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February 24, 2010

Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3837

RE: Notification of GRAS determination for Refined Arachidonic Acid-rich Oil

Dear Sir/Madame:

In accordance with proposed 21 CFR § 170.36 (Notification of a claim for exemption based on a GRAS determination published in the Federal Register (62 FR 18939-18964), enclosed are three copies of a GRAS Notification on refined arachidonic acid-rich oil for its use as an infant formula ingredient.

Sincerely,

(b) (6)

Phillip L. Casterton, M.S., D.A.B.T.
Cargill, Incorporated
Manager, Scientific and Regulatory Affairs
Food Ingredients & Systems North America
15407 McGinty Road West, MS-163
Wayzata MN 55391

Enclosures

COPY

Generally Recognized As Safe (GRAS) Notification for the Use of Refined Arachidonic Rich Oil *("RAO")* As an Ingredient in Infant Formula

Prepared for:

Cargill™

Cargill, Incorporated
15407 McGinty Road West
Wayzata, MN 55391

Prepared by:

Spherix Consulting, Inc.
6430 Rockledge Drive #503
Bethesda, MD 20817

February 18, 2010

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ABBREVIATIONS USED THROUGHOUT THIS DOCUMENT:

ALA = α-linolenic acid

ARA = arachidonic acid

bwt = body weight

cGMP = current Good Manufacturing Practices

CNS = central nervous system

DTH = delayed-type hypersensitivity

EC = European Commission

EFSA = European Food Safety Authority

EPA = U.S. Environmental Protection Agency

ERG = electroretinogram

EU = European Union

FDA = U.S. Food and Drug Administration

fl = femtaliter

fM = femtamole

g = grams

GCA = gestational corrected age (adjusted to time of normal term birth)

GLP = Good Laboratory Practices

GRAS = Generally Recognized As Safe

GRN = GRAS Review Notice/Notification, *i.e.*, documentation sent to the FDA by food ingredient manufacturers seeking concurrence with previously-reached conclusions regarding the GRAS status of their food ingredient. When used in this dossier, GRN will always represent FDA agreement (*via* issuance of a "no objection letter") with manufacturers of other commercially available arachidonic acid-rich oils on the GRAS status of their products.

JMHW = Japanese Ministry of Health and Welfare

kg = kilogram

l = liter

LA = linolenic acid

LCPUFA = Long-chain polyunsaturated fatty acid

MDA = malonyldialdehyde

MDI = mental development index

meq = milliequivalent

mg = milligram

mM = millimole

n = number

NEC = necrotizing enterocolitis

NOAEL = no observed adverse effect level

OECD = Organization of Economic Cooperation and Development

PDI = physical development index

PMA = postmenstrual age

ppm = part per million

RA = random dot stereoacuity

RAO = Refined Arachidonic acid-rich Oil (*i.e.*, the ARA-rich oil produced by Cargill Alking Bioengineering Co., Ltd, Wuhan, China)

RBC = red blood cell

SAE = serious adverse event

U = international units

μg = microgram

VEP = visual evoked potential

I. GRAS EXEMPTION CLAIM

A. NAME AND ADDRESS OF SPONSOR

Sponsor: Cargill, Incorporated
15407 McGinty Road West
Wayzata, MN 55391

Contact: Phillip L. Casterton, M.S., D.A.B.T.
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B. COMMON OR USUAL NAME OF GRAS SUBSTANCE

The substance that is the subject of this Generally Recognized As Safe (GRAS) determination is refined arachidonic acid-rich oil (hereinafter, " RAO") derived from fermentation of *Mortierella alpina* I_{49}-N_{48}. This substance is commonly referred to as "*M. alpina* oil*" or "*Mortierella alpina* oil*"[1] for the purposes of commercial infant formula ingredient labeling.

C. INTENDED USE

RAO is intended for use as an ingredient in the manufacture of infant formula that will be consumed by premature as well as full term infants.

D. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of RAO as an ingredient in infant formula is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of RAO from the intended uses specified above has been determined to be safe and GRAS, using scientific

[1] Infant formulas manufactured by Abbott Nutrition and Nestlé, S.A. identify the arachidonic acid ingredient as "*M. alpina* oil*" whereas Mead Johnson Nutrition's infant formulas identify it as "*Mortierella alpina* oil*". For both versions, the asterisk refers to an additional label statement saying "A source of arachidonic acid (ARA)".

procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). The GRAS determination is made on the basis of generally available and accepted information evaluated by independent experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food.

Cargill, Incorporated is proposing to market refined arachidonic acid-rich oil (RAO), produced by Cargill Alking Bioengineering Co., Ltd, Wuhan, China as a source of arachidonic acid-rich oil used in the manufacture of infant formula. The end-use infant formulas will be consumed by premature as well as full term infants. Consistent with currently marketed GRAS sources of ARA-rich oil, RAO is produced by the fungus *Mortierella alpina* and specifications for the oil stipulate a minimum content of 40% arachidonic acid (ARA as a percentage of the fatty acid composition).

The following safety evaluation fully considers the composition, intake, nutritional, microbiological, and toxicological properties of RAO as well as appropriate corroborative data from essentially equivalent ARA-rich oils. The proposed use of RAO in the manufacture of infant formulas has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based upon the following:

- The compositional data and product properties are consistent with carefully controlled cGMP production and purification. RAO contains no impurities or contaminants of concern for human health. The morphology, biochemistry, and physiology of *M. alpina* are well- documented and there is no concern for human or animal pathogenicity or toxigenicity. *M. alpina* is not genetically modified.

- The intended use for RAO is as an ingredient in infant formula. The roles and functional importance of long-chain polyunsaturated fatty acids (LCPUFA) in pregnancy, lactation and infancy have been the subject of numerous clinical evaluations, particularly as they relate to the n-6 and n-3 LCPUFA, ARA (C20:4n-6) and docosahexaenoic acid (DHA; C22:6n-3). In humans, the essential dietary fatty acids, linoleic (C18:2n-6) and α-linolenic (C18:3n-3) are the precursors for *in situ* desaturation and elongation reactions that result in the synthesis of ARA and DHA,

respectively. Both substances are associated with normal growth and maturation of numerous organ systems and rapid accretion of LCPUFA takes place in an infant's nervous tissue during pre- and early postnatal life. Studies have suggested that infants may not synthesize sufficient amounts of ARA and DHA *de novo* from their precursors to cover the high demand during this period of rapid accretion. Breast milk is the preferred method of providing an exogenous supply of ARA and DHA. However, when for medical or personal reasons, infant formula is chosen as a sole source or supplementary infant food, addition of ARA and DHA to the formula may support optimal nutritional status of preterm and term infants. Based on current knowledge regarding the importance of LCPUFA and their presence in human milk, guidelines and recommendations established by the World Association of Perinatal Medicine, the Early Nutrition Academy, and the Child Health Foundation collectively recommend that the level of DHA in infant formula be 0.20 to 0.50 weight percent of total fat; with the minimum amount of ARA being equivalent to the DHA content (Koletzko *et al.*, 2008). These recommendations lead to the use and approval of highly refined oils as sources of ARA and DHA for addition to infant formulas.

- An estimate of exposure to ARA from its addition to infant formula is based on mean target ARA levels of 0.75 g and 0.40 g per 100 g total fat for term and preterm infants, respectively. Assuming human infants consume about 100 kcal/kg bwt/day (term infants) to 120 kcal/kg bwt/day (preterm infants), of which fat comprises about 50%en, an infant will consume about 5.6 g (term infants) to 6.7 g (preterm infants) of fat/kg bwt/day (1 g fat = 9 kcal). These correspond to intakes of ARA of 42 mg and 27 mg ARA/ kg bwt/day (or 104 and 67 mg of RAO/kg bwt/day) for term infants and preterm infants, respectively. This consumption is in the range of intake of ARA from human breast milk (Brenna *et al.*, 2007). This intake is also consistent with intakes resulting from currently approved levels of ARA supplementation of commercially available infant formula (Generally Recognized as Safe (GRAS) in the U.S. (FDA, 2001a, 2001b, 2006), approved by the Dutch authority (NFU, 2005), and concluded to be safe by the European Food Safety Authority (EFSA, 2008).

- Numerous animal safety studies have been conducted over a period of more than a decade on ARA-rich oils derived from *M. alpina.* Submissions of these data along with production process and final product specification information and corroborative information from growth and tolerance studies in infant populations have been reviewed by scientific agencies and regulatory bodies, including those in the US, the EU and Australia/New Zealand. In all jurisdictions, the conclusions reached were that these commercially available ARA-rich oils derived from *M. alpina*, meeting appropriate food grade specifications, provide a safe ARA source for supplementation of infant formula. These decisions have led to its availability for this use in at least 50 countries worldwide.

- The safety studies and publicly-available literature for essentially-equivalent sources of ARA-rich oil support the safety of RAO. A critical aspect of the safety evaluation is bridging new ingredient information to existing information on chemically similar/essentially equivalent ingredients identified in the published literature. Animal and *in vitro* studies similar to those conducted on other commercially available ARA-rich oils were conducted using RAO to confirm its safety as an ARA source. The purpose of conducting safety studies on RAO was to establish that the safety profile for this oil matches the profile of other refined ARA-rich oils reported in the scientific literature.

- The safety of RAO was evaluated for reverse mutation, chromosome aberration and gene mutation *in vitro*, and in a 90-day Wistar rat feeding study with *in utero* exposure. The results of the genotoxicity assays were all negative. The *in utero* phase of the 90-day study involved dietary exposure to 0.5%, 1.5% and 5% RAO and two controls diets, a standard feed low-fat diet and a high-fat diet supplemented with 5% corn oil. This exposure covered four weeks prior to mating, through mating, gestation and lactation until offspring (F_1) weaning. A subsequent 90-day feeding study with the F_1 rats evaluated the same test and control diets. No adverse test article-related effects occurred at even the highest dietary RAO concentration administered in that study, equal to approximately 3,000 mg RAO/kg bwt/day. This represents a daily

RAO intake that is approximately 29-fold greater than the anticipated intake of 42 mg ARA/kg bwt/day (104 mg of RAO/kg bwt/day) for term infants and 45-fold greater than the intake of 27 mg ARA/kg bwt/day (67 mg of RAO/kg bwt/day) for preterm infants.

- Results from the toxicology studies of RAO are consistent with those from toxicology studies on other ARA-rich oils, Information from these corroborative studies establish RAO as a safe source of ARA-rich oil.

Taken together, the available data from historical studies conducted on ARA-rich oils and those studies conducted specifically on Cargill RAO establish a strong body of evidence for the safety of RAO as a source of ARA for supplementation of infant formula.

The GRAS status of RAO (compliant with the established food grade specifications for RAO), under the intended conditions of use proposed by Cargill, Inc. has been determined through the deliberations of Roger A. Clemens, Dr.PH., CNS, FACN, FIFT; Lewis P. Rubin, M.D.; and I. Glenn Sipes, Ph.D, A.T.S. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of RAO and the potential human exposure to RAO resulting from its intended use as an ingredient in infant formula, and have concluded:

There is no evidence in the available information on RAO that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when RAO is used at levels that might reasonably be expected from the proposed applications. RAO is GRAS for use as an ingredient in the manufacture of infant formula.

RAO is thus safe and GRAS at the proposed levels of ingestion. It is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

E. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., CEO, Spherix Consulting, Inc., at 6430 Rockledge Drive, Westmoreland Bldg. #503, Bethesda, MD 20817. Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ckruger@spherix.com.

F. SIGNATURE

Pursuant to the criteria provided in proposed 21 CFR 170.36, Cargill, Inc. hereby notifies the Food and Drug Administration that the use of RAO as an ingredient in the manufacture of infant formula under the intended conditions of use is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Cargill, Inc. has determined that such use is generally recognized as safe (GRAS) through scientific procedures.

(b) (6)

Signature

2/18/2010
Date

II. DESCRIPTION OF SUBSTANCE

A COMMON OR USUAL NAME(S)

RAO (Refined Arachidonic acid-rich Oil). The oil contains approximately 40% ARA. The chemical name for ARA is all-*cis*-5,8,11,14-eicosatetraenoic acid (20:4 n-6) with molecular formula of $C_{20}H_{32}O_2$ This substance is commonly referred to as "*M. alpina* oil*" or "*Mortierella alpina* oil*"[2] for the purposes of commercial infant formula ingredient labeling.

B. SOURCE

RAO is derived from a fungal strain, *Mortierella alpina* I_{49}-N_{48} (Yuan *et al.*, 2002). This production strain was derived *via* ion implantation from a wild strain isolated from soil in the People's Republic of China. Ion implantation is a routine technique used for the isolation of production strains in the fermentation industry and does not involve recombinant DNA technology. Thus, *M. alpina* I_{49}-N_{48} used for the production of RAO is not considered a genetically modified organism (GMO). *M. alpina* I_{49}-N_{48} is deposited at the China Center for Type Culture Collection (CCTCC) at Wuhan University with a CCTCC registration number of M201026.

The RAO manufacturing process meets cGMP requirements for the production of food. All growth media/raw materials/processing aids used in the RAO fermentation and manufacturing processes meet internationally-recognized specification requirements for food production.[3] The fermentation process is well-controlled and critical control points are monitored to detect insufficient controls on the process, *e.g.*, incomplete sterilization, incorrect pH or temperature ranges, insufficient fatty acid composition, etc. If any of those control parameters fail to meet internal specifications, the fermentation is terminated and the batch rejected.

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[2] Infant formulas manufactured by Abbott Nutrition and Nestlé, S.A. identify the arachidonic acid ingredient as "*M. alpina* oil*" whereas Mead Johnson Nutrition's infant formulas identify it as "*Mortierella alpina* oil*". For both versions, the asterisk refers to an additional label statement saying "A source of arachidonic acid (ARA)".

[3] The specifications followed for fermentation raw materials as well as all other raw materials used in RAO manufacturing include those established by JECFA (Joint WHO/FAO Expert Committee on Food Safety), the Food Chemicals Codex (FCC; administered by the United States Pharmacopoeia), the U.S. FDA (*via* regulation in the U.S. Code of Federal Regulations), and the European Commission (*via* 96/77/EC).

Contamination checks are also conducted in the seed and production fermenter. The main fermentation reaction is stopped when the ARA content reaches the desired percentage above 40%. The amount of ARA in RAO will always be at least 40%; however, it may be higher, depending on specific customer requirements. All finished batches of RAO undergo rigorous quality assurance testing to meet well-defined product specifications prior to release.

1. Fungal cultural characteristics

Colonies are white, flat lobate on Potato Dextrose Agar and then change to a light orange color. Colonies grow quickly and spread with a great deal of fluffy aerial mycelium. The reverse side of the colonies is colorless. After 10 days at 28° C, colonies are 5.0 – 5.5 cm in diameter.

2. Fungal microscopic characteristics

Mycelia have no septa and are colorless and transparent. The bases of the sporangia are smooth, straight and unbranched, 45 μm x 5.0 μm. Sporangia are irregular spheres, smooth and are about 12.5 μm in diameter. Sporangiospores are ellipsoidal, 2.5 – 4.0 μm x 5.0 μm. Chlamydospores are spherical and are about 10.0 – 15.0 μm in diameter.

C. MANUFACTURING PROCESS

The following section presents an overview of the manufacturing process employed by Cargill Alking Bioengineering to produce RAO.

Overview: Seed cultures of *M. alpina* I_{49}-N_{48} are scaled up sequentially in flasks followed by first and second stage fermenters; all accomplished under aerobic conditions. Microorganisms from those seed cultures are then introduced into a nutrient-rich fermentation broth containing a nitrogen (protein) source, an energy source, a fatty acid source, and process control substances. The fermentation broth is grown under aerobic conditions in a series of progressively larger stirred tank fermenters to eventually yield an ARA-rich biomass. This biomass is filtered, dried and extracted to yield crude ARA-rich oil, which is then refined *via* a series of steps that include degumming, deacidification, decolorization, deodorization and antioxidant addition. Finished RAO is packaged under vacuum in polyethylene that is approved for contact with fatty foods. Figures1 and 2 provide manufacturing flow charts for the production of crude and refined RAO, respectively.

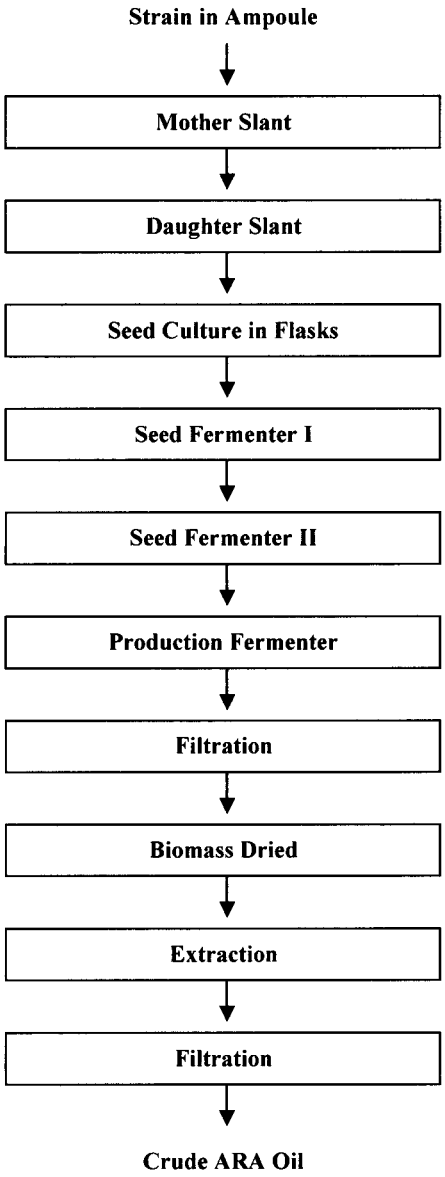

Figure 1. Crude ARA-rich oil production

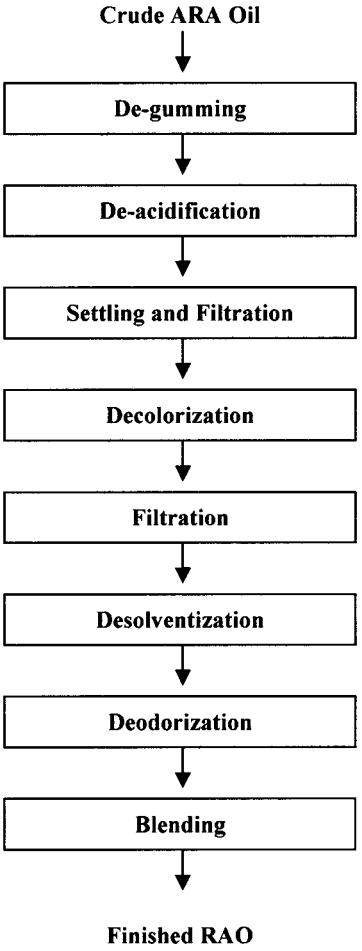

Figure 2. Refinement of crude RA-rich oil into RAO

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To assure the safety of finished RAO, Cargill Alking Bioengineering Co., Ltd has over 300 Standard Operating Procedures (SOPs) in place that cover all aspects of manufacturing. In addition, the most pivotal steps in the RAO manufacturing process are covered by a strictly-followed comprehensive Hazard Analysis and Critical Control Point (HACCP) plan.

1. Equipment and processing aids

a) Description of equipment

All equipment that has direct contact with finished RAO or its intermediates are made of food grade polyethylene, stainless steel or carbon steel.

b) Raw materials

The raw materials used in RAO manufacturing (*e.g.*, fermentation media, processing aids, antioxidants) include activated carbon, antifoam, ascorbyl palmitate, butane, citric acid, DL-α-tocopherol, glucose, hexane, sodium hydroxide, silicon dioxide, sunflower oil, and yeast powder; aluminum-coated polyethylene bags are used to package RAO. Cargill Alking Bioengineering, Ltd. requires that all of these substances be of a quality suitable for use in food manufacturing and its subsequent packaging. The suitability of each substance is established *via* supplier qualification procedures under cGMP, which include requiring their conformance to one or more of the following internationally-recognized standards/specifications: (1) specification monographs established by Food Chemicals Codex (FCC) and/or the Joint WHO/FAO Expert Committee on Food Additives (JECFA), (2) regulations established under 96/77 EC, and (3) food additive regulations established by the U.S. Food and Drug Administration.

2. Production facility

RAO is produced at Cargill Alking Bioengineering (Wuhan) Co. Ltd., located at International Corporation Center, Special No.1, Guanshan No.2 Road, Wuhan, Hubei Province, P. R. China. This facility conforms with ISO 22000:2005 (HACCP and Food GMP) and is certified by SGS-CSTC Standards Technical Services Company, Ltd., Shanghai Branch (3rd Building, No.889 Yishan Road, 200233, Shanghai, China). A Hazard Analysis and Critical Control Point (HACCP) plan to assure food safety that addresses physical, chemical, and biological hazards as a mean of prevention rather than finished product inspection is in place.

D. History And Safety Of The Production Organism

Refined ARA-rich oil (RAO) is synthesized by *M. alpina* I_{49}-N_{48}, a common soil fungus. Fungi are eukaryotic, nonphotosynthetic organisms that have a vegetative structure known as a mycelium (a multinucleate mass of cytoplasm enclosed within a rigid, multi-branched system of tubes). A mycelium normally arises by the germination and outgrowth of a single reproductive cell, or spore and the initial hypha branches repeatedly as it elongates to form the mycelium. Fungal growth is characteristically confined to the tips of the hyphae. Because the size of a single mycelium is not fixed, as long as nutrients are available, outward growth by hyphal extension can continue. Usually, asexual reproduction occurs by the formation of spores, which are pinched off at the tips of the hyphae.

Phylogenically, *M. alpina* falls under the Zygomycetes Class in the Zygomycota Order. All Zygomycetes share two properties that readily distinguish them from the remaining classes of fungi. First, their asexual spores are always endogenous, and second, their mycelium shows no cross walls except in regions where a specialized cell is formed from a hyphal tip (nonseptate mycelium). The Zygomycetes include a group of soil inhabitants known as terrestrial Zygomycetes and *M. alpina* is one of the most common species in this group. It is easily isolated from the soil and has been identified in soils from diverse geographies (Stanier *et al.*, 1980). Like many fungi, *M. alpina* is associated with common root crops and therefore, is in the direct food chain of many mammals.

Mortierella species have been widely studied in isolated laboratory culture and their morphology, biochemistry and physiology are well documented in more than 25 references spanning the last 20 years. *M. alpina* has been described in Japanese publications and patents as a potential source of ARA and consequently, it has been the subject of many intensive laboratory investigations (Bajpai *et al.*, 1991; Lindberg and Molin, 1993; Shinmen *et al.,* 1989; Totani and Oba, 1987), none of which have reported *M. alpina*-associated pathogenicity or toxigenicity to humans or animals (Domsch *et al.,* 1980; Scholer *et al.*, 1983).

Only a few of the thousands of known species of fungi cause human diseases. In a clinical setting involving an already diseased or immunocompromized host, any fungus that grows at 37° C could be considered an opportunistic organism (Cooper 1985; Smith 1966). *M.*

alpina was reported to be isolated as a secondary infection from calf liver lesions in New Zealand (Smith 1966); however, Scholer *et al.* (1983) questioned whether the isolated organism was indeed *M. alpina* or a different species of *Mortierella.*

Although some fungal species have been reported to produce mycotoxins, such fungi belong to the class of Deuteromycetes, which clearly differs from the Zygomycete fungi to which *Mortierella* belongs. There has never been any report of mycotoxin production from *M. alpina* or any other of the many species of the genus *Mortierella*. Strains of *M. alpina* used by other manufacturers of ARA-rich oil have not been reported to produce mycotoxins (FDA, 2001a; 2001b; 2006) and a mycotoxin screen of five RAO batches similarly showed no detectable mycotoxin contamination.

Within Class Zygomycetes, the *Mortierella* genus is presently classified in Order Mortierellales and Family Mortierellaceae. A few organisms in Order Mortierellales have been associated with mucormycosis, a disease resulting from an opportunistic infection. Classic risk groups for mucormycosis infection include individuals who are immunocompromised and those with uncontrolled diabetes (Chew *et al.*, 2008).

No references were found in the literature suggesting any specific toxin production by members of the genus *Mortierella*, with the exception of the pathogenic species *M. wolfii*, which has been recovered from cattle in cases of abortion and pneumonia (Di Menna *et al.*, 1972; Seviour *et al.*, 1987; Carter *et al.*, 1973; Corbel and Eades 1991; Cordes *et al.*, 1972). This organism excretes a water-soluble heat- and trypsin-labile nephrotoxin (Davey and Kalmakoff 1973; Davey and Kalmakoff 1974; Davey *et al.*, 1974).

