVANCE®), Nestle (Good Start® Supreme DHA & ARA), PBM Products Inc. (Bright Beginnings™, Vermont Organics™, Wal-Mart Parent's Choice™), Hain Celestial (Earth's Best®), and Nutricia North America (Neocate®) (Seeking Alpha, 2011). Martek sales of nutritional products increased 183% over the first nine months of 2002 as compared to the same period for 2001, with 80% of its revenues generated from sales of ARASCO® and and its DHA algal oil, DHASCO®, to infant formula companies (INFACT Canada, 2002).

Martek claims that formula supplemented with ARA and DHA oils has penetrated almost all of the U.S. infant formula market, with over 35 infant formula manufacturers using the oils in their products. The worldwide retail market for infant formula is about $15 billion and the U.S. retail market about $4.5 billion. The companies using Martek's ARA and DHA oils represent approximately 75% and nearly 100% of the estimated worldwide and U.S. retail markets for infant formula, respectively. Infant formula products containing Martek's ARA and DHA oils are currently sold in over 75 countries (Seeking Alpha, 2011).

Martek was acquired for over $1 billion by Royal DSM N.V., a global life sciences and materials sciences company based in the Netherlands, in February 2011 (DSM, 2011). The strategic rationale provided by DSM for this acquisition was that "the acquisition will create a strong platform for DSM to enter the fast growing Omega-3 and Omega-6 market through Martek's microbial DHA and ARA products." The future of ARA in food supplements developed by DSM was previously discussed in a 2006 presentation to chemical analysts at a DSM plant (van Doesum, 2006). In this presentation, a DSM representative noted that Asian and European markets were still in the "take-off" or early phase of commercial development of ARA in infant formula. As of 2005, approximately 57% of U.S infants were estimated to consume ARA, while only 2.9% of infants worldwide consumed ARA. Nonetheless, the market for ARA in infant formula had increased dramatically between 2002 and 2005 both domestically and worldwide. DSM estimates that the number of U.S. infants and toddlers consuming ARA rose from about a half million in 2002 to 4.5 million in 2005, and the number worldwide increased from about 1.2 million in 2002 to 7 million in 2005 (van Doesum, 2006).

In general, ARA Single-cell Oil has been experiencing an upward market trend since 2001, and the recent acquisition of Martek by DSM suggests that industry intends to continue to expand the international market for ARA Single-cell Oil in infant formula and other niche food products.

<u>Additional Question #11:</u> What are the naturally occurring levels of fatty acids, including ARA, in milk from cows on concentrated grain diets versus cows consuming pasture only? Is there a correlation between rate of grain supplementation and ARA content in milk?

No information on the effect of diet on the ARA content in cow's milk was identified. Studies have shown, however, that changes in the diet of cows can influence the levels of fatty acids generally in cow's milk. Dhiman et al. (1999) examined fatty acid profiles in milk produced by cows consuming diets of different ratios of pasture and forage/grain. Cows were fed either one-third pasture, two-thirds pasture, or pasture-only diets, with the diets of the one-third and two-thirds pasture-fed cows supplemented with forage and grain. The investigators concentrated on the levels of conjugated linoleic acid (CLA), or isomers of linoleic acid (also referred to by its lipid number of $C_{18:2}$) produced in the gut of cows, because of the positive health benefits associated with these conjugated fatty acids.

Dhiman et al. (1999) found that the amount of CLA in milk fat increased linearly with an increase in the percentage of the diet from pasture (Table 4). Cows feeding on pasture only produced milk with 150% and 53% more CLA than milk produced by cows feeding on one-and two-thirds pasture, respectively. Pasture-only cows produced milk with 500% more CLA than cows fed only forage and grain (Dhiman et al., 1999). Concentrations of palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$) were also significantly higher in the milk fat of cows fed pasture-only diets when compared to that of cows fed diets supplemented with grain/forage. Concentrations of medium-chain and some long-chain fatty acids (i.e., $C_{10:0}$ to $C_{16:0}$ and $C_{18:1}$), however, did not differ among treatments in this experiment (Table 4).