The *M. alpina* strain 1S- 4 is employed in the production of an ARA-rich oil that is already authorized by the EC as a novel food (EFSA, 2008) and considered GRAS in the U.S. (FDA, 2006). The manufacturing information in the associated regulatory support documents indicates that *M. alpina* 1S-4 is fermented initially at 28° C with fermentation of the main culture at 26° C. In the RAO manufacturing process, *M. alpina* I_{49}-N_{48} is fermented at similar temperatures.

The nontoxic nature of *M. alpina* I_{49}-N_{48} was also demonstrated by the results from an acute mouse study conducted by the Institute of Food Safety Control and Inspection, Ministry of Public Health, Beijing, P. R. China. In this study, cultures of *M. alpina* I_{49}-N_{48} prepared in stock solutions of (1) potato-yeast extract-saccharose, (2) malt extract-yeast extract, and (3) malt extract-peptone; and their 2.5-fold concentrates, were administered to groups of 12 Kunming mice (six/sex) at 20 ml/kg bwt *via* gastric lavage. Control animals were administered culture media that had not been inoculated with *M. alpina* I_{49}-N_{48}. The mice were observed for clinical signs, mortality and weight gain over 14 days. Toxic reactions and mortality did not occur in any of the test animals and there were no differences in weight gain compared to controls.

Strains of *M. alpina* have been historically employed to produce ARA-rich oils that are added to infant formulas by their manufacturers. Those strains include *M. alpina* Peyronel 1S-4 and *M. alpina* ATCC 32222. GRAS Review Notices (GRN94, GRN80, GRN41) and a novel food dossier (NFU2005) use ARA-rich oils produced by those two strains of *M. alpina*.

E. SPECIFICATIONS

1. Product specifications

The product specifications for RAO are shown in Table 1

2. Stability

The results of stability testing for up to 36 months on three lots of RAO manufactured in 2006 are presented in Table 2.

3. Analytical information

Five lots of RAO were subjected to analytical testing for a wide variety of parameters, which are consistent with those evaluated and presented in regulatory submissions for ARA-rich oils that are similar to RAO. Table 3 shows the various test categories and the subsequent Tables 4 to 15 contain the detailed analytical data for composition and potential chemical and microbiological impurities the 5 lots of RAO.

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Table 1. RAO specifications

Criteria	Specifications	Method
Appearance	Clear yellow oil	Visual
Arachidonic acid (wt %)	≥ 40%	AOCS Ce 1i-07*
Moisture and volatiles (wt %)	≤ 0.1	AOCS Ca 2d-25
Residual hexane (ppm)	≤ 1.0	AOCS Ca 3b-87
Residual butane (ppm)	≤ 1.0	AOCS Ca 3b-87
Peroxide value (meq/kg)	≤ 2.0	AOCS Cd 8b-90
Acid value (mg KOH/g)	≤ 1.0	AOCS Cd 3d-63
p Anisidine value (AV)	≤ 20	AOCS Cd 18-90
Free fatty acids (FFA; % oleic acid)***	≤ 0.2	AOCS Ca 5a-40
Unsaponifiable material (w/w %; LOD = 0.3)	≤ 3.0	AOCS Ca 6b-53
Lovibond color, (25.4mm cell (@ 1 inch; LOD = 1" for yellow and 0.1" for red)	≤ 50 (Yellow)	AOCS Cc 13e-92
	≤ 5 (Red)	AOCS Cc 13e-92
Lead (mg/kg; LOD = 0.02 mg/kg)	≤ 0.1	AOAC 986.15
Arsenic (as As_2O_3, mg/kg; LOD = 0.02 mg/kg)	≤ 0.1	AOAC 986.15
Cadmium (mg/kg; LOD = 0.01 mg/kg)	≤ 0.1	AOAC 986.15
Mercury (mg/kg; LOD = 0.01 mg/kg)	≤ 0.05	AOAC 986.15
Microbiological		
Total Plate Count, (APC) (CFU/g)	< 10	**
Coliforms (MPN/g)	< 3	**
Yeasts (CFU/g)	< 10	**
Molds (CFU/g)	< 10	**

AOAC = Association of Official Analytical Chemists; AOCS = American Oil Chemist's Society; CFU = Colony Forming Units; LOD = Limit of Detection; meq = milliequivalents; MPN = Most Probable Number; *this method follows an adapted version of AOCS method Ce 1i-07. The adaptation occurs when a pressure program is used at the end of the run to elute very long chain fatty acid monoesters (FAME) such as C26:0 and C26:1; **AOAC-Ch.17, Method #966.23 for APC and coliforms; Standard Methods for the Examination of Dairy Products-17th edition for yeasts and molds.

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Table 2. Results of stability testing on three lots of RAO

		Times of post-manufacturing stability testing (months)							
Batch #	Parameters	0	3	6	9	12	18	24	36
	Storage at ≈ -18 to - 13°C								
AOG060216	AV	0.2	0.16	0.17	0.19	0.16	0.19	ND	ND
	POV	0.03	0.56	0.03	0.48	0.03	0.03	ND	0.03
	% ARA	46.70	ND	47.17	ND	44.7	ND	45.7	46.57
AOG060221	AV	0.2	0.18	0.16	0.21	0.17	0.19	ND	ND
	POV	0.07	0.97	0.03	0.62	0.03	0.35	ND	0.03
	% ARA	47.20	ND	47.62	ND	44.6	ND	45.82	47.03
AOG060224	AV	0.03	0.2	0.18	0.18	0.21	0.16	ND	ND
	POV	0.03	0.85	0.03	0.17	0.03	0.03	ND	0.03
	% ARA	47.20	ND	47.41%	ND	45.35	ND	45.93	47.32
	Storage at ≈ 0 to - 10°C								
AOG060216	AV	0.2	0.15	0.13	0.18	0.33	0.12	0.19	NA
	POV	0.03	1.03	0.36	0.55	0.03	0.96	0.03	0.03
	% ARA	46.70	ND	47.07	ND	46.29	ND	45.95	46.37
AOG060221	AV	0.2	0.18	0.16	0.18	0.33	0.11	0.19	NA
	POV	0.07	1.32	0.82	0.83	0.03	1.15	0.03	0.03
	% ARA	47.20	ND	46.25	ND	47.19	ND	45.75	46.40
AOG060224	AV	0.03	0.18	0.14	0.22	0.31	0.24	ND	ND
	POV	0.03	1.86	1.5	0.7	0.03	0.9	ND	ND
	% ARA	47.20	ND	47.18	ND	46.36	ND	ND	ND

ARA = arachidonic acid (test method = AOCS Ce 1i-07, a GC method with tritricosanoin, C23:0 TAG as an internal standard. The coefficient of variation (CV) ranges from 1.01 to 3.87%); AV = *p*-anisidine value (test method = AOCS Cd 18-90, a titration method with CV ranges between 4.0 and 5.8%. Reference materials were run to make sure the method is running within acceptable control limits (1.5 times standard deviation of the mean); POV = peroxide value (test method = AOCS Cd 8b-90, a titration method with CV ranges between 1.3 and 6.7%. Reference materials were run to make sure the method is running within acceptable control limits (1.5 times standard deviation of the mean; ND = not determined.

Table 3. Analytical categories evaluated for five separate lots of RAO

Categories	Table #
Microbiological parameters	4
Mycotoxins	5
Other lipid parameters	6
Sterols	7
Total polymers, TAGs, DAGs and MAGs	8
Fatty acid composition	9
Tocopherols	10
Heavy metals	11
Phthalates and other esters	12
Polynuclear aromatic hydrocarbons (PAH)	13
Polychlorinated biphenyls (PCB) and pesticides	14
Polychlorinated dibenzodioxins and dibenzofurans (PCDD/F)	15

Five lots of RAO were tested for Total Plate Count, Coliforms, Yeasts and Molds and the results are summarized in Table 4. Methods are referenced as follows: AOAC-Ch.17, Method #966.23 for Total Plate Count and Coliforms (MPN/g); AOAC-Ch.17, Method #989.10 for Coliforms (CFU/g); Standard Methods for the Examination of Dairy Products-17th edition for Yeasts and Molds.

Table 4. Microbiological test results on five lots of RAO

Lot #	Total Plate Count	Coliforms		Yeasts and Molds	
	CFU/g	CFU/g	MPN/g	Yeasts (CFU/g)	Molds (CFU/g)
071013	<10	<10	<3	<10	<10
09022301	<10	<10	<3	<10	<10
08062101	<10	<10	<3	<10	<10
09032201	<10	<10	<3	<10	<10
09032101	<10	<10	<3	<10	<10

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A screening for mycotoxins in the five lots of RAO was conducted by Trilogy Analytical Laboratory;[4] the results are summarized in Table 5.

Table 5. Mycotoxin analysis of five RAO lots*, **

Mycotoxin	Result	Limit of Detection	Analytical Method
Aflatoxin B_1	Not Detected	1 ppb	HPLC[a]
Aflatoxin B_2	Not Detected	1 ppb	HPLC[a]
Aflatoxin G_1	Not Detected	1 ppb	HPLC[a]
AflatoxinG_2	Not Detected	1 ppb	HPLC[a]
Fumonisin B_1	Not Detected	0.1 ppm	HPLC[b]
Fumonisin B_2	Not Detected	0.1 ppm	HPLC[b]
Fumonisin B_3	Not Detected	0.1 ppm	HPLC[b]
Nivalenol	Not Detected	0.5 ppm	GC[c]
Ochratoxin A	Not Detected	1 ppb	HPLC[d]
Patulin	Not Detected	10 ppb	HPLC[e]
Sterigmatocystin	Not Detected	200 ppb	TLC[f]
Zearalenone	Not Detected	50 ppb	HPLC[g]

* Lots 071013, 09022301, 08062101, 09032201, 09032101; ** Reference standards were used by Trilogy Analytical Laboratory as positive controls for the four aflatoxins, the three fumonisins, Ochratoxin A and Zearalenone. For Nivalenol, Patulin and Sterigmatocystin, rather than reference standards, spiked recovery samples were analyzed alongside test samples to verify percent recovery of the spike. *Per* Trilogy's SOPs, if any positive control step results in a failure, the reasoning behind that failure will be investigated and resolved before any analytical report is issued to a client. Therefore, the data are considered to have been verified by positive control data; a = Association of Analytical Communities (AOAC) 994.08 with modifications (post column bromination derivatizaion using a Kobra Cell substituted for pre column TFA (trifluoroacetic acid) derivative); b = AOAC 2001.04 with modifications (NDA (naphthalene-2,3-dicarboxaldehyde) derivatization substituted for OPA (*o*-phthalatdehyde); c = Tacke and Casper (1996), Journal of AOAC International, 79: 2. 472-475; d = AOAC 2000.03; e = AOAC 995.10; f = AOAC 973.38; g = MacDonald *et al.* (2005) Journal of AOAC International, 88: 6. 1733-1740; GC = gas chromatography; HPLC = high performance liquid chromatography; TLC = thin layer chromatography.

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[4] 870 Vossbrink Drive, Washington MO 63090; 636-239-1521.

Table 6. Other lipid parameters evaluated in five lots of RAO*

	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201	Method
Peroxide value (meq/kg)	ND	ND	ND	ND	ND	AOCS Cd 8b-90
p-Anisidine Value	6.44	2.79	7.62	6.06	5.51	AOCS Cd 18-90
Free fatty acids (FFA; % oleic acid)**	0.09	0.06	0.03	0.03	0.03	AOCS Ca 5a-40
Acid value (KOH/g)	0.17	0.13	0.07	0.07	0.06	AOCS Cd 3d-63
Total sterols (mg/g)	11.0	8.8	9.9	9.7	9.4	¥
Total *trans*-isomers (w/w % FA basis)	0.51	0.44	0.40	0.47	0.44	AOCS 1h-05
Unsaponifiable matter (w/w %)***	1.26	1.30	1.02	1.14	1.21	AOCS Ca 6b-53
Lovibond color yellow (@ 1 inch)	2.0	2.0	2.0	2.0	2.0	AOCS Cc 13e-92
Lovibond color red (@ 1 inch)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	AOCS Cc 13e-92
Free fatty acids (Area %)	0.03	0.03	0.02	0.19	0.31	AOCS Ca 5a-40

* except for unsaponifiable matter, all testing was conducted by Cargill Advanced Analytics (Wayzata MN, USA); ** FFA = free fatty acid = acid value /1.99 (AOCS Method Cd 3d-63); *** unsaponifiable matter testing conducted by Eurofins, Ltd. (Hamburg, Germany); ¥ = an internal Cargill gas chromatographic method titled *Determination of sterols and sterol esters: Analysis protocol and method.* (In December, 2009, this method was submitted to the Journal of the American Oil Chemists Society for publication as an official AOCS method.)

Table 7. Detailed analysis of sterols in five lots of RAO (average w/w%, n=2)*

Sterol	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Desmosterol	0.080	0.061	0.093	0.092	0.086
Zymosterol	0.017	0.013	0.015	0.007	0.006
24-Methyl Cholesta-5,24(25)-dien-3β-ol	0.598	0.301	0.579	0.599	0.589
24-Methylene Cholesterol	0.067	0.098	0.046	0.048	0.048
Campesterol	0.013	0.013	0.011	0.02	0.009
4α-Methyl Zymosterol	0.023	0.018	0.023	0.012	0.016
24-Methyl Cholesta-5,(25)27-dien-3β-ol	0.134	0.190	0.078	0.078	0.076
Lanosterol	0.033	0.050	0.042	0.03	0.033
β-Sitosterol	0.033	0.035	0.036	0.039	0.026
31-Norlanosterol	0.031	0.028	0.033	0.026	0.028
Total Unknown Sterols	0.067	0.07	0.040	0.021	0.028
Total Sterols	1.096	0.876	0.995	0.971	0.942

* = preparation and testing of samples in duplicate was conducted by Cargill Advanced Analytics (Wayzata MN, USA) *via* a simultaneous alkaline saponification and extraction (Cargill internal method GFR SR 15-3).

Table 8. Total polymers, TAGs, DAGs and MAGs evaluated in five lots of RAO*

	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Polymers (Area %)	ND	ND	ND	ND	ND
TAG (Area %)	98.78	99.13	99.14	98.53	98.29
DAG (Area %)	1.19	0.84	0.84	1.28	1.40
MAG (Area %)	ND	ND	ND	ND	ND

* = testing conducted by Cargill Advanced Analytics (Wayzata MN, USA) using size exclusion chromatography; ND = not detected; TAG = triacylglycerides (*i.e.*,triglycerides); DAG = diacylglycerides (*i.e.*, diglycerides); MAG = monoacylglycerides (*i.e.*, monoglycerides).

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Table 9. Fatty acid compositions of five RAO lots using fatty acid methyl ester analysis*, **

Fatty Acid	Lot 071013 (w/w% FA basis)		Lot 08062101 (w/w% FA basis)		Lot 09022301 (w/w% FA basis)		Lot 09032101 (w/w% FA basis)		Lot 09032201 (w/w% FA basis)	
	BF_3 FAME*	NaMeOH FAME*	BF_3 FAME	NaMeO H FAME	BF_3 FAME	NaMeO H FAME	BF_3 FAME	NaMeO H FAME	BF_3 FAME	NaMeO H FAME
Saturates										
8:0	0.01	ND	0.01	ND	0.01	ND	ND	ND	ND	ND
10:0	0.02	0.03	0.04	0.04	0.03	0.03	0.04	0.05	0.03	0.04
11:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
12:0 (Lauric)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
13:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
iso 14:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14:0 (Myristic)	0.23	0.23	0.35	0.35	0.26	0.25	0.23	0.24	0.23	0.24
iso 15:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ant 15:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
15:0	0.10	0.10	0.09	0.09	0.08	0.08	0.08	0.08	0.08	0.08
iso 16:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ant 16:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16:0 (Palmitic)	6.54	6.58	6.87	6.86	5.84	5.73	5.43	5.58	5.44	5.48
7Me 16:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
iso 17:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ant 17:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3,7,11,15-tetra Me 16:0 (Phytanic)	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
17:0	0.17	0.17	0.20	0.20	0.18	0.17	0.18	0.18	0.18	0.19
iso 18:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:0 (Stearic)	4.48	4.48	5.98	5.94	5.21	5.12	4.90	4.99	4.98	4.98
anteiso 19:0	0.16	0.15	0.16	0.16	0.15	0.15	0.17	0.17	0.16	0.16

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Fatty Acid	Lot 071013 (w/w% FA basis)		Lot 08062101 (w/w% FA basis)		Lot 09022301 (w/w% FA basis)		Lot 09032101 (w/w% FA basis)		Lot 09032201 (w/w% FA basis)	
	BF_3 FAME*	NaMeOH FAME*	BF_3 FAME	NaMeO H FAME	BF_3 FAME	NaMeO H FAME	BF_3 FAME	NaMeO H FAME	BF_3 FAME	NaMeO H FAME
19:0	0.02	0.02	0.03	0.02	0.03	0.02	0.02	0.02	0.03	0.03
iso 20:0	0.01	ND	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
20:0 (Arachidic)	0.69	0.69	0.80	0.80	0.77	0.76	0.74	0.74	0.74	0.74
21:0	0.10	0.10	0.10	0.10	0.09	0.09	0.09	0.09	0.10	0.10
22:0 (Behenic)	3.02	3.00	3.24	3.22	3.13	3.14	3.04	3.09	3.10	3.10
24:0 (Lignoceric)	10.14	10.02	9.53	9.37	10.20	10.06	10.24	10.30	10.50	10.47
25:0	0.16	0.16	0.12	0.12	0.15	0.15	0.16	0.17	0.16	0.17
26:0	1.42	1.41	1.13	1.10	1.34	1.33	1.41	1.41	1.48	1.48
Total Saturates	27.30	27.14	28.68	28.40	27.50	27.11	26.76	27.14	27.24	27.29
Monoenes										
12:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14:1	0.01	0.01	0.01	ND	ND	ND	0.01	ND	ND	ND
15:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16:1 n9	0.03	0.04	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.02
16:1 n7	0.22	0.24	0.21	0.21	0.20	0.20	0.15	0.16	0.14	0.15
16:1 n5	0.01	0.01	0.01	0.01	0.01	ND	ND	ND	ND	0.01
16:1 n5 + *iso* 17:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
17:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:1 n9	5.74	5.76	5.61	5.58	4.77	4.70	4.39	4.48	4.33	4.34
18:1 n7	0.32	0.32	0.24	0.25	0.23	0.23	0.21	0.22	0.21	0.21
18:1 n5	0.01	0.01	ND	ND	0.01	0.01	0.01	ND	ND	0.01
19:1	0.04	0.04	0.05	0.04	0.04	0.03	0.03	0.04	0.04	0.04
20:1 n11	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
20:1 n9	0.27	0.27	0.22	0.22	0.22	0.22	0.20	0.20	0.21	0.21

Fatty Acid	Lot 071013 (w/w% FA basis)		Lot 08062101 (w/w% FA basis)		Lot 09022301 (w/w% FA basis)		Lot 09032101 (w/w% FA basis)		Lot 09032201 (w/w% FA basis)	
	BF_3 FAME*	NaMeOH FAME*	BF_3 FAME	NaMeOH FAME	BF_3 FAME	NaMeOH FAME	BF_3 FAME	NaMeOH FAME	BF_3 FAME	NaMeOH FAME
20:1 n7	0.08	0.08	0.07	0.08	0.07	0.08	0.09	0.10	0.09	0.10
22:1 n11+13	0.12	0.13	0.09	0.11	0.11	0.12	0.10	0.12	0.10	0.13
22:1 n9	0.17	0.23	0.17	0.22	0.19	0.23	0.16	0.23	0.18	0.24
22:1 n7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24:1	0.44	0.45	0.45	0.48	0.51	0.52	0.56	0.59	0.51	0.55
26:1	0.07	0.06	0.05	0.06	0.07	0.07	0.09	0.08	0.08	0.07
Total Monoenes	7.55	7.67	7.23	7.30	6.48	6.45	6.06	6.26	6.01	6.10
Dienes										
16:2 n6	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01
16:2 n4	ND	0.01	ND	0.01	ND	ND	ND	0.01	ND	ND
18:2 n7,13	0.04	0.04	0.05	0.05	0.04	0.04	0.03	0.04	0.04	0.04
18:2 n7,10	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
18:2 n6	8.69	8.78	8.40	8.38	8.05	7.96	7.28	7.48	6.92	6.99
18:2 other	0.09	0.09	0.09	0.09	0.09	0.09	0.11	0.11	0.10	0.10
18:2 n4	0.01	0.01	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.01
20:2 n6	0.45	0.46	0.45	0.45	0.46	0.45	0.42	0.43	0.42	0.43
20:2 n7	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02
22:2	0.01	0.02	0.03	0.02	0.02	0.02	0.01	0.02	0.02	0.02
Total Dienes	9.32	9.45	9.07	9.06	8.70	8.62	7.89	8.14	7.54	7.63
Trienes										
16:3 n4	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
16:3 n3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:3 n6	2.13	2.17	2.27	2.27	2.11	2.09	2.00	2.09	2.01	2.06
18:3 n4	0.04	0.05	0.05	0.05	0.04	0.05	0.05	0.06	0.05	0.05

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Fatty Acid	Lot 071013 (w/w% FA basis)		Lot 08062101 (w/w% FA basis)		Lot 09022301 (w/w% FA basis)		Lot 09032101 (w/w% FA basis)		Lot 09032201 (w/w% FA basis)	
	BF_3 FAME*	NaMeOH FAME*	BF_3 FAME	NaMeO H FAME	BF_3 FAME	NaMeO H FAME	BF_3 FAME	NaMeO H FAME	BF_3 FAME	NaMeO H FAME
18:3 n3	0.03	0.04	0.04	0.04	0.03	0.03	0.04	0.05	0.04	0.04
18:3 n1	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
18:3 other	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04
20:3 n7	0.21	0.21	0.21	0.21	0.19	0.18	0.18	0.19	0.20	0.21
20:3 n6	3.71	3.74	3.76	3.77	3.70	3.66	3.61	3.69	3.69	3.70
20:3 n3	ND	0.02	ND	0.03	0.03	0.03	0.03	0.03	0.02	0.03
22:3 n3	0.28	0.29	0.28	0.28	0.30	0.29	0.30	0.33	0.30	0.34
Total Trienes	6.48	6.60	6.69	6.72	6.47	6.40	6.28	6.51	6.38	6.50
Tetraenes										
16:4 n3	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
16:4 n1	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
18:4 n3	ND	ND	ND	ND	ND	ND	0.01	0.01	ND	0.01
18:4 n1	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01
20:4 n6	41.15	41.43	41.70	41.44	44.41	43.93	44.55	45.55	44.68	45.09
20:4 n3	0.01	0.01	ND	0.01	0.01	0.01	0.01	0.02	0.01	0.01
20:4 Unknown Isomers	2.55	2.61	2.49	2.50	2.31	2.33	2.13	2.27	2.32	2.41
22:4 n6	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
22:4 n3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total Tetraenes	43.75	44.10	44.23	43.99	46.77	46.31	46.74	47.90	47.05	47.56
Pentaenes										
18:5 n3	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
20:5 n3	0.08	0.08	0.09	0.09	0.16	0.16	0.22	0.24	0.17	0.17
21:5 n3	0.06	0.07	0.05	0.05	0.06	0.06	0.05	0.06	0.06	0.06
22:5 n6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Fatty Acid	Lot 071013 (w/w% FA basis)		Lot 08062101 (w/w% FA basis)		Lot 09022301 (w/w% FA basis)		Lot 09032101 (w/w% FA basis)		Lot 09032201 (w/w% FA basis)	
	BF_3 FAME*	NaMeOH FAME*	BF_3 FAME	NaMeOH FAME	BF_3 FAME	NaMeOH FAME	BF_3 FAME	NaMeOH FAME	BF_3 FAME	NaMeOH FAME
22:5 n3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total Pentaenes	0.16	0.17	0.16	0.16	0.24	0.24	0.29	0.32	0.25	0.25
Hexaenes										
22:6 n3	0.04	ND	0.04	ND	0.03	ND	0.04	ND	0.04	ND
Total Hexaenes	0.04	ND	0.04	ND	0.03	ND	0.04	ND	0.04	ND
Total Fat										
NLEA Total Fat (TAG w/w%)	94.57	95.17	96.06	95.65	96.17	95.15	94.03	96.23	94.45	95.34
Iodine Value										
Total calculated iodine values	179.52	179.85	178.35	178.30	185.62	185.52	188.22	188.26	187.02	187.10

* = testing conducted by Cargill Advanced Analytics (Wayzata MN, USA);

** = The dual testing using BF_3 and NaMeOH for derivatization was undertaken because acid catalyzed methylation reactions have been reported to cause isomerization, particularly when conjugated linolenic acid species are involved (Yurawecz *et al.* 1994; Costin *et al.*, 2009). FAME = fatty acid methyl ester; FA = fatty acid; BF_3 = boron trifluoride, used in the FAME analysis as a derivitizing catalyst; NaMeOH = sodium methoxide; used in the FAME analysis as the derivitizing agent; ND = not detected; NLEA = the amount of fat required by the NLEA to be on a label; Nutrition Labeling and Education Act, *i.e.*, TAG = triacylglycerol; The method was performed following an adapted version of American Oil Chemists Society (AOCS) method Ce 1i-07. (The adaptation occurred when a pressure program was used at the end of the run to elute very long chain FAME such as C26:0 and C26:1.) These data represent single runs with two different sample preparation methods used for each sample. Administering two injections of the same sample demonstrated good reproducibility. Tritricosanoin, C23:0 TAG, was used as an internal standard for both types of sample preparation. The National Institute of Standards and Technology (NIST) library and known retention time standards were used for the identification of the various fatty acids.

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Table 10. Tocopherols compositions of five RAO lots*

	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
α-Tocopherol (ppm)	445.27	884.84	1009.69	883.99	819.60
α-Tocotrienol (ppm)	ND	ND	ND	32.42	30.20
β-Tocopherol (ppm)	2.73	2.57	2.52	2.06	1.63
γ-Tocopherol (ppm)	1.72	1.77	3.25	4.22	3.46
γ-Tocotrienol (ppm)	0.24	0.51	ND	ND	ND
δ-Tocopherol (ppm)	1.92	12.07	ND	ND	ND
δ-Tocotrienol (ppm)	ND	ND	ND	1.10	0.82

* = testing conducted by Cargill Advanced Analytics (Wayzata MN, USA) with results determined using a normal phase high pressure liquid chromatography (HPLC) method with fluorescence detector; ND = Not Detected.

Regarding the wide variation and relatively high values for α-tocopherol shown in Table 10, it should be noted that the antioxidants Vitamin C – as ascorbyl palmitate - and Vitamin E - as D,L-α-tocopherol – are added to finished RAO (at target concentrations of 1000 ppm and 200 ppm, respectively) to avoid post-manufacturing oxidation. Thus, the above data for α-tocopherol simply represents evidence that Lot 071013 quenched more of the added Vitamin E than was required by the four other lots to assure a non-oxidizing product.

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Table 11. Heavy metals analysis of five RAO lots***

	LOD	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Heavy Metals (ppm as Pb)**	10	ND	ND	ND	ND	ND
Chromium (mg/kg)	0.2	ND	ND	ND	ND	ND
Copper (mg/kg)	0.04	ND	ND	ND	ND	ND
Iron (mg/kg)	0.04	ND	ND	ND	ND	ND
Manganese (mg/kg)	0.02	ND	ND	ND	ND	ND
Molybdenum (mg/kg)	0.1	ND	ND	ND	ND	ND
Nickel (mg/kg)	0.2	ND	ND	ND	ND	ND
Phosphorus (mg/kg)	2	ND	ND	ND	ND	ND
Silicon (mg/kg)	2	11	16	3	7	5
Sulfur (mg/kg)	2	ND	ND	ND	ND	ND
Arsenic (mg/kg)*	0.02	ND	ND	ND	ND	ND
Cadmium (mg/kg)*	0.01	ND	ND	ND	ND	ND
Lead (mg/kg)*	0.02	ND	ND	ND	ND	ND
Mercury (mg/kg)*	0.01	ND	ND	ND	ND	ND

* = testing conducted by Eurofins, Ltd. (Metairie LA); ** = these endpoints are also presented in Table 1; *** = for the majority of these metals analyses, inductively coupled plasma with atomic emission spectroscopy detection (ICP-AES) was used and followed AOCS method Ca 17-01. However, arsenic, cadmium, lead, and mercury determinations were accomplished by inductively coupled plasma coupled to mass spectrometry (ICPMS), which were run according to AOAC Method 986.15; LOD = limit of detection; ND = not detected.

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Table 12. Analysis of phthalates and other esters in five RAO lots*

	LOD	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Dimethyl phthalate (DMP) (mg/kg)	1	<1	<1	<1	<1	<1
Diethyl phthalate (DEP) (mg/kg)	1	<1	<1	<1	<1	<1
Dibutyl phthalate (DBP) (mg/kg)	1	<1	<1	<1	<1	<1
Butyl benzyl phthalate (BBP) (mg/kg)	1	<1	<1	<1	<1	<1
Diethyl hexyl phthalate (DEHP) (mg/kg)	1	<1	<1	<1	<1	<1
Dioctyl phthalate (D-n-OP) (mg/kg)	1	<1	<1	<1	<1	<1
DINCH (mg/kg)	5	<5	<5	<5	<5	<5
Diisononylphthalate (DINP) (mg/kg)	5	<5	<5	<5	<5	<5
Diisodecylphthalate (DIDP) (mg/kg)	5	<5	<5	<5	<5	<5
Diethylhexyl adipate (DEHA) (mg/kg)	1	<1	<1	<1	<1	<1
Triisobutyl phosphate (mg/kg)	1	<1	<1	<1	<1	<1
Acetyltributylcitrate (mg/kg)	1	<1	<1	<1	<1	<1

* = testing conducted by Eurofins, Ltd. (Hamburg, Germany); LOD = limit of detection; The analysis was accomplished using a gas chromatography method coupled to mass spectrometry (GC-MS) developed by Eurofins, Ltd. (Hamburg, Germany).

Table 13. Polynuclear aromatic hydrocarbon (PAH) analysis of five RAO lots*

	LOD (mg/kg)	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Acenapthene (mg/kg)	0.05	ND	ND	ND	ND	ND
Acenapthylene (mg/kg)	0.05	ND	ND	ND	ND	ND
Anthracene (mg/kg)	0.02	ND	ND	ND	ND	ND
Benzo(a)anthracene (mg/kg)	0.01	ND	ND	ND	ND	ND
Benzo(a)pyrene (mg/kg)	0.01	ND	ND	ND	ND	ND
Benzo(b)fluoranthene (mg/kg)	0.01	ND	ND	ND	ND	ND
Benzo(ghi)perylene (mg/kg)	0.01	ND	ND	ND	ND	ND
Benzo(k)fluoranthene (mg/kg)	0.01	ND	ND	ND	ND	ND
Chrysene (mg/kg)	0.01	ND	ND	ND	ND	ND
Dibenzo(a,h) anthracene (mg/kg)	0.02	ND	ND	ND	ND	ND
Fluoranthene (mg/kg)	0.02	ND	ND	ND	ND	ND
Fluorene (mg/kg)	0.01	ND	ND	ND	ND	ND
Indeno(1,2,3-c,d)pyrene (mg/kg)	0.02	ND	ND	ND	ND	ND
Napthalene (mg/kg)	0.05	ND	ND	ND	ND	ND
Phenanthrene (mg/kg)	0.05	ND	ND	ND	ND	ND
Pyrene (mg/kg)	0.01	ND	ND	ND	ND	ND

* = testing conducted by Eurofins, Ltd. (Metairie LA); LOD = limit of detection; ND = not detected; The analysis was completed following the FDA Pesticide Analytical Manual (PAM) 304.

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Table 14. Polychlorinated biphenyl and pesticides analysis of five RAO lots*

	LOD	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Polychlorinated Biphenyls, Total (mg/kg)	0.01	ND	ND	ND	ND	ND
Organochlorine Pesticides						
Alachlor (Lasso) (mg/kg)	0.04	ND	ND	ND	ND	ND
Aldrin (mg/kg)	0.01	ND	ND	ND	ND	ND
Benfluralin (Benefin) (mg/kg)	0.03	ND	ND	ND	ND	ND
BHC (Alpha Isomer) (mg/kg)	0.01	ND	ND	ND	ND	ND
BHC (Beta Isomer) (mg/kg)	0.01	ND	ND	ND	ND	ND
BHC (Delta Isomer) (mg/kg)	0.01	ND	ND	ND	ND	ND
Bifenox (mg/kg)	0.03	ND	ND	ND	ND	ND
Captafol (mg/kg)	0.01	ND	ND	ND	ND	ND
Captan (mg/kg)	0.02	ND	ND	ND	ND	ND
Chlorfenson (mg/kg)	0.03	ND	ND	ND	ND	ND
Chlorbenzilate (mg/kg)	0.04	ND	ND	ND	ND	ND
Chloroneb (mg/kg)	0.03	ND	ND	ND	ND	ND
Chlorothalonil (mg/kg)	0.02	ND	ND	ND	ND	ND
Chloropropylate (mg/kg)	0.04	ND	ND	ND	ND	ND
Cyfluthrin (mg/kg)	0.05	ND	ND	ND	ND	ND
DCPA (Dacthal) (mg/kg)	0.02	ND	ND	ND	ND	ND
DDD o,p isomer (mg/kg)	0.02	ND	ND	ND	ND	ND
DDD p,p isomer (mg/kg)	0.02	ND	ND	ND	ND	ND
DDE o,p isomer (mg/kg)	0.02	ND	ND	ND	ND	ND
DDE p,p isomer (mg/kg)	0.02	ND	ND	ND	ND	ND
DDT o,p isomer (mg/kg)	0.02	ND	ND	ND	ND	ND
DDT p,p isomer (mg/kg)	0.02	ND	ND	ND	ND	ND
Dichlobenil (Casoron) (mg/kg)	0.01	ND	ND	ND	ND	ND
Dichlofluanid (Euparen) (mg/kg)	0.04	ND	ND	ND	ND	ND
Dichlone (Phygon) (mg/kg)	0.07	ND	ND	ND	ND	ND
Diclobutrazol (mg/kg)	0.05	ND	ND	ND	ND	ND
Diclofop methyl (mg/kg)	0.04	ND	ND	ND	ND	ND
Dicloran (DCNA; Botran) (mg/kg)	0.02	ND	ND	ND	ND	ND
3,4-Dichloraniline (mg/kg)	0.01	ND	ND	ND	ND	ND
Dicofol (Kelthane) (mg/kg)	0.02	ND	ND	ND	ND	ND
Dieldrin (mg/kg)	0.02	ND	ND	ND	ND	ND
Dimethachlor (mg/kg)	0.05	ND	ND	ND	ND	ND
Endosulfan I (Thiodan) (mg/kg)	0.01	ND	ND	ND	ND	ND

	LOD	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Endosulfan II (mg/kg)	0.01	ND	ND	ND	ND	ND
Endosulfan Sulfate (mg/kg)	0.01	ND	ND	ND	ND	ND
Endrin (mg/kg)	0.01	ND	ND	ND	ND	ND
Endrin Aldehyde (mg/kg)	0.02	ND	ND	ND	ND	ND
Etaconazol (mg/kg)	0.03	ND	ND	ND	ND	ND
Ethalfluralin (mg/kg)	0.01	ND	ND	ND	ND	ND
Etridiazole (mg/kg)	0.01	ND	ND	ND	ND	ND
Fenarimol (mg/kg)	0.03	ND	ND	ND	ND	ND
Fenson (mg/kg)	0.03	ND	ND	ND	ND	ND
Flamprop-iso (mg/kg)	0.03	ND	ND	ND	ND	ND
Flamprop-methyl (mg/kg)	0.03	ND	ND	ND	ND	ND
Fluazifop-butyl (mg/kg)	0.03	ND	ND	ND	ND	ND
Fluchloralin (Basalin) (mg/kg)	0.05	ND	ND	ND	ND	ND
Folpet (Folpan, Phatan) (mg/kg)	0.02	ND	ND	ND	ND	ND
HCB (Hexachlorobenzene) (mg/kg)	0.05	ND	ND	ND	ND	ND
Heptachlor (mg/kg)	0.01	ND	ND	ND	ND	ND
Heptachlor epoxide (mg/kg)	0.01	ND	ND	ND	ND	ND
Iprodione (mg/kg)	0.07	ND	ND	ND	ND	ND
Lindane (gamma-BHS) (mg/kg)	0.01	ND	ND	ND	ND	ND
Linuron (Lorox) (mg/kg)	0.14	ND	ND	ND	ND	ND
Methoxychlor (Marlate) (mg/kg)	0.03	ND	ND	ND	ND	ND
Metolachlor (mg/kg)	0.03	ND	ND	ND	ND	ND
Mirex (mg/kg)	0.01	ND	ND	ND	ND	ND
Nitrapyrin (mg/kg)	0.01	ND	ND	ND	ND	ND
Nuarimol (mg/kg)	0.05	ND	ND	ND	ND	ND
Ofurace (mg/kg)	0.05	ND	ND	ND	ND	ND
Ovex (mg/kg)	0.05	ND	ND	ND	ND	ND
Oxyfluorfen (mg/kg)	0.07	ND	ND	ND	ND	ND
PCNB-Quintozene (Avicol) (mg/kg)	0.01	ND	ND	ND	ND	ND
Pentchloroaniline (mg/kg)	0.01	ND	ND	ND	ND	ND
Permethrin (Ambush) (mg/kg)	0.06	ND	ND	ND	ND	ND
Perthane (Ethylan) (mg/kg)	0.02	ND	ND	ND	ND	ND
Piperalin (Pipron) (mg/kg)	0.05	ND	ND	ND	ND	ND
Procymidon (Sumilex) (mg/kg)	0.02	ND	ND	ND	ND	ND
Profluralin (Tolban) (mg/kg)	0.05	ND	ND	ND	ND	ND
Pronamide (mg/kg)	0.05	ND	ND	ND	ND	ND
Propanil (mg/kg)	0.02	ND	ND	ND	ND	ND

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	LOD	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Propiconazole (mg/kg)	0.04	ND	ND	ND	ND	ND
Terbacil (mg/kg)	0.04	ND	ND	ND	ND	ND
Tetradifon (Tedion (mg/kg)	0.02	ND	ND	ND	ND	ND
Thiobencarb (Bolero) (mg/kg)	0.04	ND	ND	ND	ND	ND
Tolyfluanid (mg/kg)	0.05	ND	ND	ND	ND	ND
Triadimefon (Bayleton) (mg/kg)	0.07	ND	ND	ND	ND	ND
Trichlorfon (mg/kg)	0.02	ND	ND	ND	ND	ND
Triforine (mg/kg)	0.09	ND	ND	ND	ND	ND
Trifluralin (Treflan) (mg/kg)	0.04	ND	ND	ND	ND	ND
Vegedex (Sulfallate) (mg/kg)	0.04	ND	ND	ND	ND	ND
Vinclozin (mg/kg)	0.04	ND	ND	ND	ND	ND
Organophosphorus Pesticides						
Acephate (Orthene) (mg/kg)	0.01	ND	ND	ND	ND	ND
Azinphos-ethyl (mg/kg)	0.01	ND	ND	ND	ND	ND
Azinphos-methyl (Guthion) (mg/kg)	0.01	ND	ND	ND	ND	ND
Azinphos-methyl oxon (mg/kg)	0.01	ND	ND	ND	ND	ND
Bromophos-methyl (mg/kg)	0.01	ND	ND	ND	ND	ND
Carbophenothion (Trithion) (mg/kg)	0.005	ND	ND	ND	ND	ND
Chlorfenvinphos (Supona) (mg/kg)	0.005	ND	ND	ND	ND	ND
Chloropyrifos (Dursban/Lorsban) (mg/kg)	0.005	ND	ND	ND	ND	ND
Chloropyrifos-methyl (mg/kg)	0.005	ND	ND	ND	ND	ND
Chlorthiophos (mg/kg)	0.005	ND	ND	ND	ND	ND
Coumaphos (Co-Ral) (mg/kg)	0.02	ND	ND	ND	ND	ND
Crotoxyphos (Ciodrin) (mg/kg)	0.01	ND	ND	ND	ND	ND
Cyanophos (mg/kg)	0.005	ND	ND	ND	ND	ND
DEF (Butifos) (mg/kg)	0.005	ND	ND	ND	ND	ND
Demeton (Systox) (mg/kg)	0.005	ND	ND	ND	ND	ND
Demeton-s (mg/kg)	0.005	ND	ND	ND	ND	ND
Dialifor (Torak) (mg/kg)	0.02	ND	ND	ND	ND	ND
Diazinon (O Analog) (mg/kg)	0.005	ND	ND	ND	ND	ND
Diazinon (Spectracide) (mg/kg)	0.005	ND	ND	ND	ND	ND
Dichlorvos-DDVP (Vapona) (mg/kg)	0.005	ND	ND	ND	ND	ND
Dicrotophos (Bidrin) (mg/kg)	0.005	ND	ND	ND	ND	ND
Dimethoate (Cygon) (mg/kg)	0.005	ND	ND	ND	ND	ND
Dioxathion (Delnav) (mg/kg)	0.005	ND	ND	ND	ND	ND
Disulfoton (Disyston) (mg/kg)	0.005	ND	ND	ND	ND	ND

	LOD	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Disulfoton Sulfone (mg/kg)	0.005	ND	ND	ND	ND	ND
Edifenphos (mg/kg)	0.01	ND	ND	ND	ND	ND
EPN (mg/kg)	0.01	ND	ND	ND	ND	ND
Ethion (Nialate) (mg/kg)	0.005	ND	ND	ND	ND	ND
Ethoprop (Mocap) (mg/kg)	0.005	ND	ND	ND	ND	ND
Ethyl Parathion (mg/kg)	0.005	ND	ND	ND	ND	ND
Fenamiphos (Nemacur) (mg/kg)	0.005	ND	ND	ND	ND	ND
Fenitrothion (Sumithion) (mg/kg)	0.005	ND	ND	ND	ND	ND
Fensulfothion (Dasanit) (mg/kg)	0.01	ND	ND	ND	ND	ND
Fenthion (Baytex) (mg/kg)	0.005	ND	ND	ND	ND	ND
Fonofos (Dyfonate) (mg/kg)	0.005	ND	ND	ND	ND	ND
Formothion (mg/kg)	0.02	ND	ND	ND	ND	ND
Heptenophos (mg/kg)	0.005	ND	ND	ND	ND	ND
Imidan (Phosmet) (mg/kg)	0.01	ND	ND	ND	ND	ND
Isazophos (Triumph) (mg/kg)	0.005	ND	ND	ND	ND	ND
Isofenphos (Amaze) (mg/kg)	0.005	ND	ND	ND	ND	ND
Leptophos (Phosvel) (mg/kg)	0.01	ND	ND	ND	ND	ND
Malaoxon (mg/kg)	0.005	ND	ND	ND	ND	ND
Malathion (Celthion) (mg/kg)	0.005	ND	ND	ND	ND	ND
Merphos (Tribufos) (mg/kg)	0.005	ND	ND	ND	ND	ND
Methamidophos (Monitor 4) (mg/kg)	0.01	ND	ND	ND	ND	ND
Methidathion (mg/kg)	0.005	ND	ND	ND	ND	ND
Methyl Parathion (Metacide) (mg/kg)	0.005	ND	ND	ND	ND	ND
Mevinphos (Phosdrin) (mg/kg)	0.005	ND	ND	ND	ND	ND
Monocrotophos (mg/kg)	0.005	ND	ND	ND	ND	ND
Naled (mg/kg)	0.005	ND	ND	ND	ND	ND
Omethoate (Dimethoate O analog) (mg/kg)	0.01	ND	ND	ND	ND	ND
Parathion (Alkron) (mg/kg)	0.005	ND	ND	ND	ND	ND
Paroxon (mg/kg)	0.005	ND	ND	ND	ND	ND
Phorate (Thimet) (mg/kg)	0.005	ND	ND	ND	ND	ND
Phorate-o (mg/kg)	0.005	ND	ND	ND	ND	ND
Phorate Sulfone (mg/kg)	0.005	ND	ND	ND	ND	ND
Phorate Suloxide (mg/kg)	0.01	ND	ND	ND	ND	ND
Phosalone (mg/kg)	0.01	ND	ND	ND	ND	ND
Phosphamidon (Dimecron) (mg/kg)	0.005	ND	ND	ND	ND	ND
Piperophos (mg/kg)	0.005	ND	ND	ND	ND	ND

	LOD	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
Pirimiphos-ethyl (mg/kg)	0.005	ND	ND	ND	ND	ND
Pirimiphos-methyl (mg/kg)	0.005	ND	ND	ND	ND	ND
Profenofos (Curacron) (mg/kg)	0.01	ND	ND	ND	ND	ND
Propetamphos (Safrotin) (mg/kg)	0.005	ND	ND	ND	ND	ND
Pyrazophos (Afgan) (mg/kg)	0.001	ND	ND	ND	ND	ND
Quinalphos (mg/kg)	0.005	ND	ND	ND	ND	ND
Ronnel (Ectoral) (Fenchlorphos) (mg/kg)	0.005	ND	ND	ND	ND	ND
Sulprofos (Bolstar) (mg/kg)	0.005	ND	ND	ND	ND	ND
Terbufos (Counter) (mg/kg)	0.005	ND	ND	ND	ND	ND
Tetrachlorvinphos (Gardona) (mg/kg)	0.005	ND	ND	ND	ND	ND
Thionazin (Zinophos) (mg/kg)	0.01	ND	ND	ND	ND	ND
Triazophos (Hostathion) (mg/kg)	0.01	ND	ND	ND	ND	ND
Cypermethrin (mg/kg)	0.002	ND	ND	ND	ND	ND
Oxadiazon (mg/kg)	0.01	ND	ND	ND	ND	ND
Propyzamide (mg/kg)	0.01	ND	ND	ND	ND	ND
Prothiofos (mg/kg)	0.05	ND	ND	ND	ND	ND
Tecnazene (mg/kg)	0.01	ND	ND	ND	ND	ND
Dichlofenthion (mg/kg)	0.01	ND	ND	ND	ND	ND
Chlordane (mg/kg)	0.01	ND	ND	ND	ND	ND
Toxaphene (mg/kg)	0.05	ND	ND	ND	ND	ND

* = testing conducted by Eurofins, Ltd. (Metarie LA); LOD = limit of detection; ND = not detected; The analyses were accomplished following FDA's Pesticide Analytical Manual (PAM) 304, which applies gas chromatography coupled to either electron capture detection (ECD) or flame photometric detection (FPD) depending on the analyte of interest.

Table 15. Polychlorinated dibenzodioxin and dibenzofuran (PCDD/F) analysis of five RAO lots*

	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201
2,3,7,8-TetraCDD (pg/g)	<0.07	<0.04	<0.04	<0.04	<0.04
1,2,3,7,8-PentaCDD (pg/g)	<0.11	<0.10	<0.10	<0.10	<0.10
1,2,3,4,7,8-HexaCDD (pg/g)	<0.11	<0.11	<0.10	<0.11	<0.11
1,2,3,6,7,8-HexaCDD (pg/g)	<0.11	<0.11	<0.10	<0.11	<0.11
1,2,3,7,8,9-HexaCDD (pg/g)	<0.11	<0.11	<0.10	<0.11	<0.11
1,2,3,4,6,7,8-HeptaCDD (pg/g)	<0.57	<0.57	<0.55	<0.57	<0.56
OctaCDD (pg/g)	<8.52	<8.50	<8.26	<8.50	<8.33
2,3,7,8-TetraCDF (pg/g)	<0.12	<0.12	<0.11	<0.12	<0.11
1,2,3,7,8-PentaCDF (pg/g)	<0.09	<0.09	<0.09	<0.09	<0.09
2,3,4,7,8-PentaCDF (pg/g)	<0.09	<0.09	<0.09	<0.09	<0.09
1,2,3,4,7,8-HexCDF (pg/g)	<0.1	<0.1	<0.09	<0.1	<0.09
1,2,3,6,7,8-HexaCDF (pg/g)	<0.09	<0.09	<0.09	<0.09	<0.09
1,2,3,7,8,9-HexaCDF (pg/g)	<0.1	<0.1	<0.09	<0.1	<0.09
2,3,4,6,7,8-HexaCDF (pg/g)	<0.11	<0.10	<0.10	<0.10	<0.10
1,2,3,4,6,7,8-HeptaCDF (pg/g)	<0.22	<0.22	<0.22	<0.22	<0.22
1,2,3,4,7,8,9-HeptaCDF (pg/g)	<0.12	<0.12	<0.11	<0.12	<0.11
OctaCDF (pg/g)	<0.63	<0.62	<0.61	<0.62	<0.61
Toxic Equivalent (TEQ) per Sample					
WHO(1998)-PCDD/F TEQ excl. LOQ (pg/g)	ND	ND	ND	ND	ND
WHO(1998)-PCDD/F TEQ incl. LOQ (pg/g)	0.320	0.290	0.282	0.280	0.284
WHO(2005)-PCDD/F TEQ excl. LOQ (pg/g)	ND	ND	ND	ND	ND
WHO(2005)-PCDD/F TEQ incl. LOQ (pg/g)	0.302	0.272	0.264	0.272	0.266

* = testing conducted by Eurofins, Ltd. (Hamburg, Germany); LOQ = limit of quantification; ND = not detected; The analysis was conducted in accordance with Method EN1948 using gas chromatography coupled to high resolution mass spectrometry (GC-HRMS).