Table 4. Consumption of Grain/forage Supplement, Milk Yield, and Fatty Acid Composition of Milk from Cows Grazing on 1/3, 2/3, and Only Permanent Pasture [1] (Dhiman et al., 1999)

	Treatment[2]				
Item	1/3PS	2/3PS	PS	SEM	P^3
Supplement intake	11.6^{a}	6.0^{b}	. . .	0.2	0.01
Grass intake,[4] kg/d	4.5^{c}	9.0^{b}	14.1^{a}	0.3	0.01
Milk yield, kg/d	24.5^{a}	17.5^{b}	14.5^{c}	0.9	0.01
3.5% FCM Yield, kg/d	24.5^{a}	18.0^{b}	14.2^{c}	0.9	0.01
Milk fat, %	3.51	3.64	3.37	0.1	0.2
Milk protein, %	2.90	2.73	2.86	0.06	0.09
Milk fat yield, kg/d	0.86^{a}	0.64^{b}	0.49^{c}	0.03	0.01
Fatty acid composition, mg/g of fatty acids					
$C_{10:0}$	21.1	18.0	18.0	1.1	0.09
$C_{12:0}$	26.0	22.3	23.3	1.3	0.2
$C_{14:0}$	94	89	91	3	0.5
$C_{16:0}$	247	240	251	5	0.2
$C_{16:1}$	12.3^{b}	13.1^{b}	17.6^{a}	0.6	0.001
$C_{18:0}$	152^{a}	151^{a}	121^{b}	6	0.001
$C_{18:1}$	314	333	326	7	0.12
$C_{18:2}$	42.7^{a}	27.1^{b}	14.0^{c}	1.5	0.001
CLA[5]	8.9^{c}	14.3^{b}	22.1^{a}	0.9	0.001
$C_{18:3}$	8.1^{c}	14.6^{b}	20.2^{a}	0.5	0.001
SFA[6]	540^{a}	520^{b}	505^{b}	7	0.002
UFA[7]	386	402	400	7	0.2
Others[8]	74^{b}	78^{b}	95^{a}	3	0.001

[a,b,c]Means with unlike superscripts within row differ according to P value indicated.

[1]Milk yield data are from only those cows whose milk was sampled for fatty acid analysis and represents the 1-wk period during which milk was sampled for fatty acid analysis.

[2]Cows on one-third pasture (1/3PS), two-third pasture (2/3PS), and all pasture (PS) treatments consumed one-third, two-third or all of their daily feed from pasture, respectively. The balance of feed for treatments 1/3PS and 2/3PS was supplied by a supplement.

[3]$P < 0.001$ is shown as $P = 0.001$.

[4]Estimated from net energy intake from grass. The NE_L intake from grass was calculated as milk energy output (33) + energy spent for maintenance and BW gain (24) – energy intake from the supplement. The NE_L value used for grass was 1.36 Mcal/kg of grass DM.

[5]Conjugated linoleic acid (*cis*-9, *trans*-11 $C_{18:2}$).

[6]Sum of $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$.

[7]Sum of $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, CLA, and $C_{18:3}$.

[8]100-sum of $C_{10:0}$ through $C_{18:3}$.

An advocate for grass-fed products processed the data from the study by Dhiman et al. (1999) to create a graph displaying the relative concentrations of omega-3 and omega-6 fatty acid contents in the milk fat of the cows fed the different ratios of pasture:grain/forage (Figure 3; Robinson, undated). With increasing rate of grain supplementation, the ratio of omega-3 to omega-6 fatty acids changes from approximately 1:1 to 1:5. The recommended ratio of omega-3 to omega-6 is between 1:2 and 1:4.

<u>Additional Question #12:</u> How much fish oil can be added to milk before an "off flavor" is noted?