F. COMPARISON OF COMPONENTS OF RAO WITH OTHER ARA-RICH OILS USED IN SUPPLEMENTED INFANT FORMULA

1. Fatty acid profiles

From a safety and nutritional perspective, it is important that ARA-rich oil used to supplement infant formula has no substantive impact on the fatty acid profile of that infant formula, other than delivering the intended target level of ARA. Because SUNTGA40S has already been determined GRAS in the US and authorized by the EC for use in infant formula, it

is useful to compare its fatty acid profile to those for several RAO lots. That comparison is presented in Table 16. Despite the clear demonstration of natural batch-to-batch variability associated with fermentation processes, this table shows the similarity of fatty acid profiles contained in the two ARA-rich oil products.

Given that ARA and DHA are used together to supplement infant formula, a complete analysis of the impact of ARA-rich oil on finished formula necessitates an understanding of the fatty acid distribution in a typical DHA-rich source. Table 17 provides the fatty acid distribution for a DHA-rich oil derived from tuna.

Because only small quantities of ARA- and DHA-rich oils are used in infant formula, the natural variability in their fatty acid profiles becomes negligible in the final formulas as consumed by infants. To demonstrate this "dilution" effect, Similac Special Care formula was chosen as a representative infant formula. Table 18 shows the fatty acid profiles of Similac Special Care formula (1) without ARA or DHA oil supplementation, (2) supplemented with RAO and DHA-rich tuna oil, and (3) supplemented with SUNTGA40S and DHA-rich tuna oil. For perspective, the fatty acid distributions of RAO, SUNTGA40S and DHA-rich tuna oil are also shown.

The last two columns of Table 18 show the relative percentages of major fatty acids in the finished product after supplementation with DHA from tuna oil and ARA sourced from either RAO or SUNTGA40S. When the data in those two columns are compared to the unsupplemented Similac Special Care formula, it can be seen that regardless of the ARA-rich oil source, its addition has virtually no effect on the final formula fatty acid composition, with the exception of the intentional increase in levels of ARA and DHA.

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Table 16. Fatty acid compositions of RAO and SUNTGA40S

	Fungal oil SUNTGA40S[a]				Refined ARA-rich oil (RAO) [b]					
	Lot 98041751	Lot 98971451	Lot 98100651	Cargill analysis	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201	Average
Fatty acid (FA)	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**
Saturated FA										
C6:0										
C8:0					0.01	0.01	0.01			0.006
C10:0					0.02	0.04	0.03	0.04	0.03	0.032
C12:0 (Lauric)					0.01	0.01	0.01	0.01	0.01	0.01
C14:0 (Myristic)	0.35	0.35	0.4	0.44	0.23	0.35	0.26	0.23	0.23	0.26
C15:0 (Pantadecanoic)	0.13	0.15	0.15		0.1	0.09	0.08	0.08	0.08	0.086
C16:0 (Palmitic)	11.5	13.1	11.7	12.15	6.54	6.87	5.84	5.43	5.44	6.024
C17:0 (Heptadecanoic)	0.23	0.27	0.27		0.17	0.2	0.18	0.18	0.18	0.182
C18:0 (Stearic)	7.02	7.88	7.23	7.2	4.48	5.98	5.21	4.9	4.98	5.11
C19:0 (Total)					0.18	0.19	0.18	0.19	0.19	0.186
C20:0 (Total)	0.76	0.84	0.78	0.77	0.7	0.81	0.78	0.75	0.75	0.758
C21:0					0.1	0.1	0.09	0.09	0.1	0.096
C22:0 (Behenic)	2.91	3.07	2.95	2.94	3.02	3.24	3.13	3.04	3.1	3.106
C24:0 (Lignoceric)	8.01	8.36	8.07	8.19	10.14	9.53	10.2	10.24	10.5	10.122
C25:0					0.16	0.12	0.15	0.16	0.16	0.15
C26:0 (Hexacosanoic)	0.1	0.08	0.08	1.02	1.42	1.13	1.34	1.41	1.48	1.356
Monounsaturated FA										
C14:1					0.01	0.01		0.01		0.006
C16:1 n5					0.01	0.01	0.01			0.006
C16:1 n7 (Palmitoleic)	0.07	0.1	0.1		0.22	0.21	0.2	0.15	0.14	0.184
C16:1 n9					0.03	0.03	0.03	0.02	0.02	0.026

	Fungal oil SUNTGA40S[a]				Refined ARA-rich oil (RAO)[b]					
	Lot 98041751	Lot 98971451	Lot 98100651	Cargill analysis	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201	Average
Fatty acid (FA)	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**
C18:1 n5					0.01		0.01	0.01		0.01
C18:1 n7 (Vaccenic)	0.31	0.35	0.32		0.32	0.24	0.23	0.21	0.21	0.24
C18:1 n9 (Oleic)	5.41	6.38	5.65	6.85	5.74	5.61	4.77	4.39	4.33	4.97
C19:1					0.04	0.05	0.04	0.03	0.04	0.04
C20:1 n7					0.08	0.07	0.07	0.09	0.09	0.08
C20:1 n9 (Eicosenoic)	0.39	0.44	0.36	0.44	0.27	0.22	0.22	0.2	0.21	0.22
C20:1 n11					0.02	0.02	0.02	0.02	0.02	0.02
C22:1 n9 (Erucic)	0.09	0.12	0.09	0.09	0.17	0.17	0.19	0.16	0.18	0.17
C22:1 n11+13					0.12	0.09	0.11	0.1	0.1	0.10
C24:1 n9 (Nervonic)	0.45	0.46	0.39	0.26	0.44	0.45	0.51	0.56	0.51	0.49
C26:1					0.07	0.05	0.07	0.09	0.08	0.07
Omega 6 FA										
C16:2 n6					0.01	0.01	0.01	0.01	0.01	0.01
C18:2 n6 (Linoleic acid)	8.28	9.85	9.14	9.88	8.69	8.4	8.05	7.28	6.92	7.87
C18:3 n6 (γ-Linoleic acid)	2.29	2.34	2.34	2.25	2.13	2.27	2.11	2	2.01	2.10
C20:2 n6 (Eicosadienoic)	0.66	0.84	0.66		0.45	0.45	0.46	0.42	0.42	0.44
C20:3 n6 (Eicosatrienoic)	3.88	3.86	3.96	3.32	3.71	3.76	3.7	3.61	3.69	3.69
C20:4 n6 (ARA)	46.2	40.3	44	37.37	41.15	41.7	44.41	44.55	44.68	43.30
C22:4 n6	0.45	0.41	0.39	0.48	0.01	0.01	0.01	0.01	0.01	0.01
C22:5 n6										
Omega 3 FA										
C16:4 n3					0.01	0.01	0.01	0.01	0.01	0.01

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	Fungal oil SUNTGA40S[a]				Refined ARA-rich oil (RAO) [b]					
	Lot 98041751	Lot 98971451	Lot 98100651	Cargill analysis	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201	Average
Fatty acid (FA)	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**
C18:3 n3 (α-linoleic acid)	0.22	0.34	0.35	0.35	0.03	0.04	0.03	0.04	0.04	0.04
C18:5 n3					0.02	0.02	0.02	0.02	0.02	0.02
C20:3 n3							0.03	0.03	0.02	0.02
C20:4 n3					0.01		0.01	0.01	0.01	0.01
C20:5 n3 (EPA)	0.14	0.15	0.11	0.16	0.08	0.09	0.16	0.22	0.17	0.14
C21:5 n3					0.06	0.05	0.06	0.05	0.06	0.06
C22:3 n3					0.28	0.28	0.3	0.3	0.3	0.29
C22:5 n3										
C22:6 n3 (DHA)					0.04	0.04	0.03	0.04	0.04	0.04
Others										
C18:2 n7,13					0.04	0.05	0.04	0.03	0.04	0.04
C18:2 n7,10					0.01	0.01	0.01	0.01	0.01	0.01
C18:2 other					0.09	0.09	0.09	0.11	0.1	0.1
C18:2 n4					0.01	0.02	0.01	0.01	0.01	0.01
C20:2 n7					0.01	0.01	0.01	0.01	0.01	0.01
C22:2					0.01	0.03	0.02	0.01	0.02	0.02
C16:3 n4					0.03	0.02	0.02	0.02	0.02	0.02
C18:3 n4					0.04	0.05	0.04	0.05	0.05	0.05
C18:3 n1					0.01	0.01	0.01	0.01	0.01	0.01
C18:3 other					0.04	0.05	0.04	0.04	0.04	0.04
C20:3 n7					0.21	0.21	0.19	0.18	0.2	0.2
C16:4 n1					0.01	0.01	0.01	0.01	0.01	0.01
C18:4 n1					0.01	0.01	0.01	0.01	0.01	0.01

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	Fungal oil SUNTGA40S[a]				Refined ARA-rich oil (RAO) [b]					
	Lot 98041751	Lot 98971451	Lot 98100651	Cargill analysis	Lot 071013	Lot 08062101	Lot 09022301	Lot 09032101	Lot 09032201	Average
Fatty acid (FA)	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**	**w/w% FA**
C20:4 Unknown Isomers					2.55	2.49	2.31	2.13	2.32	2.36
Total Fatty acids	**99.85**	**100.04**	**99.49**	**96.52**	**94.58**	**96.09**	**96.18**	**94.02**	**94.42**	**95.06**

a = Source is EFSA (2008); b = data are the same as those presented in Table 9; * empty cells means the fatty acid was not reported for SUNTGA40S by EFSA (2008), not detected by Cargill analysis, or not detected for RAO.

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Table 17. Representative fatty acid composition (g/100 g total fatty acids) of a tuna-derived DHA-rich oil

Fatty Acid	Relative quantity	Fatty Acid	Relative quantity	Fatty Acid	Relative quantity	Fatty Acid	Relative quantity
12:0	0.1	14:1	0.1	16:2n4+phytanic	0.9	20:5n3	5.1
14:0	3.8	16:1n9	0.1	18:2n6	1.2	21:5n3	0.3
ISO 15:0	0.1	16:1n7	5.9	18:2n4	0.1	22:5n6	1
15:0	0.9	16:1n5	0.4	20:2NMID	0.1	22:5n3	1.3
ISO 16:0	0.1	18:1n9	18.6	20:2n6	0.3	22:6n3 (DHA)	18.3
16:0	20.8	18:1n7	2.4	16:3n4	0.9		
7ME16:0	0.1	18:1n5	0.1	18:3n4	0.1		
ISO 17:0	0.2	20:1n11	0.5	18:3n3	0.4		
ANT 17:0	0.1	20:1n9	2.1	20:3n6	0.2		
17:0	0.9	20:1n7	0.2	20:3n3	0.2		
ISO 18:0	0.2	22:1n11+13	1.1	16:4n3	0.6		
18:0	4.5	22:1n9	0.5	20:4n6	1.9		
19:0	0.3	22:1n7	0.2	20:4n3	0.5		
20:0	0.3	24:1	0.7	22:4n6	0.5		
22:0	0.3			22:4n3	0.2		
24:0	0.2						
Total Saturates	**32.9**	**Total monoenes**	**32.9**	**Total di, tri and tetraenes**	**8.1**	**Total pentanes and hexaenes**	**26**

Source: GRAS notification to the US FDA (GRN94), Batch # 971105 on Pages 45 and 46

Table 18. Fatty acid compositions of an unsupplemented infant formula (UIF), RAO, SUNTGA40S, and UIF supplemented with RAO and with SUNTGA40S

Fatty Acid (FA)	UIF [a]	SUNTGA40S only [b]	RAO only [b]	DHA-rich tuna oil [c]	UIF supplemented with SUNTGA40S at 1.0% TFA and DHA-rich tuna oil at 1.5% TFA [d]	UIF supplemented with RAO at 0.86% TFA and DHA-rich tuna oil at 1.5% TFA [d]
Saturated FA						
C6:0	0.38				0.37	0.37
C8:0	30.4		0.006		29.6	29.7
C10:0	21.3		0.032		20.8	20.8
C12:0 (Lauric)	9.4		0.01	0.1	9.2	9.2
C14:0 (Myristic)	3.6	0.44	0.26	3.8	3.6	3.6
C15:0 (Pantadecanoic)			0.086	0.9	<0.02	<0.02
C16:0 (Palmitic)	5.3	12.15	6.024	20.8	5.6	5.5
C17:0 (Heptadecanoic)	0.06		0.182	0.9	0.07	0.07
C18:0 (Stearic)	2.6	7.2	5.11	4.5	2.7	2.7
C19:0 (Total)			0.186			0.002
C20:0 (Total)	0.12	0.77	0.758	0.3	0.13	0.13
C21:0			0.096			<0.001
C22:0 (Behenic)	0.11	2.94	3.106	0.3	0.14	0.14
C24:0 (Lignoceric)	0.27	8.19	10.122	0.2	0.35	0.35
C25:0			0.15			0.001
C26:0 (Hexacosanoic)		1.02	1.356		0.01	0.012
Monounsaturated FA						
C14:1			0.006			<0.001
C16:1 n5			0.006			<0.001
C16:1 n7 (Palmitoleic)	0.22		0.184	5.9	0.3	0.3
C16:1 n9			0.026			<0.001
C18:1 n5			0.006			<0.001
C18:1 n7 (Vaccenic)			0.242	2.4	<0.04	<0.04
C18:1 n9 (Oleic)	8.2	6.85	4.968	18.6	8.3	8.3
C19:1			0.04			<0.001
C20:1 n7			0.08			<0.001

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Fatty Acid (FA)	UIF [a]	SUNTGA40S only [b]	RAO only [b]	DHA-rich tuna oil [c]	UIF supplemented with SUNTGA40S at 1.0% TFA and DHA-rich tuna oil at 1.5% TFA [d]	UIF supplemented with RAO at 0.86% TFA and DHA-rich tuna oil at 1.5% TFA [d]
C20:1 n9 (Eicosenoic)	0.08	0.44	0.224	2.1	0.11	0.11
C20:1 n11			0.02			<0.01
C22:1 n9 (Erucic)		0.09	0.174		<0.01	0.01
C22:1 n11+13			0.104	1.1	0.017	0.017
C24:1 n9 (Nervonic)		0.26	0.494		<0.01	<0.01
C26:1			0.072			<0.001
Omega 6 FA						
C16:2 n6			0.01			<0.01
C18:2 n6 (Linoleic acid)	16	9.88	7.868	1.2	16	16
C18:3 n6 (γ-Linoleic acid)		2.25	2.104		0.02	0.02
C20:2 n6 (Eicosadienoic)			0.44	0.3	0.01	0.01
C20:3 n6 (Eicosatrienoic)		3.32	3.694	0.2	0.04	0.04
C20:4 n6 (ARA)		37.37	43.298	1.9	0.40	0.40
C22:4 n6		0.48	0.01		<0.01	<0.01
C22:5 n6			0	1	0.015	0.015
Omega 3 FA						
C16:4 n3			0.01			<0.001
C18:3 n3 (α-linoleic acid)	2.4	2.71	0.036	0.4	2.4	2.4
C18:5 n3			0.02			<0.01
C20:3 n3			0.016			<0.001
C20:4 n3			0.008			<0.001
C20:5 n3 (EPA)		0.16	0.144	5.1	0.08	0.08
C21:5 n3			0.056			<0.001
C22:3 n3			0.292			0.003
C22:5 n3			0	1.3	0.02	0.02
C22:6 n3 (DHA)			0.038	18.3	0.3	0.3
Others						
C18:2 n7,13			0.04			<0.001
C18:2 n7,10			0.01			<0.001
C18:2 other			0.096			<0.001

Fatty Acid (FA)	UIF [a]	SUNTGA40S only [b]	RAO only [b]	DHA-rich tuna oil [c]	UIF supplemented with SUNTGA40S at 1.0% TFA and DHA-rich tuna oil at 1.5% TFA [d]	UIF supplemented with RAO at 0.86% TFA and DHA-rich tuna oil at 1.5% TFA [d]
C18:2 n4			0.012			<0.001
C20:2 n7			0.01			<0.001
C22:2			0.018			<0.001
C16:3 n4			0.022			<0.001
C18:3 n4			0.046			<0.001
C18:3 n1			0.01			<0.001
C18:3 other			0.042			<0.001
C20:3 n7			0.198			<0.001
C16:4 n1			0.01			<0.001
C18:4 n1			0.01			<0.001
C20:4 Unknown Isomers			2.36			<0.001
Total	**100.44**	**96.52**	**95.058**	**91.6**	**100.3**	**100.3**

a = Source: Similac Special Care formula. Table III-6 on Pages 72 (GRN94); b = data are from Table 16; c = data are from Table 17; d = ARA rich oil is supplemented (1% SUNTGA40S or 0.86% RAO) to provide an ARA level of about 0.4% total fatty acids; 1.5% of fatty acids is supplemented with DHA rich oil to provide a DHA level of about 0.27% total fatty acids; % of TFA (total fatty acids) = % of ARA-rich oil derived fatty acids based on the % of total fatty acids in the entire infant formula. Presented data are based on calculation.

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2. Sterol profiles of RAO and ARA rich oils in finished infant formula

Terpenes are a diverse family of compounds that are assembled from the basic unit *isoprene.* Members of this family include the monoterpenes, sesquiterpenes, diterpenes, steroids, carotenes, polyprenoids such as ubiquinone side chain, and the polymer rubber. Although almost all organisms make and use terpenes, available studies suggest that the terpenes produced by *M. alpina* are sterols. Therefore, the sterol profile for ARA-rich oil products (ARASCO and SUNTGA40S) from *M. alpina* were quantified. (*M. alpina* Peyronel 1S-4 (GRN94[5]) or that from *M. alpina* ATCC 32222 (GRN41[6]). The sterol profile for RAO has also been quantified and is presented in this dossier.

Because sterols are natural products of botanical species, many of which are consumed as food (Clifton, 2009; Patch *et al.*, 2006), humans are commonly exposed to sterols. *M. alpina* is a fungus known to produce sterols which will remain in finished, refined ARA-rich oil. RAO contains sterols; on average, at approximately 1% (*i.e.*, 1 g/100 g oil). The three predominant sterols in RAO are desmosterol, 24-methyl desmosterol and codisterol, all of which are phytosterols/intermediates in the biosynthesis of steroids such as cholesterol, vitamin D_2 and phytosterols. Table 19 presents a comparison of sterol content among three ARA-rich oils derived from *M alpina*: RAO, SUNTGA40S and ARASCO oils.

The sterols found in RAO are commonly found in nature and are part of normal metabolic pathways. Its principal sterol is 24-methyl desmosterol (24-methylcholesta-5, 24-dien-3β-ol). 24-Methyl sterols are commonly found in fungi, including edible fungi such as mushrooms (Nes and Le, 1990), and in plants such as green alga (Jo *et al.*, 2004), tomato shoots (Takatsuto and Narumi, 2001 and pollens of sunflower (Takatsuto and Omote, 1989a) and Vicia faba bean (Takatsuto and Omote, 1989b). In plants, 24, 25-reductase normally uses 24-methyl and 24-ethyl desmosterol as a substrate to generate campesterol and sitosterol, respectively (Yamada *et al.*, 1997; Fujimoto *et al.*, 1997).

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[5] April 18, 2006. Agency Response Letter GRAS Notice No. GRN 000094. The subjects of the notice were arachidonic acid-rich oil from *Mortierella alpina* (AA-rich fungal oil) and docosahexaenoic acid-rich oil from tuna (DHA-rich tuna oil).

[6] May 17, 2001, Agency Response Letter GRAS Notice No. GRN 000041. The subjects of the notice were DHASCO (docosahexaenoic acid-rich single-cell oil) and ARASCO (arachidonic acid-rich single-cell oil).

Table 19. Sterol compositions in RAO, SUNTGA40S, and ARASCO oils

	RAO oil [a]	SUNTGA40S (Cargill analysis)	ARASCO oil [b]
Sterol	**% w/w in oil**	**% w/w in oil**	**% w/w in oil**
Desmosterol	0.082	0.172	0.532
Cholesta-5,25-dien-3β-ol		0.011	0.016
Zymosterol	0.012	0.002	
24-Methyl Cholesta-5,24(25)-dien-3β-ol	0.533	0.034	0.111
24-Methylene Cholesterol	0.061	0.017	
Campesterol	0.013		
4α-Methyl Zymosterol	0.018	0.003	
24-Methyl Cholesta-5,(25)27-dien-3β-ol	0.111	0.037	0.114
Lanosterol	0.038	0.003	0.009
Ergosterol		0.003	
β-Sitosterol	0.034	0.007	
31-Norlanosterol	0.029	0.003	
Total Unknown Sterols	0.045	0.011	
Total	**0.977**	**0.303**	**0.782**

[a] An average of five batches derived from Table 7; [b] Derived from Food Standards Australia New Zealand (FSANZ) Technical Report Series No. 22 (June 2003), "DHASCO and ARASCO oils as sources of long-chain polyunsaturated fatty acids in infant formula", available from FSANZ at http://www.foodstandards.gov.au.

The second most abundant sterol in RAO is codisterol (24-methylcholesta-5, 25-dien-3β-ol), a common end product of sterol metabolism in plants (Garg and Nes, 1985), which has been found in commonly consumed food plants such as cucumber and yew. The third most abundant sterol in RAO, desmosterol (cholesta-5, 24-dien-3β-ol), is found in the normal metabolic pathway of cholesterol biosynthesis and is commonly found in several common food sources including animal fat, vegetable oils, and human milk (GRN41).

Other sterols with much smaller quantities in RAO include lanosterol (4α, 4β, 14α-trimethyl-5α-cholesta-8, 24-dien-3β-ol), zymosterol (5α-cholesta-8, 24-dien-3β-ol), 4α-methylzymosterol (4α-methyl-5α-cholesta-8,24-dien-3β-ol), β-sitosterol, campesterol (24α-methyl-5-cholesten-3β-ol), 24-methylenecholesterol, ergosterol and 31-norlanosterol (4, 14-dimethyl-5α -cholesta-8, 24-dien-3β-ol).

The first three sterols listed above (lanosterol, zymosterol and 4α-methylzymosterol) are intermediates in the biosynthesis of plant steroids. Like desmosterol, lanosterol is a precursor of cholesterol and is the compound from which all steriods are derived. Zymosterol and 4α-methylzymosterol are both intermediates in the biosynthesis of cholesterol from lanosterol (Bae and Paik, 1997; Echevarria *et al.*, 1990). Because they are intermediates or precursors in the biosynthesis of cholesterol, they would be expected to be present in many plants, including those that are part of the diet.

β-Sitosterol is one of several phytosterols with chemical structures similar to that of cholesterol. It is widely distributed in the plant kingdom and the most abundant phytosterol, occuring in legumes, oil seeds, and unrefined plant oils as found in foods such as peanut butter, pistachios, and sunflower seeds (Awad *et al.*, 2007). β-Sitosterol is a major component of plant sterols and stanols that are approved for food use in the US (GRN39, GRN48, GRN53, GRN61, GRN112, GRN176, GRN177, GRN181, GRN206 and GRN250).

Campesterol (24α-methyl-5-cholesten-3β-ol), along with β-sitosterol and stigmasterol, is one of the three principle phytosterols found in nature (Sorenson and Sullivan, 2007). Phytosterols containing campesterol are used in dietary supplements in the US. Campesterol is a component of plant sterols and stanols that are approved for food use in the US (GRN39, GRN48, GRN53, GRN61, GRN112, GRN176, GRN177, GRN181, GRN206 and GRN250). 24-Methylenecholesterol is a precursor of campesterol and is abundant in plants like African rice (Omoloye and Vidal, 2007).

Ergosterol (ergosta-5,7,22-trien-3β-ol) is present in small amounts in higher plants, such as certain mushroom (Jasinghe and Perera, 2005) and is converted by ultraviolet irradiation ultimately into vitamin D_2.

31-Norlanosterol (4, 14-dimethyl-5α -cholesta-8, 24-dien-3β-ol) is also widely distributed in nature, such as in vegetable oils (Jeong *et al.*, 1975). Cholesta-5, 25-dien-3β-ol is present in SUNTGA40S, but is not in RAO oil (under the detection limit, *i.e.*, < 0.005%).

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In conclusion, all the sterols found in RAO are naturally existing substances, either as intermediates in steroid synthesis or as widely distributed plant phytosterols, to which humans are commonly exposed; and with the exception of campesterol, all the sterols identified in RAO were also reported in SUNTGA40S (GRN94). Campesterol is naturally present in infant formula with a reported range of 0.03 to 0.1 mmol/l (Huisman *et al.*, 1996); equivalent to 12 to 40 ppm. Calculation shows that the RAO-derived campesterol content in infant formula is approximately 0.1 ppm, which is substantially lower than the amount of campesterol naturally present in infant formula (Huisman *et al.*, 1996). The calculation is based on the following: 1) RAO is supplemented in infant formula to provide target ARA levels of 0.75 g/100g total fat for term infants and 0.40 g/l00 g total fat for preterm infants; 2) fat comprises about 50%en of total calories in infant formula; 3) RAO contains about 40% ARA and approximately 0.0132% campesterol (Table 20), and 4) infant formula will be dissolved in water with a dilution ratio of about 7 before it is fed to infants. Thus, supplementation with RAO will not substantially affect the level of campesterol in the finished product.

Sterol Profile Comparison: As shown in Table 19, small sterol profile differences exist when comparing RAO and SUNTGA40S; however, *per* the above discussion, there should be no concern for adverse safety or nutritional effects associated with ingestion of RAO. In addition and as shown in Table 20, RAO's sterol contribution to finished infant formula is negligible.

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Table 20. Contribution of sterols from RAO and SUNTGA40S to total sterols in infant formula

	Sterols in SUNTGA40S (Cargill analysis) [a]	Sterols in RAO oil [a]	Sterol content in an average formula [b]	Sterol content in an average formula supplemented with SUNTGA40S at 1.0% of total fatty acids [c]	Sterol content in an average formula supplemented with RAO at 0.86% of total fatty acids [c]
Sterols	**g/100 g ARA-rich oil**	**g/100 g ARA rich oil**	**mg/g total fatty acids**	**mg/ g total fatty acid**	**mg/ g total fatty acid**
Cholesterol			0.5	0.5	0.5
Desmosterol	0.172	0.082		0.017	0.007
Cholesta-5,25-dien-3β-ol	0.011			0.001	
Zymosterol	0.002	0.012		<0.001	<0.001
24-Methyl Cholesta-5,24(25)-dien-3β-ol	0.034	0.533		0.003	0.046
24-Methylene Cholesterol	0.017	0.061		0.002	0.005
Campesterol		0.013	0.55	0.55	0.55
4α-Methyl Zymosterol	0.003	0.018		<0.001	0.002
24-Methyl Cholesta-5,(25)27-dien-3β-ol	0.037	0.111		0.004	<0.001
Lanosterol	0.003	0.038		<0.001	0.003
Ergosterol	0.003			<0.001	
β-Sitosterol	0.007	0.034	1.45	1.44	1.44
31-Norlanosterol	0.003	0.029		<0.001	0.003
Total Unknown Sterols	0.011	0.045		0.001	0.004
Stigmasterol			0.22	0.22	0.22
Fucosterol (including 28-iso-fucosterol)			0.074	0.073	0.073
Total	**0.303**	**0.977**	**2.794**	**2.796**	**2.854**

a. Data are the same as those in Table 19.
b. In infant formula (powder, concentrate or ready-to-feed), the cholesterol content varies. An average of 0.5 mg cholesterol/gram of fat is used in the calculation. For example, about 0.3 mg cholesterol/gram of fat presents in Similac ready-to-feed formula (http://www.nutritiondata.com/facts/baby-foods/457/2); about 0.63 mg cholesterol/gram of fat presents in Enfamil powder (http://www.quitehealthy.com/nutrition-facts/enfamil/03871Z1.html); an average of 22.4 mg/l of cholesterol was found in 10 infant formula milks (Huisman *et al.*, 1996), which is equivalent to 0.57 mg cholesterol/gram of total fatty acid, assuming that an average water content in infant formula milk is 87% and fatty acids accounts for an average of 30% of total dry weight; In addition to cholesterol, infant formula also contain phytosterols, mainly β-sitosterol and campesterol (Huisman *et al.*, 1996). Based on the data of 10 infant formula milks, average concentrations of β-sitosterol, campesterol, stigmasterol, and fucosterol (including 28-iso-fucosterol) are 56 mg/l, 21.3 mg/l, 8.7 mg/l and 2.9 mg/l, respectively, which are equivalent to 1.45, 0.55, 0.22 and 0.074 mg/gram fatty acid, assuming that an average water content in infant formula milk is 87% and an average of 30% of total dry weight is fatty acids.
c. ARA rich oil is supplemented (1% SUNTGA40S or 0.86% RAO) to provide an ARA level of about 0.4% total fatty acids. Data presented are based on calculation.

G. MICROBIOLOGICAL INFORMATION ON THE FOOD INGREDIENT

Analysis for five lots of RAO (Table 4) demonstrated that the microbiological limits given in the product specifications summarized in Table 1 were met.

III. HISTORY OF USE AND INTENDED USE

A. HISTORY OF EXPOSURE TO ARA FROM HUMAN MILK

The studies included in this section update information on infant exposure to ARA since the review by the FDA and International authorities 2005(FDA, 2005; NFU, 2005; EFSA, 2008). This information corroborates the conclusions reached by FDA and EU authorities who reviewed literature published prior to 2005 regarding the extent of previous human exposure to ARA and ARA-rich oils used to supplement infant formula (FDA, NFU, 2005; EFSA, 2008).

This chapter: (1) provides information on the levels of previous human exposure to ARA from human milk, including new data available since 2005; (2) summarizes the current scientific authority recommendations/regulations for addition of ARA to infant formula and intake of ARA from supplementation at these levels to existing commercial infant formulas; and (3) summarizes new clinical trials documenting infant exposure to ARA-rich supplemented formulas and provides results from these trials that corroborate the safety of ingestion of ARA-rich oil from these exposures.

Studies documenting infant exposure to ARA from human milk:

In a GRAS exemption claim (GRN94) for ARA-rich fungal oil made from *M. alpina* Peyronel 1S, levels of ARA in human milk were reported from 27 published studies that showed a mean ARA content of 0.31% to 1.22% (GRN94). More recently, Mitmesser and Jensen (2007) evaluated data within a similar timeframe and corroborated the variability of ARA content of human milk reported in GRN94. The data cited in Table 21 from this review shows the overall range of ARA concentration in human milk across the global population to be 0.34 to 1.22%. The authors suggested that the high levels of ARA in breast milk indicated a strong influence of the maternal diet on maximum levels of infant exposure to ARA.

Several other studies (Table 22) have also reported ARA levels in human milk. Bahrami and Rahimi (2005) found that milk collected in western Iran from 40 lactating mothers, ages 17 to 39 years, had a mean ARA content of 1.4% ± 0.35% of total milk fatty acids. The authors noted that these levels are higher in levels from milk of American and European women, likely

influenced by consumption of partially hydrogenated vegetable oils with high levels of *trans*-fatty acids. Kuipers *et al.* (2007) reported that in nine regions of Tanzania where mothers have a high consumption of freshwater fish as their only source of animal protein and fat, the range for median ARA breast milk concentrations was 0.37% to 0.80% (Table 22). The minimum and maximum values for these same women were 0.27% in the Maasai (inland) region (n=27) and 1.07% in the Nyiramba (lake) region (n=36), respectively. In Portugal, breast milk was collected from 31 women at various intervals from Day 7 to Week 16 of lactation, and analyzed for ARA content (Ribeiro *et al.*, 2008). ARA levels were found to decrease progressively during lactation from a mean of 0.67% ± 0.16 for "transitional milk" to 0.42% ± 0.12 at 16 weeks for mature milk; there were no significant differences in ARA levels in mature milk from lactation Weeks 4 to 16. Decreases in ARA in mature milk compared with human colostrum have been reported: declines in ARA levels by Week 6 of lactation noted from women from Hong Kong (0.71% to 0.52%) and from women from Chongqing (0.23% to 0.10%). Data from women in Italy were similar, where initial levels of 0.91% ARA declined to 0.50% after 12 months of lactation (Chen *et al.* 1997; Agostoni *et al.* 1999).

Brenna *et al.* (2007) conducted a meta-analysis of ARA concentrations in mature human milk based on published data from 65 studies spanning 1986 to 2006 and involving 2474 women. The mean (±SD) concentration of ARA as a percentage of total fatty acids was 0.47% ± 0.13% (range: 0.24% to 1.0%) (Table 23). The authors noted that the highest concentrations of ARA in human milk were seen in coastal regions; possibly associated with marine-rich diets. This evaluation reveals a broad range of ARA levels in human milk on a worldwide basis and shows the range of possible infant exposure to ARA, which provides a guide for levels of ARA supplementation in infant formulas.

Table 21. ARA levels in human milk (Data adapted from studies reviewed by Mitmesser and Jensen, 2007)

Diet / Location	# of women sampled	% mean fatty acid from ARA	Reference
Marine/ China	45	1.17	Jensen *et al.* (1999)
Japan	53	0.36	Jensen *et al.* (1999)
Urban/ China	22	1.01	Jensen *et al.* (1999)
Rural/ China	16	0.80	Jensen *et al.* (1999)
Israel	26	0.58	Jensen *et al.* (1999)
Nigeria	20	0.56	Jensen *et al.* (1999)
Spain	40	0.5	Jensen *et al.* (1999)
France	10	0.5	Jensen *et al.* (1999)
U.S.	220	0.40 – 0.67	Brenna *et al.* (2007)
Australia	23	0.45	Jensen *et al.* (1999)
Germany	38	0.45	Jensen *et al.* (1999)
Netherlands	99	0.34	Jensen *et al.* (1999)
Pastoral/ China	24	1.22	Jensen *et al.* (1999)
Sudan	77	0.50	Jensen *et al.* (1999)

Table 22. ARA levels in human milk (studies not included in Mitmesser and Jensen, 2007)

Diet / Location	# of women sampled	% mean fatty acid from ARA	Reference
Japan	36	0.86 ± 0.16 (S.E.) (Colostrum)	Young *et al.* (1997)
China (Hong Kong)	51	0.76 ± 0.24 (S.D.)	Chen *et al.* (1997)
China (Chongquing)	33	0.61 ± 0.18 (S.D.)	Chen *et al.* (1997)
Italy	10	1.05% colostrum; 0.64% 1mo.; 0.50% 12 mo.	Agostoni *et al.* (1999) and Marangoni *et al.* (2000)
The Netherlands	20/20	0.39% to 0.46% (atopic/ non-atopic)	Thijs *et al.* (2000)
Nepal	36	0.43%	Glew *et al.* (2001)
Italy	54	0.95% colostrum; 0.50% 3 mo.	Marangoni *et al.* (2002)
High trans-fat diets/ Iran	52	1.4% ± 0.35	Bahrami and Rahimi (2005)
Marine/Tanzania	208	0.37% to 0.80% (median range)	Kuipers *et al.* (2007)
Spain	6	0.71 ± 0.14 (colostrum) 0.49 ± 0.11 (mature milk)	Sala-Vila *et al.* (2006)
Brazil	16	16 Female infants: 0.69% ± 0.22 (colostrum); 0.48% ± 0.11 (mature milk) 21 Male infants: 0.65% ± 0.26 (colostrum); 0.43% ± 0.17 (mature milk)	Tinoco *et al.* (2008)
Portugal	31	0.67% ± 0.16 (transitional milk) 0.42% ± 0.12 (mature milk)	Ribeiro *et al.* (2008)

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Table 23. ARA concentrations in mature human milk; adapted from Brenna *et al.* (2007)

Country	Infant age (months)	# of women sampled	ARA % of total fatty acids	Reference
Hungary	1	18	0.59	Minda *et al.* (2004)
Spain	3	11	0.41	Sala-Vila *et al.* (2004)
Australia	3	36	0.38	Stoney *et al.* (2004)
U.S. (N. Carolina)	3	22	0.41	Bopp *et al.* (2005)
U.S. (Texas)	4	77	0.44	Jensen *et al.* (2005)
Denmark	4	39	0.30	Kovacs *et al.* (2005)
China	3	23	0.51	Xiang *et al.* (2005)
Iceland	2	59	0.32	Olafsdottir *et al.* (2006)
Spain	0.5-1	10	0.49	Sala-Vila *et al.* (2006)
U.S,	1-12	49	0.45	Yuhas *et al.* (2006)
U.K.	1-12	44	0.36	Yuhas *et al.* (2006)
Philippines	1-12	54	0.39	Yuhas *et al.* (2006)
Mexico	1-12	46	0.42	Yuhas *et al.* (2006)
Japan	1-12	51	0.40	Yuhas *et al.* (2006)
China	1-12	50	0.49	Yuhas *et al.* (2006)
Chile	1-12	50	0.42	Yuhas *et al.* (2006)
Canada	1-12	48	0.37	Yuhas *et al.* (2006)
Australia	1-12	48	0.38	Yuhas *et al.* (2006)

B. REGULATORY AND EXPERT GUIDANCE ON INTAKE OF ARA FROM INFANT FORMULA

According to FAO/WHO's guidance (FAO/WHO, 1994), formula for preterm babies should provide a mean of 60 mg of ARA and its associated long chain n-6 fatty acids/kg bwt/day. For term infants, this provision is 40 mg/kg bwt/day of ARA and its associated n-6 fatty acids. To meet these intake levels, ARA should be added to preterm infant formula and term infant formula at levels of 0.90% and 0.72% of total fatty acid, respectively. The conversions are based on the assumptions that human infants consume about 100 (term infants) to 120 (preterm infants) kcal/kg bwt/day, of which fat comprises about 50% (caloric value of fat is 9 kcal per gram).

Following a 1999 National Institutes of Health (NIH) workshop on the essentiality of n-6 and n-3 fatty acids and their Recommended Dietary Intakes (RDI), it was recommended that the

Adequate Intake (AI) of ARA from infant formula be 0.5% of total fatty acids; there was no differentiation between preterm and term infant formulas (Simopoulos *et al.*, 1999). Based on the same assumptions above, that AI corresponds to ARA intakes of 33 and 28 mg/kg bwt/day for preterm and term infants, respectively.

Similarly, the British Nutrition Foundation (1992) recommended fortification of ARA in infant formula at a level of 0.40% of total fatty acids, which corresponds to ARA intakes of 27 and 22 mg/kg bwt/day for preterm and term infants, respectively.

In a safety assessment of ARA- and DHA-rich oils, Food Standards Australia New Zealand (FSANZ) reviewed a number of toxicology studies conducted using ARA-rich and DHA-rich oil sources, administered either singly or in combination. They indicated that the treatment related findings were not accompanied with any associated histopathological, biochemical or hematological changes that would be indicative of toxicity at doses up to 2500 mg ARA-rich oil/kg bwt/day and 1250 mg DHA-rich oil/kg bwt/day. The most frequent changes observed (*e.g.*, increased liver weights, decreased serum cholesterol and triglycerides) were consistent with the physiological changes observed in response to the administration of high levels of LCPUFAs, irrespective of source, and were not concluded to be a manifestation of toxicity specific to the administration of ARA- rich or DHA-rich oils. In addition, a large number of clinical studies with pre-term and term infants were reviewed. A number of the studies examined how well the supplemented formulas were tolerated and whether their use correlated with any adverse effects (*e.g.*, reduced growth, changes in serological markers of spleen and liver function). These studies indicated that formulas supplemented with ARA-rich and DHA-rich oils are well tolerated by human infants and not associated with any apparent adverse effects.

FSANZ (2003) thus concluded that ARA-rich oil and DHA-rich oil were safe sources of ARA and DHA for use in infant formula. A maximum use level of 1.25% of formula fat was noted for both LCPUFA-rich oils, equal to maximum ARA and DHA use levels of 0.50% (both oils are standardized to contain 40% ARA or DHA, respectively) and intakes of 70-85 mg each of ARA- and DHA-rich oil/kg bwt/day.

A summary of the recommendations and expert guidance on safe and appropriate levels of ARA supplementation of infant formula mentioned above is presented in Table 24. Table 25 summarizes the European and U.S. regulatory approvals and opinions concerning ARA supplementation of infant formula (using ARA-rich oils produced by *M. alpina* Peyronel 1S-4 and *M. alpina* ATCC 32222). The ARA percentage range encompassed by those approvals is 0.4% to 1% of total fatty acids.

Table 24. Recommended levels of ARA supplementation and intake of ARA from RAO

	ARA (% total fatty acids)		ARA Intake* (mg/kg bwt/day)		ARA-rich oil (% total fatty acids)		ARA-rich oil intake (mg/kg bwt/day)	
	Preterm	Term	Preterm	Term	Preterm	Term	Preterm	Term
British Nutrition Foundation, 1992	NA	0.40	27	22	--	--	--	--
FAO/WHO 1994	0.90	0.72	60	40	--	--	--	--
Simopoulos *et al.* (1999)**	0.50	0.50	33	28	--	--	--	--
Child Health Foundation, Germany***	NA	≥ 0.35	NA	≥ 20	--	--	--	--
FSANZ 2003	NA	0.50	NA	28	--	--	--	--
RAO	0.40	0.75	27	42	1	1.9	67	104

*Assumptions for the conversion between ARA intake and ARA level: (1) preterm and term infants consume 120 kcal/kg bwt/day and 100 kcal/kg bwt/day, respectively, (2) fatty acids comprise 50% of the available energy in breast milk or infant formula, and (3) one gm of fat contains nine kcal;

**Data from Simopoulos *et al.*, 1999. The workshop was sponsored by the National Institute on Alcohol Abuse and Alcoholism-NIH; the Office of Dietary Supplements-NIH; the Center for Genetics, Nutrition and Health; and the International Society for the Study of Fatty Acids and Lipids; and co-sponsored by several industry groups.

***Koletzko *et al.*, (2001); NA = not available.

Table 25. Regulatory approvals/opinions for ARA supplementation of term (preterm) infant formula

EU and US						
Levels of Use of ARA in Formula for Term (Preterm) Infants						
	Martek FDA GRAS[1] Panel (Term)	**GRN41[2] (Term)**	**GRN80[3] (Term)**	**GRN94[4] Term[Preterm in Hospital/Preterm Post-Discharge]**	**Novel Foods Unit The Netherlands (2005)[5] Term (Preterm)**	**Commission Decision (2008) Commission Decision 2008/968/EC Term (Preterm)**
Percent of total fat content	1.0	0.5	0.75	0.40 [0.40/0.40]	0.40 (0.40)	≤1 (≤1)
Percent of calories	0.5	0.25	0.375	NA	NA	NA
Estimated ARA Intake (mg/kg bwt/day)	60	30	45	26 [32/27]	30 (30)	30 (30)[6]

[1] Generally Recognized as Safe; [2]GRAS notification submitted to FDA for Martek ARA-rich oil; [3]GRAS notification submitted to FDA for Mead Johnson ARA-rich oil; [4]GRAS notification submitted to FDA for Abbot Laboratories (SUNTORY) ARA-rich oil; [5]Abbott (2005) dossier to NFU (2005); [6]EFSA (2008); NA: not available

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C. History of Exposure to ARA in Infant Formulas Supplemented with Fungal Oils Derived from *M. alpina*

The use of long-chain ARA-rich polyunsaturated oils derived by fermentation of the fungus *M. alpina* for supplementation of infant formula has been assessed by various international bodies. Fungal oils have been used in commercially available infant formulas in at least 50 countries since the early 1990s. The information provided in GRN94 for ARA-rich oil (SUNTGA40S) and also in the published Opinion on the novel food authorization for this oil (EFSA 2008) noted that the production of long chain polyunsaturated fatty acids by micro-organisms, including *M. alpina* has been employed for several years. In addition, *M. alpina* is not a known human pathogen and has not been reported to produce mycotoxins.

Similar to the ARA-rich oil that was the subject of review by FDA in GRN41, GRN80, and GRN94, and by EFSA (2008), RAO is produced by *M. alpina* I_{49}-N_{48} by submerged fermentation to yield a triglyceride oil containing approximately 40% ARA. A comparison of the fatty acid profiles for RAO and the ARA-rich oil, SUNTGA40S, that was reported by EFSA (2008) is shown in Table 26.

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Table 26. Constituent fatty acids and respective percentages in nine lots of RAO manufactured over a 28-month period, and those reported in the scientific literature for the ARA-rich oil from EFSA (2008)

	Analysis of nine lots of RAO*		Fatty acid distribution reported for the 3 lots of ARA-rich oil (EFSA 2008)	
	Range (Area %)	Avg. (SD) (Area %)	Range (Area %)	Avg. (SD) (Area %)
Myristic (C14:0)	0.28-0.41	0.34 (0.04)	0.35-0.40	0.37(0.03)
Palmitic (C16:0)	5.87-7.45	6.69 (0.58)	11.5-13.1	12.1(0.87)
Palmitoleic (C16:1n-7)	0.19-0.29	0.22 (0.03)	0.07-0.10	0.09(0.02)
Stearic (C18:0)	5.26-5.89	5.58 (0.25)	7.02-7.88	7.38(0.45)
Oleic (C18:1n-9)	5.48-9.27	7.48 (1.50)	5.41-6.38	5.81(0.51)
Linoleic (C18:2n-6)	4.69-9.81	7.21 (1.66)	8.28-9.85	9.09(0.79)
γ-Linolenic (C18:3n-6)	2.16-2.45	2.33 (0.10)	2.29-2.34	2.32(0.03)
Arachidic (C20:0)	0.80-0.89	0.83 (0.03)	0.76-0.84	0.79(0.04)
Eicosenoic (C20:1n-9)	0.24-0.45	0.34 (0.09)	0.36-0.44	0.40(0.04)
Eicosadienoic (C20:2n-6)	0.41-0.51	0.44 (0.03)	0.66-0.84	0.72(0.10)
Eicosatrienoic (C20:3n-6)	3.54-4.06	3.78 (0.15)	3.86-3.96	3.9(0.05)
Arachidonic (C20:4n-6)	41.81-43.66	42.91 (0.66)	40.3-46.2	43.5(2.98)
Eicosapentaenoic (C20:5n-3)	0.08-0.40	0.15 (0.11)	0.11-0.15	0.13(0.02)
Behenic (C22:0)	3.18-3.42	3.27 (0.10)	2.91-3.07	2.98(0.08)
Lignoceric (C24:0)	9.41-11.98	10.50 (0.82)	8.01-8.36	8.15(0.19)
Hexacosanoic (C26:0)	1.11-1.72	1.40 (0.19)	0.08-0.10	0.09(0.01)

* Reported in Casterton *et al.* (2009); SD = standard deviation

There are four current approvals by regulatory bodies in the US and EU for the addition of ARA-rich oils derived from the fermentation of *M. alpina* to infant formula:

- Suntory Ltd, EC novel food authorization for SUNTGA40S (Commission Decision 2008/968/EC).
- Abbot Laboratories; FDA approval for SUNTGA40S (GRN94; FDA, 2006).
- Martek Biosciences; FDA approval for ARASCO (GRN41, FDA, 2001a).
- Mead Johnson; FDA approval for ARASCO. (GRN80; FDA, 2001b).

In addition, the infant formula use of the fungal oil ARASCO was reviewed by FSANZ, which concluded that ARASCO provides a safe source of LCPUFA for supplementation of infant formula (FSANZ, 2003).

Table 27 summarizes the levels of ARA approved by US and EU regulatory bodies for supplementation of infant formula using various approved ARA-rich fungal oil products

In addition to approval by US and EU regulatory agencies, various expert panels, FSANZ and consensus from scientific workshops have published recommendations for levels of ARA and DHA in infant formula (Table 24). For preterm infant formula, these recommendations are in the ranges of 20-60 mg/kg bwt/day for ARA and 20-40 mg/kg bwt/day for DHA. For term infant formulas, the recommended intakes are 20-40 mg/kg bwt/day for ARA and 40 mg/kg bwt/day for DHA.

Based on scientific consensus and current knowledge regarding the importance of LCPUFA in the infant diet and its presence in human milk, supplementation of infant formula with ARA together with DHA has been recommended by the World Association of Perinatal Medicine, the Early Nutrition Academy, and the Child Health Foundation (Koletzko *et al.*, 2008). In situations where infants are not breast-fed, those organizations collectively recommend that the level of DHA in infant formula be 0.2 to 0.5 weight percent of total fat; with the minimum amount of ARA being equivalent to the DHA content.