Studies or reports that evaluated fish oil additive best practices with regard to "off flavors" were not identified. Research was found that addressed issues of analyzing and preventing off-flavors in milk enriched with fish oil. Though the amount of fish oil added does influence the presence or absence of "off flavors" in milk, factors such as the type and quality of the oil, the degree of oxidation of the oil, storage conditions, temperature, and pressure all influence the presence and amount of "off flavors" detected in enriched milk. Pure milk and fish oil-enriched milk (containing 0.5% cod liver oil by weight) were evaluated for volatile compounds using gas chromatographic methods by Venkateshwarlu and colleagues (2004). The resulting chromatograms showed 14 volatile compounds present for the fresh milk, and 60 volatile compounds for the fish oil-enriched milk. The volatile compounds found in the enriched milk, but

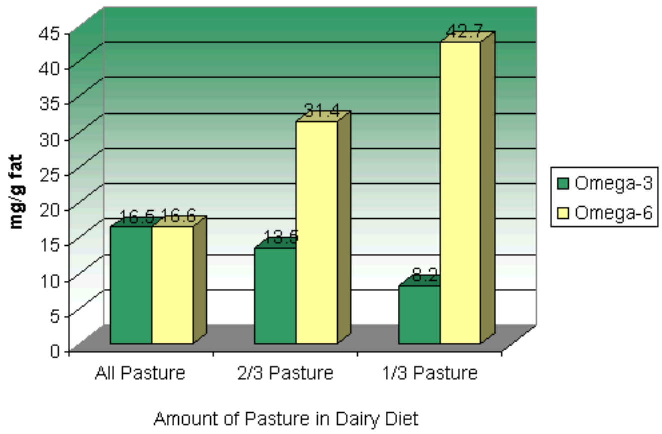

Figure 3. Relatively Quantities of Omega-3 and Omega-6 Fatty Acids in the Milk of Cows Fed Diets of 1/3, 2/3 and Only Pasture (Robinson, undated)

not in the pure milk were assumed to be due to the oxidation of the added fish oil. Sensory evaluation of the milk samples showed that the enriched milk had a distinctly fishy taste one day after the milk was enriched. The intensity of the fishy odor and taste increased each day, and was significantly higher than the pure milk at days four and eight of the evaluation period. These results indicate that at the levels tested, oxidation of added fish oil during storage of milk can increase fishy off-flavors in milk, and that off-flavors can be detected at 0.5% fish oil by weight (Venkateshwarlu et al., 2004). Studies that incorporated fish oil into milk at less than 0.5% by weight were not found.

The type and quality of fish oil added to milk can affect the potential for off-flavors. Fish oil quality is usually measured by peroxide value (PV), and the PV can significantly affect oxidative flavor deterioration in milk. In a study with 0.5% fish oils added to milk, two fish oils were compared, cod liver oil and tuna oil. The cod liver oil had a PV of 1.5 meq/kg and the tuna oil had a PV of 0.1 meq/kg. The cod liver oil oxidized significantly faster than the tuna oil and had significantly more fishy off-flavors. Temperature and pressure of processing can also affect oxidation and the production of off-flavors. Several antioxidants have been investigated for use as additives in fish oil-enriched milk to prevent oxidation and development of off-flavors (Jacobsen, 2010).

In a petition to the FDA by Unilever United States, Inc., the "Future Intended Use Levels" of fish oil in milk products is 2.9% by weight (FDA, 2002). According to 21 CFR 184.1472(a)(3), menhaden oil (a source of fish oil), may be added to milk at a maximum level of 5.0% to ensure that the intake of EPA and DHA does not exceed 3.0 grams per person, per day. Krill oil, a substitute for fish oil, has "a strong taste that begins to be detected at levels between 300 and 500 milligrams per serving, depending on the type of food," according to an FDA agency response letter to a notice from GRAS Associates, LLC (FDA, 2008).

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