Table 27. Maximum approved ARA use levels in infant formulas approved or recommended for term and/or preterm infants

	GRN041	GRN080	GRN094			Commission Decision 2008/968/EC	
	Term	Term	Term	Hospitalized, preterm	Preterm, post-discharge	Term	Preterm
% of total fat	0.5	0.75	0.40	0.40	0.40	≤1	≤1
Estimated intake (mg/kg bwt/day)	30	45	26.3	32.4	27.7	30*	30*
GRN = GRAS Review Notice; *EFSA (2008).							

D. INTENDED USE

The intended use of RAO is as a source of ARA-rich oil for addition to infant formula in order to produce a product whose ARA concentration is consistent with that of human milk. Tables 21 and 23 show that the ARA content in human milk varies from 0.34% to 1.22% of total fatty acids among different populations. Therefore, the proposed use of 0.75% and 0.40% ARA by weight of fatty acids in term and preterm infant formulas, respectively, is within the range of ARA percentages found in human milk. Assuming human infants consume about 100 kcal/kg bwt/day (term infants) to 120 kcal/kg bwt/day (preterm infants), of which fat comprises about 50%, an infant will consume about 5.6 g (term infants) to 6.7 g (preterm infants) of fat/kg bwt/day (1 g fat = 9 kcal). These correspond to intakes of ARA of 42 mg and 27 mg ARA/ kg bwt/day (or 104 and 67 mg of RAO/kg bwt/day) for term infants and preterm infants, respectively. This method is the same as that used by Food Standards Australia New Zealand (FSANZ, 2003).

The supplementation of infant formula with ARA at levels consistent with those in human milk is important because the n-6 and n-3 fatty acids present in human milk have critical roles in membrane structure and as precursors of potent and highly reactive eicosanoids (Mandal *et al.*, 2008). Together, ARA (n-6) and DHA (n-3) are involved in brain development and have been noted to be of particular importance for preterm infants due to an insufficient intra-uterine supply of ARA and DHA and low fat reserves (Fleith and Clandinin, 2005; Heird and Lapillonne, 2005; Eilander *et al.*, 2007; Gibson and Makrides, 2001; Innis, 2007; Hadders-Algra *et al.*, 2007; Koletzko *et al.*, 2008). Although preterm infants are capable of endogenous synthesis of ARA from precursor fatty acids, this capacity appears to be sub-optimal to meet the demands of the developing tissues. Thus, there is a particular benefit to preterm infants who consume a diet containing pre-formed long-chain polyunsaturated fatty acids (LCPUFA) (FSANZ, 2003).

IV. SAFETY

A. HUMAN CLINICAL TRIALS WITH ARA-RICH FUNGAL OILS

1. Summary

FDA was notified of the safety and GRAS status of ARA-rich oils in previous GRAS notifications with closure in 2006 (FDA 2006). Additionaly, in 2006 the EU concluded deliberations on safety of ARA-rich oil. Therefore, the following section provides an update of clinical studies (presented in chronological order) published since this last evaluation of infants exposed to ARA-rich oil supplemented formulas. These studies demonstrate the safety of exposure to ARA-rich oil supplemented infant formulas and corroborate the conclusions reached by the FDA in 2006 as well as by International regulatory authorities (EC, 2006; Commission Decision 2008/968/EC), which support the safe, well-tolerated and nutritional role of ARA supplementation in infant. The studies summarized in this document provide endpoints relevant to the corroboration of safety, including information related to the assessment of safety of ingestion of ARA-rich oils from the perspective of its physiological effects such as nutritional support. Information pertinent to the discussion of safety of exposure, such as evaluation of tolerance, anthropometric measures, clinical chemistry and hematology, adverse events, and drop-outs are included in this section and are summarized in Table 28. Results and summaries of these studies that are relevant to the discussion of physiological effects such as support of sound infant nutrition are provided in Section IV.B.

2. Human clinical trials of exposure to ARA-rich oil from supplementation of infant formula

A double-blind, randomized, comparative study in larger (> 2000 g body weight) preterm infants of gestational ages 30-37 weeks and in full feeding status was conducted to evaluate the effect of 6 months feeding with formula supplemented with ARA and DHA (Fang *et al.*, 2005). Infants were entered into an active six-month intervention where infant formula with ARA (0.10%) and DHA (0.05%) (Neoangelac plus) and without ARA and DHA supplementation (Neoangelac) was consumed. The infants had scheduled physical examinations in which cognitive development, visual activity, and vital signs were evaluated. Observed adverse events,

including vomitus, diarrhea, abdominal distension, skin rash, and allergy were recorded. Adverse events were also recorded in response to an open, standardized question during hospitalization. No serious adverse effects were reported from supplementation of formula during the study period (no details were reported). The physical examination showed that height, weight and head circumference increased steadily during 12 months in both groups, but no significant differences existed between groups by repeated measures ANOVA. No adverse effects of ARA and DHA supplementation of infant formula were noted in this trial of preterm infants.

In a controlled, double-blind randomized study, Groh-Wargo et al. (2005) evaluated growth and body composition of premature infants fed formulas supplemented with ARA and DHA for up to one year of gestation-corrected age (GCA). Preterm infants (750–1800 g birth weight and <33 wks GA) were assigned to one of three formulas within 72 hrs of first enteral feeding. The three formulas included: 1) control, 2) formula supplemented with ARA from fungal oil and DHA from fish oil; 3) formula supplemented with ARA from egg-derived triglyceride and DHA from fish oil. Breast feeding was encouraged before initiating formula feeding and throughout the entire study for infants in each group. When the amount of milk produced by the mother was insufficient to meet the volume and energy needs of the infant, the randomly assigned study formulas was used to make up the shortfall. Infants were categorized as exclusively formula-fed or as receiving mixed formula-breast milk feedings. Exclusive formula feeding was defined as less than a total of 100 mL/kg birth weight of in-hospital breast milk intake and formula for >80% of feedings at GCA. Infants were fed breast milk and/or preterm formulas with or without 0.42% ARA and 0.26% DHA to GCA followed by breast milk or post-discharge preterm formulas with or without 0.42% ARA and 0.26% DHA to12 months GCA. Birth weight, length, and head circumference measurements were obtained from the medical record. In-hospital weights were measured daily, and length and head circumference were measured weekly using standardized procedures. Post-discharge weight, length and head circumference were recorded at 35 and 40 wk GCA and at four- and twelve-month GCA. Dual energy x-ray absorptiometry measurements were made at these same time points to evaluate total body bone mineral content, total body bone mineral density, total body fat and total lean mass. The results of the study showed no significant differences in weight, length, head circumference or bone mineral content and density at any time point among the three study groups. Development of significant clinical complications was noted during the hospital stay; these

included patent ductus arteriosus, bronchopulmonary dysplasia or intraventricular hemorrhage. No differences in the percentage of infants with these conditions were found between the treated and control groups. No infants developed confirmed necrotizing enterocolitis (NEC). There were no significant differences among the three study groups in the longitudinal analyses of weight, length or head circumference. At 12 months GCA, infants who were fed ARA/DHA-supplemented formulas had significantly greater lean body mass and significantly less fat mass than infants fed the unsupplemented control formula. The ARA/DHA-supplemented formulas also supported normal bone mineral content and bone mineral density in premature infants who were born at <33 wks gestation. Based on their findings, the authors concluded that supplementation with ARA and DHA at the levels studied had a beneficial effect on growth and body composition.

To evaluate the safety and benefit of feeding preterm infants formulas containing ARA and DHA, Clandinin et al. (2005) conducted a prospective, randomized, double blinded study of 361 preterm infants (≤ 35 weeks postmenstrual age (PMA)) randomized across three formula groups (1) Control, no supplementation; (2) ARA from fungal oil and DHA from algal oil; and (3) ARA from fungal oil and DHA from fish oil; ARA levels were 34 mg /100 kcal from fungal oil and 17 mg DHA/100 kcal was from algal oil (group 2) or fish oil (group 3). The authors noted that these levels of ARA and DHA are similar to median worldwide amounts reported for mature human milk of approximately 0.3% by weight of fatty acids as DHA and 0.6% as ARA. Each study group was provided with premature, discharge and term ready-to-use formulas, with the only differences being the LCPUFA profiles due to absence of ARA and DHA in control formulas and the different sources of DHA in the supplemented formulas. Study formulas were to be the sole source of nutrition for preterm infants until 57 weeks PMA (4 months after term) and the primary source of nutrition until 92 weeks PMA. Study formula was stopped at 92 weeks PMA. Subjects in the second phase were monitored until 118 weeks PMA (18 months after term). Term infants breast-fed ≥4 months (n=105) were a reference group. Endpoints of the study included growth, tolerance, adverse events, and Bayley development scores (Bayley 1993). Weight, length, and head circumference were measured by standardized procedures at time points up to 118 weeks PMA. For preterm infants, general intake and formula tolerance were recorded daily during hospitalization. Parents provided 24-hour diet and tolerance data at time points up to 57 weeks PMA. Similar data were collected for breast-fed term reference subjects. Blood samples were

collected from preterm subjects at 57 weeks PMA, the age when exclusive formula feeding ended. Analyses included hematology, serum glucose, cholesterol, high-density lipoproteins, triglyceride, mineral and electrolyte measurements, and liver and kidney function tests. Data on morbidity and adverse events were collected. Results indicated that there were no differences among groups in discontinuation rates or distribution of reasons for discontinuation. Analysis of a wide spectrum of clinical data, including serum chemistry and hematology values and incidence and severity of medical conditions related to prematurity found no safety issues related to the supplemented formulas. Mean weight, length and head circumference and mean weight, length and head circumference growth rates in the first phase and at the start of the second phase did not differ among preterm groups. Longer term growth enhancement was associated with ARA and DHA supplementation from fungal and algal sources. The very and extremely low birth weight infants fed formulas with ARA and DHA from the start of enteral feeding up to 12 months after term achieved body weights and lengths comparable to breast-fed term infants by 18 months after term, whereas infants fed unsupplemented formulas or formulas with DHA from fish oil did not achieve similar growth indices.

Birch et al. (2005) evaluated safety and benefit of LCPUFA administration using amounts typical for human milk (ARA 0.72% and DHA 0.36% of total fatty acids in formula) in a double-masked, randomized controlled clinical trial of 103 infants. Infants were randomly assigned to either an ARA/DHA supplemented formula or a control formula. The overall study was composed of two substudies, the visual evoked potential (VEP) substudy and the electroretinogram (ERG) substudy. In the VEP study, infants were evaluated at four time points for growth, VEP acuity, and random dot stereoacuity (RD). In the ERG substudy, infants were evaluated identically except that they underwent an ERG rather VEP at the initial visit. Blood samples were collected from the infants in the VEP substudy at 17 and 39 weeks and from the infants in the ERG substudy at six and 39 weeks of age. For both groups, all anthropometric outcome data were normally distributed. No significant effect of diet was found on weight, length, or head circumference. Both diets were reported to be well tolerated.

Hoffman et al. (2006) conducted a randomized, double-blind, prospective trial to assess the effects of two different formula levels of LCPUFA on blood phospholipid ARA and DHA in term

infants at 120 days of age. Healthy, formula-fed infants (n = 78) were randomized to receive either 1) lower LCPUFA formula: routine milk-based formula with 21 mg ARA, 8 mg DHA, 110 mg alpha-linolenic (ALA), and 1,000 mg linoleic acids (LA) per 100 kcal; n = 39; or 2) higher LCPUFA formula: supplemented milk-based formula with 34 mg ARA, 17 mg DHA, 85 mg ALA, and 860 mg LA per 100 kcal; n = 39. Infants in both groups consumed study formula exclusively until 120 days of age. Body weight, length, and head circumference were recorded at 14, 30, 60, 90, and 120 days of age. Weight, length and head circumference at birth were obtained from birth records. Fatty acid methyl esters from red blood cell (RBC) and plasma phospholipid fractions were assessed using capillary column gas chromatography. Growth rates (body weight, length, and head circumference) were similar in the two groups, although this study was not powered to assess minor differences. Both formulas were well tolerated by parental assessment of stool frequency, stool characteristics, fussiness, and gas. One serious adverse event (SAE) was reported in each group: cows' milk intolerance in one infant in the lower LCPUFA group and acute gastroenteritis in the one infant in the higher LCPUFA group. Number and reason for drop-outs in both groups did not indicate any difference between groups with different LCPUFA dose levels.

The findings from a multi-center, prospective, open-label, 12-month observational study, which assessed the effects of infant formula supplemented with 0.64% ARA and 0.32% DHA compared to control formulas (containing either low ARA + DHA or no ARA + DHA), on the incidence of respiratory illnesses in 1342 term infants was reported by Pastor et al. (2006). Anthropometrics, medical history, and illnesses were evaluated in this study. There were no statistically significant differences between study groups for growth rate (weight, length, and head circumference). There was no difference between groups for dropout rate and reasons for dropout. Tolerance was similar among formula products.

Preterm infants are prone to oxidative stress due to deficient antioxidant defense mechanisms. Free radical generation and oxidative injury contribute to some of the early complications of prematurity including necrotizing enterocolitis. Thus a harmful or protective effect of dietary LCPUFA on oxidative stress in preterm infants is of particular interest. Siahanidou et al. (2007) conducted a prospective, randomized, controlled study in healthy premature infants fed from birth with formulas that differed only in the LCPUFA content (12 mg

ARA and 7.1 mg DHA/100 mL of formula) to determine if LCPUFA have an impact on oxidative stress. Serum malonyldialdehyde (MDA), total peroxide concentrations, and total antioxidant capacity were measured. Anthropometric measurements and clinical characteristics, including erythrocyte transfusions, iron supplementation, days needed for full enteral feeding, caloric intake and growth rates (weight, length, and head circumference) were evaluated. Results showed no difference in clinical and anthropometric characteristics between the two groups. Serum MDA levels did not differ significantly between groups. The concentrations of total peroxides were below the detection limits of the assay in 41 of 50 infants of the control group and 43 of 54 infants of the ARA/DHA group and results were not significantly different between the two infant groups. In addition, no difference was observed in serum total antioxidant capacity between groups. The authors concluded that supplementation of infant formulas with LCPUFA did not affect lipid peroxidation in healthy preterm infants in this study and thus, under these conditions, did not contribute to oxidative stress.

Burks et al. (2008) reported results from two studies comparing the effect on growth in healthy infants of a new amino acid-based formula (AAF) and an extensively hydrolyzed formula (EHF) as a control, both with 0.64% ARA and 0.32% DHA supplementation relative to total fatty acids at levels similar to those in human milk worldwide. In addition, the hypoallergenicity of this new AAF in infants and children with confirmed cow's milk allergy was evaluated. In the first study, a total of 165 healthy, full-term, formula-fed infants randomly received the new AAF or the EHF control formula. Anthropometric measurements, tolerance, and adverse events were recorded throughout the study. Plasma amino acid profiles were evaluated in a subset of the infants. In the second study, the hypoallergenicity of the new AAF was evaluated in 32 infants and children using a double-blind, placebo-controlled food challenge; an open challenge; and a 7-day feeding. Acceptance and tolerance measures and any adverse events were recorded. The results of the first study showed that overall growth (weight, length and head circumference) of infants from both groups were similar. The authors concluded that an isolated difference between the two groups in rate of length increase from 14 to 120 days and achieved length at 120 days was not considered clinically relevant; all other measures were not statistically significantly different because mean length measurements in both groups were between the 50th and 90th percentiles on the CDC reference charts. No difference was noted between groups in the number of subjects who

experienced at least one adverse event. A statistically significant difference in diarrhea reported as an adverse event was detected between the two groups, with diarrhea reported for nine infants in the EHF control group versus no infants in the experimental group. In the second study, acceptance and tolerance of the new AAF was reported as well tolerated. Results from this study demonstrate that the new AAF with ARA and DHA was safe and supported growth and tolerance.

The importance of consuming ARA and DHA on safety and immune function in healthy term infants fed either a standard term formula (n =14) or the same formula supplemented with the 0.34% ARA and 0.20% DHA (w/w as % of total fatty acids) (n=16) was studied by Field et al. (2008). Infant weight, length, and head circumference were measured at study entry (designated as two weeks) and after four weeks of treatment (designated as six weeks) in the study; blood samples from these time points were taken for standard blood chemistry and hematological measures. Peripheral blood collected at these time points was also used to measure immune cell response (the rate of [3H]thymidine uptake and cytokine production after stimulation with phytohaemagglutinin (PHA). Measurement of parameters relevant to the assessment of safety indicted that ARA was well tolerated and supported normal growth. There was no significant difference among diet groups in weight, length, or head circumference at either age two weeks or six weeks. All infants tolerated the diets and there was no difference among groups in the incidence of adverse effects that were reported. Blood chemistry (standard blood chemistry and hematology, details not given) results indicated that all values were within normal ranges for healthy term infants at both time points and did not differ among diet groups.

The effects of ARA/DHA supplementation were reported by Henriksen et al. (2008) for a randomized, double blinded, placebo-controlled study of preterm infants (n=141; birth weights <1500 g). The intervention with 31 mg of ARA and 32 mg of DHA per 100 mL of human milk started one week after birth and lasted until discharge from the hospital (on average, nine weeks). Infants received human milk from the first or second day after birth. As enteral feeding was increased, the milk was fortified with proteins, mineral, vitamins, iron and folic acid. Control infants received a daily dose of 0.5 ml of study oil per 100 ml of human milk. The intervention group received study oil with ARA and DHA. Cognitive development was the primary end point evaluated at six months of age by using the Ages and Stages Questionnaire and event-related

potentials, a measure of brain correlates related to recognition memory. Growth data on infants were obtained from medical charts each week during hospitalization. All adverse events were recorded throughout the study. There was no significant difference in registered adverse outcomes intentionally assessed between groups. No significant differences in energy and nutrient intakes (apart from ARA and DHA) or growth (weight, length, head circumference) were found between the two groups.

Hoffman et al. (2008) conducted a randomized, double blinded, controlled parallel group study in healthy 14-120-day-old infants who consumed either: (1) control, soy-based formula without supplementation or (2) soy-based formula supplemented with at least 34 mg ARA/100 kcal and 17 mg DHA/100 kcal. The objective of this study was to evaluate the safety and benefit of LCPUFA supplementation in the matrix of a soy-based formula compared to a cow's milk-based formula. Anthropometric measurements (body weight and length, head circumference and atopic dermatitis scores) were taken at 14, 30, 60, 90, and 120 days of age and 24-h dietary and tolerance recall were recorded at 30, 60, 90, and 120 days of age. Adverse events were recorded throughout the study. Blood samples were drawn from subsets of 25 infants in each group. Capillary column gas chromatography was used to analyze the percentages of fatty acids in RBC lipids and plasma phospholipids. Growth rates did not differ significantly between feeding groups at any assessed time point. Formula intake, stool frequency, and stool characteristics were similar for infants in both groups. Both formulas were well tolerated as reported by parental assessment of fussiness, diarrhea, and constipation, with the only difference between groups being a higher incidence of excessive gas in the control group than the ARA + DHA group at 60 days of age. Minimal incidence of atopic dermatitis was noted for infants in each study group at all time points. There were no significant differences between groups for total discontinuation rates or for discontinuation due to feeding-related issues. Serum chemistry values for a wide variety of metabolic parameters were similar for infants in both groups at 14 and 120 days of age. Soy-based infant formula supplemented with ARA and DHA supported growth rates and tolerance of term infants as well as commercially available soy-based formulas without these fatty acids.

Using the methodology outlined by the Cochrane Collaboration, Makrides et al. (2005) reviewed all known randomized controlled trials and performed meta-analyses of data from studies

that involved LCPUFA supplementation of infant formula fed to term infants to determine effects on growth. Doses of n-3 LCPUFA in the supplemented formulas ranged from 0.1% to 1.0% of total fatty acids, and the dose of n-6 LCPUFA ranged from 0 to 0.72% of total fatty acids. Sources of LCPUFA varied. Outcome measures were weight, length, and head circumference. Comparison of supplemented formula with control groups for these outcomes was completed at four and twelve months of age; when data were available, comparisons were also done at three, six and nine months of age. Fourteen eligible randomized controlled trials were identified; in three of these, no ARA was included in any of the LCPUFA supplemented formula groups. Original data obtained from the investigators of the published trials were used. Outcomes were analyzed with fixed-effects or random-effects model meta-analyses and were reported as weighted mean differences with 95% confidence intervals. Subgroup analyses assessed whether there were differential effects of supplementation on growth depending on the type and source of LCPUFA and whether there were differential effects of LCPUFA supplementation between boys and girls because of their differing growth patterns. Meta-analysis of results from the 14 eligible trials with high quality data available (1846 infants) showed no significant effect of LCPUFA supplementation on infant weight, length, or head circumference at any assessment age. The source of LCPUFA for supplementation did not significantly affect infant growth. No significant differences in weight, length or head circumference between the groups at either four or twelve months of age in the subgroup analyses was related to sex or sources of LCPUFA. The subgroup analyses; however, must be interpreted with caution because of the comparatively smaller sample sizes and increased number of comparisons. The authors concluded that there was no evidence that LCPUFA supplementation of infant formula had an adverse effect on the infant growth parameters measured.

Simmer et al. (2008a) reviewed fifteen randomized trials to assess the safety and intellectual benefit of feeding formula supplemented with LCPUFA on preterm infants. Trials were identified by MEDLINE (February 2007), Oxford Database of Perinatal Trials, Cochrane Central Register of Controlled Trials (CENTRAL, The Cochrane Library, Issue 1, 2007) and by checking reference lists of relevant articles and conference proceedings. All randomized trials of formula supplemented with LCPUFA and with clinical endpoints (visual development, intellectual development, growth parameters (a measure of safety) were reviewed. Infants enrolled in the trials were relatively mature and healthy preterm infants. Assessment schedule and methodology, dose

and source of supplementation and fatty acid composition of the control formula varied between trials. Of the fifteen trials included in the review, four of these were not classified as of high quality, due to low follow-up, uncertainty regarding concealment of patient allocation and randomization, and problems with assessment methodology. The composition and dose of LCPUFA supplement varied. Of the fifteen studies reviewed, growth was measured in infants at different postnatal ages in thirteen studies. Nine of these included n-3 and n-6 LCPUFA supplementation (most often as ARA plus DHA, however, precursors to these fatty acids were administered in some trials); four evaluated trials using DHA supplementation without supplemented ARA. There was no reported adverse negative effect on growth in studies with n-3 and n-6 LCPUFA supplementation with the exception of one study that reported mild reductions in absolute length and weight at 18 months post-term. Contrary to these results, all other studies of n-3 and n-6 LCPUFA supplementation reviewed showed no adverse effect of supplementation on weight, length or head circumference at different postnatal ages. Four of these studies reported benefits of LCPUFA on growth of supplemented infants at different postnatal ages. A meta-analysis of five studies (one of which included a DHA-only supplementation group) also reported increased weight and length at two months post-term in supplemented infants. There was no evidence that supplementation of formula with n-3 and n-6 LCPUFA impaired the growth of preterm infants.

Simmer et al. (2008b) identified eligible clinical trials to assess the safety and benefit of LCPUFA to term infants. Eligible studies were identified by searching MEDLINE (March 2007), EMBASE 1980 - 2007, Cochrane Central Register of Controlled Trials (The Cochrane Library, Issue 1, 2007) and CINAHL (December 1982 - March 2007). Abstracts of the Society for Pediatric Research were hand searched from 1980 to 2006 inclusive. Reference lists of published narrative and systematic reviews were also reviewed. All randomized and quasi randomized trials comparing LCPUFA supplemented formula milk vs. non-supplemented formula milk and with clinical endpoints were reviewed. Methodological quality of eligible studies was assessed according to allocation concealment, blinding of intervention, blinding of outcome assessment and completeness of follow up. The main outcomes assessed were visual acuity, neurodevelopmental and physical growth. When appropriate, meta-analysis was performed to provide a pooled estimate of effect. Continuous data were analyzed using weighted mean difference. There were no

categorical outcomes in this review. Twenty randomized studies were identified. Fourteen studies were included (n = 1719), six were excluded and eleven of the included studies were considered of good quality. The trial formula in all studies was commenced within the first two week so life. The duration of use of the study formula varied among studies from two months to one year. Twelve of the included studies used supplementation of ARA plus DHA. Two studies compared DHA only enriched formula vs. unenriched formula. Physical growth (weight, length and head circumference) was measured at various ages throughout first three years of life by twelve studies. Some studies reported the actual measurements while some reported the rate of growth over a time period. Some studies used z scores. Irrespective of the type of LCPUFA supplementation, duration of supplementation and method of assessment, none of the individual studies found beneficial or harmful effects of LCPUFA supplementation on growth parameters. Meta-analysis of relevant studies also did not show any effect (beneficial or harmful) of LCPUFA supplementation on growth of term infants.

Rosenfeld et al. (2009) examined the effects of ARA given together with DHA in LCPUFA supplemented infant formula on growth at 18 months. The authors performed a meta-analysis based on individual patient data of 901 children from four large, randomized, controlled intervention trials of term (two trials) and preterm (two trials) infants given infant formula supplemented with both ARA and DHA ARA (range 0.30 to 0.45%) and DHA (range 0.17 to 0.30%). Anthropometrics were assessed by z-scores based on weight for age, length for age, head circumference for age and body mass index (BMI) for age at 18 months. The meta-analysis included data from four major studies that used a similar LCPUFA diet and infant characteristics. Multivariate regression analyses including the possible confounders, sex, gestational age, birth weight, smoking in the last trimester and maternal age, as well as interaction terms showed no significant effects of LCPUFA supplementation on any z-score. The results showed no significant beneficial or adverse effects of ARA and DHA given in LCPUFA supplements on any z-score based on weight for age, length for age, head circumference for age and BMI for age at 18 months. In addition, subgroup analyses of trials with supplement duration of six months or longer also indicated no significant effect on growth.

Table 28. Clinical studies: summary of safety, physical, cognitive and visual effects from nutritional supplementation of infant formulas with ARA and DHA

Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Groh-Wargo *et al.* (2005)	• Control: milk formula; • Test group 1: milk formula supplemented with 0.42% ARA (egg-derived triglyceride and 0.26% DHA (fish oil); (% based on grams/100 grams total fatty acids) • Test group 2: milk formula supplemented with 0.42% ARA (fungal oil) and 0.26% DHA (fish oil); (% based on grams/100 grams total fatty acids)	A controlled, double blind study conducted up to 40-weeks gestational corrected age of infants	Sixty preterm infants with birth weights from 750 to 1800 g and gestational age at birth <33 wk, • Control group (n=22); • Test group 1 (n= 18); • Test group 2 (n=20)	No significant differences were seen among the three groups were seen at any time point in weight, length, or head circumference. Bone mineral content and bone mineral density did not differ among groups. At 12 mo. Term corrected age (TCA) infants who were fed ARA/DHA -supplemented formulas had significantly greater lean body mass and significantly less fat mass than infants who were fed the unsupplemented control formula. The ARA/DHA-supplemented formulas supported normal growth and bone mineralization in premature infants who were born at <33 wk gestation. No differences among the groups were seen in the percentage of infants with adverse clinical complications. At 12 months TCA, preterm infants that were fed the ARA + DHA supplemented formula had increased lean body mass and significantly less fat mass by one year of age than infants fed unsupplemented formula. The authors concluded supplementation of infant formula with ARA and DHA had a beneficial effect on growth and lean body mass.

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Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Fang *et al.* (2005)	• Control group given standard infant formula; • Test group: Standard formula supplemented with 0.10% ARA and 0.05% DHA given 110 kcal/kg per day during the first 4 months and more than 70 kcal/kg per day from 4 to 6 months; (Source of LCPUFA not specified.)	Double blind, randomized comparative study; 6-months	Preterm infants (n=28) Infants >2000 g body weight, over 32 weeks of gestation and in full feeding status selected for the active intervention period	Results showed that mean scores of the Mental Development Index (MDI) for the ARA/DHA supplementation group were better than the group given non-supplemented formula for six months and one year The mean scores for the Physical Development Index (PDI) of the supplemented group were better than the unsupplemented group at six months and at one year. Statistical evaluation of the data showed significant difference between both groups for MDI and PDI. There were no differences in visual acuity, physical examination variables or vital signs between these two groups. Height, weight and head circumference increased progressively during the study period but there were no significant differences between the control and supplemented groups. Although incidences of vomitus, diarrhea, abdominal distension, skin rash and allergy were observed, the authors concluded there were no serious adverse effects in either of the groups during the study period.

Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Clandinin *et al.* (2005)	• Control (un-supplemented infant formula); • Test group 1: Formula plus 34 mg ARA (fungal) + 17 mg DHA (algal)/100 kcal • Test group 2: Formula plus 34 mg ARA(fungal) + 17 mg DHA (algal)/100 kcal Concentrations of ARA (0.6%) and DHA (0.3%) of total fatty acids chosen to be similar to concentrations in human milk.	A prospective, randomized double-blind study; 92 weeks post-menstrual age (PMA) with follow up in second phase at 118 weeks PMA	361 preterm infants ≤ 35 weeks PMA randomized over the control and test groups; Reference Group: term infants (n=105) breast-fed for ≥ four months	Results showed that weight of the infant group given fungal ARA together with algal-DHA was significantly ($p<0.05$) greater than the control group from 66 to 118 weeks PMA but did not differ from infants in the reference group at 118 weeks PMA. Bayley mental (MDI) and psychomotor development (PDI) scores at 118 weeks PMA (18 months after term) were higher in infants given ARA/DHA supplemented formula compared to the control group. Mean weight, length and head circumference and respective growth rates did not differ among the preterm groups. The MDI and PDI scores for the infants in the breast-fed term reference group were near the reference norm and significantly higher than the preterm groups. Analysis of clinical data including severity of medical conditions relating to prematurity, serum chemistry and hematology found no safety issues related to the supplemented formulas. There were no increases in morbidity or adverse events in the groups given supplemented formulas relative to the control.

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Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Bouwstra *et al.* (2005)	• Control group: commercial infant formula; • Test group: LCPUFA supplemented formula 0.45% ARA; 0.30% DHA (Source of LCPUFA and basis of percentage in diet not specified.)	Prospective, double-blind, randomized controlled study with18 month follow up of infants fed supplemented formula for 2 mo.; All formula fed infants were given unsupplemented formula from 2 to 6 months of age	Healthy term infants: • 169 infants given un-supplemented formula • 146 infants given LCPUFA supplemented formula • Reference group: 159 infants (73 breast-fed infants received LCPUFA supplemented formula after breast feeding terminated at 2 months).	No statistically significant differences were seen in clinical neurological condition, neurological optimality score, fluency score and psychomotor and mental development indices. Multivariate analysis confirmed that there was no effect of type of feeding on neurological condition. There were no adverse or beneficial effects on neurological conditions resulting from supplementation with ARA and DHA in observations at 2 months. No associations were found between ARA, DHA or total trans-fatty acids with Psychomotor Developmental Index (PDI) or motor development index (MDI). The authors concluded that a beneficial neurodevelopmental effect of 2 months of LCPUFA supplementation in healthy term infants can not be detected at the age of 18 months.
Morale *et al.* (2005)	• Test group: Infants given formula supplemented with LCPUFA containing 0.72% ARA and 0.36% DHA (Basis of percentages in diet not specified.)	Data from 4 randomized clinical trials conducted at a single research center were combined to determine to determine the relationship between duration of dietary supply of LCPUFA and visual acuity at 52 weeks of age.	Preterm infants (n=243); Reference groups: • Healthy term infants breast-fed for average of 37 weeks; • Healthy term infants breast-fed for a minimum of 52 weeks	Results indicated that a longer duration of LCPUFA supply was associated with significantly better ($r=-0.286$; $p<0.005$) mean acuity at 52 weeks of age as measured by swept visual evoked potentials (sweep VEP). The relationship between duration of dietary LCPUFA supply and sweep VEP acuity at 52 weeks was similar whether the LCPUFA were provided via formula containing 0.72% ARA and 0.36% DHA or human milk. The authors concluded there was a continued benefit from a supply of ARA and DHA from LCPUFA in infants through 52 weeks of age, suggesting that the brain may not have sufficient stores of LCPUFA from an early postnatal supply to support the optimal maturation of the visual cortex.

Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Birch *et al.* (2005)	• Control group: given unsupplemented infant formula • Test group: infant formula supplemented with 0.72% ARA (fungal oil) and 0.36% DHA (algal oil). (Percentages in diet given as % of total fatty acids.) Control and test formulas also provided ~15% linoleic acid and 1.5% α-linolenic acid.	Doubly masked, randomized controlled trial with 39-week duration and follow up until 52 weeks following study initiation.	Term infants (n=103); n=52 in control and n=51 in test group at day 1; at 52 weeks, n=44 in control and n=42 in test group.	Evaluation of sweep visual evoked potential (VEP) acuity in the LCPUFA supplemented group was significantly better than that in the non-supplemented control group at all time points measured ($p<0.001$ to 0.01). Red blood cell concentration of ARA was 15-18% higher in the LCPUFA supplemented group than in the control group. Red blood cell DHA concentrations in the LCPUFA group were 215% higher than in the control group by 39 weeks. Both increases were statistically significant ($p<0.001$ to 0.01). LCPUFA supplementation of term infant formula during the first year of life yielded differences in visual function and total red blood cell lipid composition in this study. For both groups, all anthropometric outcome data were normally distributed. No significant effect of diet was found on growth evaluated by weight, length, or head circumference and both diets were well tolerated.

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Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Pastor *et al.* (2006)	Three control groups fed standard formula with: • a.) No added ARA or DHA; • b.) 6 mg of ARA + 16 mg of DHA/ 100 kcal; • c). 13 mg of ARA + 8 mg of DHA/ 100kcal • Test group: Infants were fed standard formula supplemented with 34 mg ARA (0.64%) and 17 mg of DHA (0.32%)/100 kcal. (Source of LCPUFA not specified.)	Multi-center, prospective, open-label, 12-month observational study.	Term non-breast-fed infants (n=1342)	No statistically significant differences were found between test and control groups for growth rate (weight, length, and head circumference). There was no difference between groups for dropout rate or reasons for dropout. Tolerance was similar among formula products. Weight, length, and head circumference were similar for all groups. Among the infants, a significantly higher incidence of bronchiolitis was observed in control infants at 5 months ($p<0.0001$), 7 months ($p<0.01$), and 9 months ($p<0.01$) fed either non-supplemented formula or with lower levels of ARA/DHA in the first year of life, compared to those in the test group who consumed supplemented infant formula. The authors concluded that ARA/DHA supplementation of infant formula may have a positive impact in reducing respiratory tract diseases such as bronchiolitis/bronchitis, upper airway infection and rhinitis.

Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Agostoni *et al.* (2006)	• Control group: standard phenylalanine-free infant formula; • Test group: standard formula supplemented with ARA (0.7g) and DHA (0.3g)/100g fatty acids (Source of LCPUFA not specified.)	Prospective, double blind, randomized study with intervention until 20-weeks of age and follow up at one year of age.	42 term healthy infants diagnosed by routine neonatal screening to have type I hyper-phenylalaninaemia of normal birth weight (>2500g); completed the study. Control group n=18, test group n=21.	Between entry and 20 weeks of age, median DHA levels in erythrocyte membrane phospholipids decreased by 15% in the LCPUFA supplemented group and by 61% in the control group. At one year, the between-group differences in ARA levels from baseline were insignificant. A dietary supply of LCPUFA in infants with PKU prevented the decline in DHA levels associated with a diet supplying minimal sources of LCPUFA. There was no evidence that either formula had an effect on either cognitive or psychomotor development.

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Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Hoffman *et al.* (2006)	Milk based formula supplemented with ARA/DHA at 2 levels: • Control (Low LCPUFA Group): ARA 21 mg, DHA 8 mg, 110 mg alpha-linolenic acid (ALA) and 1000 mg linoleic acid (LA) /100 kcal; • High LCPUFA Group: ARA 34 mg, DHA 17 mg, ALA 85 mg and LA 860 mg/100 kcal (Source of LCPUFA not specified.)	Randomized, double-blind, prospective trial; 120 days duration	Healthy formula-fed term infants, 38 to 42 weeks gestation and 14 to 120 days of age (n=78), were randomized into each of the low (control) and high LCPUFA supplemented formula groups	Infants in the high-LCPUFA group had significantly ($p < 0.001$) greater percentages of fatty acids as DHA in red blood cell (RBC) phosphatidylethanolamine (PE), RBC phosphatidylcholine (PC), total RBCs, and plasma phospholipids compared to the control group Infants in the control group fed the lower-LCPUFA formula had higher percentages of precursor omega-6 fatty but lower percentages of ARA (RBC PE, RBC PC, and plasma phospholipid ($p < 0.001$); total RBC ($p = 0.017$) compared with the higher-LCPUFA group. Blood ARA content was lower in infants fed the low LCPUFA formula compared to the high LCPUFA formula. The results indicated that conversion of ALA in the low LCPUFA group did not produce as great and increase in DHA in blood lipids as those of the higher LCPUFA formula and did not compensate for lower intakes of preformed ARA and DHA from infant formula. These results suggested that in vivo formation from precursor fatty acids may not provide adequate ARA and DHA for incorporation into neural or retinal tissues in infants Growth rates (body weight, length and head circumference) were similar in the two groups and both formulas were well tolerated by assessments of stool frequency and characteristics as well as amounts of gas. One incident of intolerance to cows' milk was reported in the low LCPUFA group and acute gastroenteritis in one infant in the high LCPUFA group.

Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Sala-Vila *et al.* (2006)	Infant formula containing: • Test group 1: 10% of the fat in formula from egg phospholipids containing 1.9% ARA and 1.25% DHA • Test group 2: 0.47% n-6 fungal oil containing 40% ARA and 0.46% of a high DHA and low EPA fish oil (Analyses of supplemented diets showed concentrations of 0.2% ARA and 0.12% DHA as a % of total fatty acids.)	Comparative study of infants fed test formulas from birth to 3-months	Full term neonates (15 males, 14 females) Reference group: infants fed breast milk (n = 9)	At 3 months, infants on formula milk showed significantly lower ($p<0.05$) concentrations of ARA in phosphatidylcholine and DHA in phosphatidylcholine and in phosphatidylethanolamine compared to infants receiving breast milk. Incorporation of the two fatty acids into erythrocyte phospholipids depends mainly on the lipid composition of the diet received rather than the chemical form in which they are delivered.

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Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Schultz *et al.* (2006)	Infant formula with or without supplementation with ARA and DHA (Concentration and source of LCPUFA in supplemented formula not specified.)	Case-control study using data from parental assessments entered in the Autism Internet Research Survey 4 year cutoff used for comparative analyses	Overall database of 861 children with autistic disorder and 123 control children from 2 to 18 years of age; Subset of database was used in current comparisons of 150 cases and 38 controls of children within range of 2 to 4 years.	When compared to breast feeding for more than six months, feeding with infant formula without ARA/DHA supplementation was associated with a significant increase in the odds of autistic disorder The results of this study indicated that absence of breast feeding, when compared to breast feeding for ≥6 months, was significantly (P=0.022) associated with an increase in the odds of having autistic disorder. In addition, in comparison to infants that received exclusive breast feeding, use of infant formula without ARA and DHA supplementation was associated with a significant increase in the odds of autistic disorder in all cases investigated. The authors concluded that children who were breast-fed or were fed infant formula with ARA/DHA supplementation were less likely to have autistic disorder than children fed non-supplemented formula (non-supplemented groups significantly higher likelihood; p=0.022 compared to breast-fed infants)
Siahanidou *et al.* (2007)	• Control group infants fed non-supplemented formula; • Test group: infants fed formula supplemented with ARA 12.0 mg, and DHA, 7.1 mg, per 100 mL of formula (Source of LCPUFA not specified.)	Prospective, randomized, controlled study with determinations for a mean age (SD) of 34.7 (10.9)	104 healthy preterm infants gestational age, 32.6 weeks; birth weight: 1605 g (averages). Control group n=50; test group n=54.	Clinical and anthropometric characteristics did not differ significantly between the two groups fed LCPUFA containing ARA and DHA. Mean serum MDA levels did not differ significantly between both groups. The concentrations of total peroxides were below the detection limits of the assay and were not significantly different between the two infant groups. No difference was observed in serum total antioxidant capacity between both the groups Supplementation of infant formulas with LCPUFA did not affect lipid peroxidation in healthy preterm infants and, under the conditions in this study, did not contribute to oxidative stress.

Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Jing *et al.* (2007)	• Test group: commercial infant formula supplemented with ARA/DHA (Source and concent-rations of LCPUFA in infant formula not specified.)	Longitudinal comparative study; 6-months duration	Full term healthy infants (n=41) weighed a minimum of 2700 g at birth. Test group consisted of 9 males and 12 females Reference group of 11 males and 9 females were exclusively breast-fed	Results for event-related potentials (ERPs) to two synthesized consonant-vowel stimuli at 3 and 6 months of age in infants indicated that auditory sensory and discrimination responses in breast-fed and LCPUFA supplemented formula-fed infants during the first 6 months of life were similar between the two groups. The neurophysiologic correlates of these processes were similar and are positively correlated with the behavioral scores of Bayley Index of Infant Development. The absence of significant differences in the processing of speech sounds in the groups supports an indication that LCPUFA in infant formula can improve the development of auditory functions.
Carnielli *et al.* (2007)	• Control group: standard infant formula; • Test group: standard formula supplemented with 0.84% ARA (fungal oil) and 0.64% DHA (fish oil) (% based on wt of ARA or DHA/wt of total fat in diet.)	Comparative dietary intervention study conducted as part of a randomized clinical trial. Infants were exclusively fed control and test formulas for 7 months before weaning to local food diets.	Healthy, non-breast-fed, preterm infants (n=22) randomly assigned equally into control (4 males/3 females and test groups (6 males/5 females)	Results showed that endogenous LCPUFA biosynthesis measured by ^{13}C content of major selected plasma fatty acids was higher in infants fed an LCPUFA supplemented formula than in infants given a standard infant formula. Measurement of absolute endogenous LCPUFA synthesis and the percentage of LCPUFA endogenous synthesis relative to dietary intake showed that ARA and DHA decreased significantly ($p<0.01$) over time (1, 3 and 7 months) in both groups compared to values in blood samples taken at birth. ARA and DHA in plasma phospholipids of infants fed the control formula were significantly lower ($p<0.01$) than those in infants fed the formula containing ARA and DHA.

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Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Birch *et al.* (2007)	• Control group infants fed commercial un-supplemented formula; • Test group: Infants fed supplemented formula with 0.72% ARA (fungal oil) and 0.35% DHA (algal oil) (% based on total fatty acid content.)	Randomized trial with follow up study at 4 years of children that had been fed supplemented diets for 17 weeks during infancy	26 term infants in both the control and test groups Reference group: 32 breast-fed infants that had been enrolled in the same 17 week randomized trial	The children were evaluated in a follow-up assessment of visual acuity at 4 years of age. Outcome measures were visual activity (visual evoked potential; VEP) and the Wechsler Preschool and Primary Scale of intelligence-Revised. At 4 years of age, the group that had received the control formula as infants had significantly poorer visual activity ($p<0.03$) and verbal IQ ($p<0.003$) than the children who were breast-fed for an average of 43 weeks or those who had been fed formula containing ARA/DHA during the first 17 weeks of life. The formula supplemented group had visual acuity and verbal IQ scores that did not differ significantly from breast-fed children at 4 years of age. The control formula group (-ARA/DHA) had Verbal IQ scores poorer than the breast-fed group when assessed at 4 years. The result of this trial suggested that ARA/DHA supplementation of infant formula for at least the first 4 months after birth supports visual activity and IQ maturation similar to that of breast-fed infants.
Hoffman *et al.* (2008)	• Control: soy-based infant formula: • Test group: Soy based formula supplemented with 34 mg ARA/100 kcal from fungal oil and 17 mg DHA/100 kcal from algal oil	Randomized, double-blind, controlled parallel group study 120-days	Healthy term infants (n=244) 12-14 days of age. Minimum birth weight of 2,500 g, and solely received formula at least 24 hr prior to randomization.	Infant percentages of ARA and DHA in total RBC and plasma phospholipids were significantly higher (p,0.05) in infants in the ARA + DHA group at 120 days of age when compared to the control group that received unsupplemented formula Growth rates (body weight, length and head circumference) did not differ significantly between feeding groups when assessed at 14, 30, 60, 90 or 120 days of age. Supplementation did not affect the tolerance of formula recorded by parent or the incidence of atopic dermatitis seen in both groups. Formula intake, stool frequency and characteristics were similar in both groups Feeding soy-based formula supplemented with ARA and DHA from single cell oil sources at concentrations similar to human milk significantly increased (p,0.05) circulating blood levels of ARA and DHA when compared with the control group.

Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Burks *et al.* (2008)	• Control amino acid-based formula (AAF) • Extensively hydrolyzed formula (EHF) containing ARA (0.64% of total fatty acids; 34 mg/100 kcal); DHA (0.32%) of total fatty acids; 17 mg/100 kcal)	First study: double-blind, randomized controlled parallel-design prospective study with 120-days duration; Second study: double-blind, placebo-controlled food challenge; an open challenge; and a 7-day feeding	Study 1: 164 healthy, term infants 14 ± 2 days of age in first study on nutritional effects; Study 2: 32 term infants and children (8 months to 2 years of age) with confirmed allergy to cow's milk evaluation of hypoallergenicity of supplemented formula	Results of the first study showed that overall growth, tolerance and safety outcomes were similar in infants receiving supplemented formula and the control group. Rates of weight increase, growth rate of head circumference and achieved body weights were similar in both groups. A significant difference was seen between the two groups only in fussiness (p=0.028) with no differences in the number of subjects who experienced at least one adverse event. In the second hypoallergenicity study, no allergic reactions were seen in any of the 32 subjects The authors concluded that formula supplemented with ARA and DHA at levels similar to those in human milk worldwide is hypoallergenic. It also is safe and supports growth in healthy, term infants.

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Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Henriksen *et al.* (2008)	• Control group received human milk; • Intervention group: received human milk supplemented with 31 mg of ARA 32 mg of DHA/ 100 mL (Source of ARA and DHA oil unspecified but contained 6.9% of each LCPUFA.)	Randomized, double blinded, placebo-controlled study with 6-months (average) duration	Preterm infants (n=141) with birth weights of <1500 g that received human milk from day one or two after birth.	No differences in adverse events or growth between the intervention group that received human milk supplemented with ARA and DHA and the group receiving unsupplemented human milk. At the 6-month follow-up evaluation, the intervention group performed better on the problem-solving subscore, compared with the control group. There was also a nonsignificant higher total score. The event-related potential data revealed that infants in the intervention group had significantly lower responses after the standard image, compared with the control group. There was no significant difference in registered adverse events between groups. No significant differences in energy and nutrient intakes (apart from ARA and DHA) or growth (weight, length, head circumference) were found between the 2 groups. Supplementation with ARA and DHA for preterm infants fed human milk in the early neonatal period was associated with better recognition memory and higher problem-solving scores at 6 months.

Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Field *et al.* (2008)	• Control group: fed standard term infant formula • Test group: fed standard formula supplemented with ARA (fungal) 0.34% and DHA (algal) 0.20%; % based on wt/wt of total fatty acids.	Comparative study of infants fed control, test or reference diets for 2 and 6 weeks	Full term infants exclusively fed commercial formula since birth randomized into control and treatment groups. Control group: n=14; Supplemented formula: n= 16; Reference group: Infants fed human milk from birth; n=16	Compared with cells from infants receiving only human milk (HM), the rate of [^{3}H] thymidine uptake in response to PHA (phytohaemagglutinin), but not IL-2 production, was lower for infants receiving the ARA/DHA supplemented formula ($P < 0.05$). Formula-fed infants (without ARA/DHA) produced more TNF-alpha (unstimulated) and had a fewer CD3+CD44+ cells before stimulation and fewer CD11c+ cells post-stimulation ($P < 0.05$) compared with HM-fed infants. Infants fed the ARA/DHA supplemented formula had an immune cell distribution (higher percentage CD3+CD44+ and CD4+CD28+ cells) and cytokine profile (lower production of TNF-alpha post-stimulation) that did not differ from that found in HM fed infants. There were no significant differences among diet groups in weight, length, or head circumference at either age 2 weeks or 6 weeks indicating that the ARA/DHA supplemented formula was well tolerated and supported normal growth. All infants tolerated the diets and there was no difference among groups in the incidence of minor adverse effects that were reported. Blood chemistry results also indicated that all values were within normal ranges for healthy term infants at both time points and did not differ among diet groups. The authors concluded that early diet influences both the presence of specific cell types and function of infant blood immune cells and that these immune changes may be of physiological importance to the developing infant.

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Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Meta-Analyses of Multiple Studies on Safety and Nutritional Support of Formula Supplemented with ARA and DHA				
Makrides *et al.* (2005)	• Control group received un-supplemented commercial formula; • Test groups in various trials received from 0.1% to 1.0% of n-3 LCPUFA and 0% to 0.72% n-6 LCPUFA in terms of total fatty acids (Sources of LCPUFA not specified.)	Meta-analysis of 14 eligible trials with supplementation and evaluation periods from 3 to 12 months.	A total of 1846 term infants given ARA/DHA supplemented formula for ≥12 weeks in comparison with control groups receiving unsupplemented formula	A total of 14 randomized, placebo controlled intervention trials in term infants were found in the MEDLINE database that met eligibility criteria for meta-analysis of effects of LCPUFA supplemented infant formula compared to formula fed infants. No significant differences in weight, length or head circumference between the groups was observed at either 4 or 12 months of age or in subgroup analyses comparisons of effects of sex or sources of LCPUFA. The authors concluded that there was no evidence that LCPUFA supplementation of infant formula had either a beneficial or adverse effect on the infant growth parameters measured.
Simmer *et al.* (2008a)	Formulas supplemented with n-3 and n-6 LCPUFA from fish and fungus oil; some studies involved supplementation with n-3 fatty acids only.	Meta-analysis of data from 15 randomized trials involved a minimum follow up of 6 weeks from start of supplementation up to evaluations at 52 weeks.	Preterm infants <37 weeks gestation	Results from studies of supplementation of infant formula with LCPUFA typically found no significant differences in any visual parameters except one study that found that visual evoked potential (VEP) was more mature in at six months in a subgroup of infants given supplemented formula. Of seven trials that assessed neurodevelopment, three studies reported modest benefits at different postnatal ages but meta-analyses of Bayley Scales of Infant Development at 12 months (4 studies) or at 18 months (3 studies) showed no significant effect of supplementation on neurodevelopment. Meta-analysis of five studies (four studies with n-3 and n-6 supplement and one study with only n-3 supplement) reported increased weight and length at two months post-term. There was no evidence that supplementation of infant formula impaired growth of preterm infants.

Reference	Test Material and Dose	Type and Duration of the Study	Subjects	Results and Conclusions
Simmer *et al.* (2008b)	Formulas supplemented with LCPUFA from various sources including egg yolk, milk fat, vegetable oils, fish or fungus oil; some studies involved DHA supplementation without ARA	Meta-analysis of data from 14 randomized, controlled trials for various ARA/DHA formula supplementation periods from 2 months to 1 year; At various ages: Visual acuity was measured during first three years of life in nine studies; Neurodevelopmental outcome was measured for up to two years in 11 studies; physical growth measured for up to three years in twelve studies.	A total of 1719 term infants ≥37 weeks gestation at birth. Inclusion criteria required that infants commenced intake of formula within two weeks after birth and formula was the only source of milk from the time of randomization until a minimum of eight weeks of age.	Overall results from studies evaluating effects of ARA/DHA supplementation of infant formula for term infants did not show beneficial effects of LCPUFA supplementation of formula milk on the physical, visual and neurodevelopmental outcomes of infants born at term. One group of researchers found some beneficial effects on visual evoked potential acuity, while 2 groups of researchers have shown some beneficial effect on mental development. Supplementation of infant formula to attain increased mental development was not supported by the current analysis None of the individual studies or results from meta-analysis of relevant studies showed adverse or beneficial effects of LCPUFA supplementation on growth of term infants.
Rosenfeld *et al.* (2009)	LCPUFA containing ARA and DHA	18-months	Data analyzed for 901 children from four large, randomized clinical trials of formula milk with and without LCPUFA.	Multivariate regression analyses including the possible confounders, sex, and gestational age, birth weight, smoking in the last trimester and maternal age, as well as interaction terms showed no significant effects of LCPUFA supplementation on any z-score. Subgroup analyses on trials with high amounts of DHA and on studies with duration of supplementation of at least 6 months each resulted in a lack of LCPUFA supplementation when assessed at 18 months of age. Lack of effects of diet cannot be explained by the lack of statistical power.

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B. PHYSIOLOGICAL EFFECTS

1. Introduction

ARA is a well characterized fatty acid, currently used along with DHA, to supplement infant formulas intended for ingestion by preterm and term infants (Directive 89/389/EEC). ARA and DHA are not considered to be essential fatty acids because linoleic and linolenic acids function as precursors for their synthesis. Studies suggest, however, that ARA and DHA may be conditionally essential in cases when dietary intake becomes an important issue for proper nutrition at early stages of infant development (Birch *et al.*, 2005; Morale *et al.*, 2005).

ARA is a key constituent of all cell membranes in the body and it is especially abundant in neural tissues (Hadders-Algra *et al.*, 2007; Heird and Lapillonne, 2005). It is also a precursor of eicosanoids, which have an important role as structural components of nervous tissue and in cellular signal transfer (Gibson and Makrides, 2001; Heird and Lapillonne, 2005; Eilander *et al.*, 2007) and is also a precursor for prostaglandins, thromboxanes, and leukotrienes, which have important roles in immunological function and other physiologic processes (Mandal *et al.*, 2008).

The importance of sufficient levels of ingestion of ARA and DHA for sound infant nutrition is underscored by the rapid accretion of ARA and DHA in infant brain during early prenatal life (Fleith and Clandinin, 2005). ARA and DHA quickly incorporate in nervous tissue of the retina and brain during the brain's growth spurt, which takes place mainly from the last trimester of pregnancy up to age two (Eilander *et al.*, 2007). The physiologic supply of preformed ARA and DHA to the premature infant may be limited by the early termination of maternal-to-fetal transfer of these fatty acids (Martinez, 1992; O'Connor *et al.* 2001). Endogenous supply of ARA and DHA in the preterm infant may also be limited by an immature synthesis capability from essential precursor fatty acids. This could result in an insufficient supply to allow for normal or optimal LCPUFA accretion in body tissues (Fleith and Clandinin, 2005; Sala-Vila, 2006; O'Connor *et al.*, 2001). While full-term infants synthesize LCPUFA from dietary precursors, as Sala-Vila *et al.* (2006) noted, dietary ARA and DHA intake via either breast milk or supplemented in infant formula may enhance the concentration of these fatty acids in blood, brain or retinal lipids.

Huang *et al.* (2007) noted that early studies using greater intake of ARA and DHA precursors (*i.e.*, ALA or LA) have not proven effective in increasing ARA and DHA levels in infants. In addition, ARA levels are suppressed by high ALA/LA ratios. Circulating levels of both ARA and DHA in breast-fed infants can only be matched with the addition of both of these fatty acids to formula (Koletzko *et al.*, 2008). In addition, supplementation of DHA in infant formulas without supplemental ARA may reduce circulating ARA in preterm and term infants ingesting these formulas (Hoffman *et al.*, 2000) and has been associated in some studies with a negative effect on infant growth (Carlson *et al.*, 1996; Ryan *et al.*, 1999; Koletzko *et al.*, 2008).

Reviews published since 2005 on the role and importance of LCPUFA in preterm and term infant nutrition (Agostoni, 2008; Fleith and Clandinin, 2005; Heird and Lapillonne, 2005; Eilander *et al.*, 2007; Hadders-Algra *et al.*, 2007; Koletzko *et al.*, 2008; Hoffman *et al.*, 2009; Smithers *et al.*, 2009) have highlighted the importance of a diet supplemented with LCPUFA (DHA of at least 0.3% of fatty acids and ARA equal to or greater than the DHA level) as a recommended and sound nutritional practice to ensure a sufficient source of these important fatty acids. In terms of functional endpoints, reviews of recent randomized controlled trials of ARA and DHA supplementation have indicated that evidence for benefits on visual development at <6 months of age in preterm infants is inconclusive, while there are indications for a beneficial effect of supplementation early in life on cognitive development at > 12 months of age.

Non-breast-fed term infants need an exogenous supply of ARA and DHA to achieve similar accretion of fatty acid in plasma and RBC in comparison to breast-fed infants, however, there are inconsistent reports of significant beneficial effects at current recommended levels of supplementation of LCPUFA on visual, mental, or psychomotor functions (Fleith and Clandinin, 2005). In term infants, LCPUFA supplementation at high doses (200 mg ARA and 100 mg DHA/day) provides some evidence for a beneficial effect on visual development during the first year of life, but no evidence for beneficial effects on cognitive development (Eilander *et al.*, 2007). Accretion in plasma and RBC for ARA may not be predictive of change in CNS concentration or function. Studies in prenatal animal models suggest that while DHA concentrations in the CNS are related to levels of dietary supplementation, ARA levels in neural tissues are refractory to ARA in the diet (Diau *et al.*, 2005; Hsieh *et al.*, 2007). This suggests that

ARA concentrations may be more tightly controlled than DHA, a fact that may derive from ARA's role as a precursor of potently bioactive eicosanoids.

Collectively, the body of literature available and also considered in review articles of the data suggests that supplementation of infant formula with LCPUFA may be an important nutritional component in the growth and development of infants. The purpose of supplementing infant formula with ARA and DHA is not for caloric content, but rather for providing dietary levels sufficient to support optimal infant development and health. To achieve this, recommendations on levels of formula supplementation with ARA and DHA are designed to provide infants with ranges of exposure and ratios of inclusion for these LCPUFA that are consistent with human milk levels and demonstrated to be safe by evaluation in animal and clinical trials.

Rapid accretion of LCPUFA takes place in an infant's nervous tissue during pre- and early postnatal life when studies have suggested that infants may not synthesize sufficient amounts of LCPUFA from their precursors to cover growth periods with high demand (O'Connor *et al.* 2001). ARA is a key constituent of all cell membranes in the body and it is especially abundant in neural tissues (Hadders-Algra *et al.*, 2007; Heird and Lapillonne, 2005). It is also a precursor of eicosanoids, which have an important role as structural components of nervous tissue and in cellular signal transfer (Gibson and Makrides, 2001; Heird and Lapillonne, 2005; Eilander *et al.*, 2007) and is also a precursor for prostaglandins, thromboxanes, and leukotrienes, which have important roles in immunological function and other physiologic processes (Mandal *et al.*, 2008). In addition, supplementation of DHA in infant formulas without supplemental ARA may reduce circulating ARA in preterm and term infants ingesting these formulas (Hoffman *et al.*, 2000) and has been associated in some studies with a negative effect on infant growth (Carlson *et al.*, 1996; Ryan *et al.*, 1999; Koletzko *et al.*, 2008).

Supplementation of term infant formula with ARA and DHA on cognitive and visual parameters have shown mixed results in the majority of meta-analyses. However, the effects of ARA/DHA supplementation may be subtle and difficult to discern in tests with infants. Addition of a dietary source of ARA and DHA may support optimal nutritional status of preterm and term

infants. Summaries of the parameters from clinical studies relevant to demonstrating nutrition support of ARA supplementation of infant formula are presented in this section (in chronological order) and in Table 28.

This section summarizes the scientific literature relating to nutritional support from exposure to ARA-rich oils that has been published since 2005. This information corroborates the 2006 conclusions reached by the FDA and International authorities (EC, 2006; Commission Decision 2008/968/EC), which supported the safe, well-tolerated and nutritional role of the ARA-rich oil, SUNTGA40S, in infant formula. RAO is another safe source of ARA for use in infant formulas.

2. Animal Studies

Neonatal animal models (piglet, baboon and rat) have been used to corroborate safety of ARA ingestion, establish tissue levels of ARA resulting from dietary supplementation with ARA-rich oils, and elucidate the role of ARA in physiology of CNS function, gastrointestinal health and immune system function. Summaries of pivotal studies published since 2005 are included below and summarized in Table 29.

ARA and DHA are major components of the cerebral cortex and visual system, playing a critical role in neural development. Although studies have focused on the cerebral cortex, concentrations of these fatty acids in most deep CNS structures, such as the basal ganglia, globus pallidus, caudate nucleus and putamen, may be vital since these structures are involved in a wide array of integrative functions involving motor coordination, integration of visual signals and other personality phenomena. To address the question of LCPUFA concentration in these regions, Diau *et al.* (2005) quantitatively mapped fatty acids in 26 regions of the central nervous system of 4-week-old breast-fed baboons. The objective of this study was to determine the influence of dietary ARA and DHA supplementation and prematurity on the concentration of these LCPUFA in CNS tissues. Baboons were randomized into a breast-fed control group and four formula-fed groups. The four formula fed groups consisted of two groups of neonates that were delivered vaginally after spontaneous labor (term) and two groups delivered approximately 24 days preterm by caesarian section (preterm). One term and one preterm group received

formula with no LCPUFA. The other term and preterm groups received formula fortified with LCPUFA (1.2% ARA and 0.6% DHA in the preterm group; 0.55% ARA and 0.30 % DHA in the term group as % of total dietary fat). All groups were euthanized at 28 days GCA, including the breast-fed group. At study termination, brains were dissected and total lipids were extracted from tissue homogenates. Fatty acid methyl esters were prepared and analyzed by GC-MS for ARA and DHA concentrations expressed as weight percentages of total fatty acids from 14 to 24 carbons. The influence of LCPUFA supplementation on ARA concentrations in the brain was very different from that for DHA. Most CNS regions were not affected by supplementation of ARA at levels twice that in current infant formulas. In addition, the breast-fed (reference) group tended to have the lowest ARA, while the group longest exposed to ARA-free formula tended to have the highest ARA concentrations. DHA concentration, however, increased in all tissues, restoring levels in the supplemented group to levels that were comparable to breast-fed baboon neonates in all regions except the cerebral cortex and cerebellum. Prematurity did not exert a strong influence on ARA or DHA concentrations in CNS tissues. The authors reported the presence of ARA and DHA in high concentration throughout the primate CNS, particularly in gray matter (*e.g.*, basal ganglia). However, results showed that while DHA concentrations dropped across most CNS structures in neonates consuming formulas with no DHA, ARA levels in the CNS were found to be refractory to ARA in the diet. This suggests that ARA concentrations may be more tightly controlled than DHA, a fact that may derive from ARA's role as a precursor of potently bioactive eicosanoids.

Recognizing ARA and DHA as ingredients in infant formula could add potential benefit on measures of visual acuity, and retinal and cognitive function, Hsieh *et al.* (2007) investigated what level of DHA, in combination with ARA, is sufficient in infant formula to normalize cerebral cortex DHA levels in breast-fed baboon neonates. The importance of ARA included in formula with DHA is associated with insuring normal growth, and for this specific outcome, ARA was included at a target level of 0.67% (w/w % of total fatty acids). Fourteen pregnant baboons delivered spontaneously around 182 days of gestation; neonates were transferred to the nursery within 24 hours of birth and randomized to one of three diet groups: control (4 F, 1M; non-LCPUFA-supplemented); moderately supplemented (3F, 1M; 0.67% ARA, 0.33% DHA), and highly supplemented (3F, 2M; 0.67% ARA, 1.00% DHA). The test diets were consumed for

12 weeks after which animals were euthanized and lipid analyses done on liver, heart, RBC and plasma, retina and CNS. No significant effects on LCPUFA administration were noted for anthropometric measures (body, weight, head circumference and crown-rump length). Relative organ weights of brain, liver, thymus, spleen heart, lungs, right kidney and pancreas were not significantly different in treated vs. control animals. In liver, RBC and plasma, ARA rises significantly in the moderately and highly supplemented groups (except or liver which was significantly different only in the moderately supplemented group). No changes in ARA concentration were found in the cerebral cortex, retina, putamen, caudate and amygdala. ARA concentration was reduced compared with control in the superior colliculus and globus pallidus in the highly supplemented and moderately supplemented groups, respectively. Tissue DHA concentrations were significantly increased in liver, heart, plasma, RBC and the CNS regions. Result showed that cerebral cortex DHA was increased with higher levels of DHA than are currently present in commercial infant formulas. These data are consistent with results from other studies (Diau, 2005) suggesting that tissue ARA concentrations, particularly in the CNS are more refractory to formula ARA than DHA.

The dietary concentrations of ARA and DHA needed to enhance tissue levels were evaluated by Huang *et al.* (2007) in order to assess impact of fortification levels in infant formula on LCPUFA accretion. A randomized block design was used. Twenty-four male piglets of normal gestation were randomly assigned to four different formulas and bottle-fed for 28 days. Doses of ARA and DHA used were multiples of those used in U.S. infant formulas (1x, 2x, and 5x the amount in infant formulas, corresponding to ARA/DHA of 8.1/4.1, 16.2/8.1, and 40.6/20.3 mg/100 kJ formula, respectively). The mean ARA content of the supplemented formulas in g/100 g total fatty acids was 0.63, 1.29 and 3.15, respectively. ARA was increased in liver, adipose tissue, plasma, and erythrocytes (*i.e.*, 1.1 to 6.0-fold higher than controls), brain and retinal ARA were unresponsive to dietary ARA. DHA results showed increased DHA in brain, retina, liver, adipose tissue, plasma, and erythrocytes by 1.1- to 25.8-fold relative to controls. Plasma and erythrocyte DHA were positively associated with DHA in neural (brain and retina) and visceral (liver and adipose) tissues. The authors concluded that (1) dietary ARA/DHA supplementation affords an effective strategy for enhancing tissue DHA, and (2) erythrocyte and plasma DHA can be used as proxies for prediction of tissue DHA changes. On the other hand,

plasma and erythrocyte ARA did not correlate with ARA in neural tissue, brain and retina; they were only significantly correlated with peripheral tissues such as liver and adipose. Because ARA concentrations were increased from dietary ARA in visceral but not neural tissues, plasma and erythrocyte ARA was not considered an indicator of ARA in neural tissues, brain or retina. Because ARA serves as an immediate substrate for bioactive eicosanoids, ARA levels may be highly regulated in neural-related tissues. Thus, elevation of blood ARA in response to dietary supplementation may be only transient due to the role of blood as a nutrient transport medium, however, neural tissue concentrations remain constant.

To gather mechanistic information on the role of ARA and DHA in the primate cerebral cortex, Kothapalli *et al.* (2007) investigated global gene expression from cerebral cortex of infant baboons consuming two levels of LCPUFA that are higher than typically found in infant formulas but within normal levels in human breast milk. Twelve neonatal baboons born spontaneously around 182 days gestation were randomized into three groups (n=4 per group, gender not given) for twelve weeks, consuming either a control diet, one supplemented with 0.67% ARA and 0.33% DHA, or one supplemented with 0.67% ARA and 1.00% DHA; ARA and DHA levels were all as % total fatty acids in the infant formula diets. Animals were euthanized at 84.4 ± 1.1 days. Tissue collected from the precentral gyrus of the cerebral cortex was processed for microarray analysis and validation of microarray results. Several novel differentially-expressed transcripts in the cerebral cortex were modulated by dietary LCPUFA. The majority of probe sets showed subtle changes in gene transcription. Among effects noted in comparison to control were: increased expression of the mitochondrial proton carrier, UCP2; differential expression of PLA2G6, implicated in childhood neurodegeneration; and increased expression of several genes involved in neural development and visual perception. Results demonstrated that LCPUFA supplementation of infant formula within the normal range of human milk induced changes in gene expression across an array of processes in neonatal baboons.

The question of the effect of dietary ARA and DHA on the composition of bone mineral, including calcium, phosphorus, magnesium, and zinc during infancy was investigated by Mollard and Weiler (2006) who examined the effect of ARA and DHA supplementation on calcium

absorption, femur mineral composition and urinary mineral excretion in piglets. Three groups of five-day-old male piglets (10/group) were fed formula for 15 days supplemented with ARA and DHA at four ratios (0.5:0.1 g, 1.0:0.2 g, or 2.0:0.4 ARA:DHA g/100 g of fat) or a control formula. Dietary ARA:DHA did not alter femur growth or mineral content when added in amounts (0.5:0.1 g/100 g fat) similar to that observed in human milk. Higher amounts of ARA:DHA at 1.0:0.2 g/100 g fat , but not at ARA:DHA of 2.0:0.4 g/100 g fat, produced significantly elevated levels of calcium and reduced zinc, but had no effect on magnesium or phosphorus in the femur. No significant effects on calcium absorption or urinary mineral excretion were seen. Thus, ARA:DHA supplementation in this animal model did not affect mineral accretion.

Non-human primates provide valuable models to study the effects of dietary LCPUFA on human health and have been used to examine LCPUFA consumption on central nervous system development and retinal function (Diau *et al.*, 2005). While reference ranges for biochemistry and white cell parameters have been published for baboon neonates, effects of dietary LCPUFA on white cell and clinical chemistry data during the first weeks of the postnatal period had not been established; therefore, Hsieh *et al.* (2008) determined the effect of formulas with moderate and high dietary LCPUFA on selected biochemical parameters and white blood cell profiles of baboon neonates to corroborate the safety of ingestion of these fatty acids. Fourteen baboon neonates (10 F, 4 M), delivered spontaneously around 182 days gestation were randomized within 24 hours of birth to control (no ARA/DHA) or test formulas (0.67% ARA/0.33% DHA (w/w%); and 0.67% ARA/1.00% DHA (w/w % of total fatty acids) during the first 12-weeks of life. Blood chemistries were assessed at six and twelve weeks and complete blood counts were performed at Weeks 2, 4, 8, 10, 12 of age. Results indicated that serum triglyceride levels were significantly lower for both groups of LCPUFA fed neonates compared with controls. Calcium was significantly higher in the low DHA test group compared with controls and the high DHA test group. No other significant effects of LCPUFA administration were detected in other clinical chemistry parameter or in any of the white cell parameters measured. All clinical chemistry parameters were within published ranges for baboons of this age group. Results from this study support a lack of adverse effects from ARA/DHA on clinical chemistry or white cell parameters.

Optimal nutritional support for the preterm infant is a challenge, complicated by several factors, including the underdeveloped state of the digestive system. Enteral nutrition is needed to promote health and development of the intestine; however, enteral nutrition is a risk factor for the development of NEC (Hess *et al.*, 2008). ARA-derived prostaglandins stimulate recovery of gut barrier function after injury. Because ARA status may be impaired in preterm infants, Hess *et al.* (2008) evaluated whether supplementation with supraphysiological levels of ARA (up to 5% of total fatty acids) was safe and would result in increased ARA concentration in mucosal phospholipids in a neonatal animal model. One-day-old piglets (n = 6 per treatment per day; gender not stated) were fed a milk-based formula for 4, 8, or 16 days after which there were euthanized and tissues collected for mucosal histology, enzymology, and fatty acid analysis. Five feeding groups included: control (no LCPUFA); 0.5% ARA; 2.5% ARA; 5% ARA, or; 5% eicosapentaenoic acid (EPA); all percentages were of total fatty acids (w/w). A sow milk reference control was included. Piglets on all treatments grew rapidly and daily body weight gains were unaffected by dietary content of LCPUFA. Clinical hematology measurements were unaffected by treatment. Small intestinal morphology (ileal villus height and area) did not differ among tested groups. Fatty acid analysis of intestinal mucosa showed a dose-response relationship between dietary ARA concentration and ARA content of mucosal phospholipids, with an enrichment plateau at eight days of feeding. By Day 8, compared to controls, ARA concentrations in jejunal mucosa were enriched by 47, 272, and 428%, at levels of ARA supplementation of 0.5, 2.5 and 5%, respectively. Maximal degree of enrichment in the ileum exceeded that in the jejunum. Congruent with the concentration of ARA measured in sow milk, ARA enrichment of mucosal phospholipid from the 0.5% ARA formula-fed pigs was similar to that of the sow milk controls. Thus, elevated levels of dietary ARA are well tolerated in a neonatal piglet model and produce an increase in ARA content of intestinal mucosal phospholipids.

A clinical trial in preterm infants given formula with egg phospholipids (Carlson *et al.*, 1998) indicated a reduction of the incidence of necrotizing enterocolitis (NEC; a common acute gastrointestinal disease in human neonates). Other work (Fewtrell *et al.*, 2002) did not report this same finding. The mechanism by which LCPUFA might reduce the incidence of NEC was investigated by Lu *et al.* (2007) using a neonatal rat model. Neonatal rats were randomly

assigned to three treatment groups and one control group immediately after cesarean section. Controls received formula without LCPUFA supplementation. Treated rats were given formula supplemented with: ARA + DHA; egg phospholipids; or only DHA (at a level of 0.7% ARA and/or 0.5% DHA as a percentage of total fatty acids). Experiments were carried out for 72 hours or until clinical signs of NEC occurred. Incidence of NEC was compared among LCPUFA-supplemented groups and the control. Compared to the control group, LCPUFA supplementation reduced the incidence of NEC and inhibited intestinal platelet activating factor receptor (PAFR) and toll-like receptor 4 (TLR4) gene expression. (PAFR and TLR4 are both important receptors in the pathogenesis of epithelial cell NEC). These *in vivo* observations were corroborated using IEC-6 cells exposed *in vitro* to platelet activating factor (PAF) (PAF has been implicated in the development of NEC, acting through PAFR to transduce signaling to downstream cellular targets) after pretreatment with ARA or DHA by the finding that both LCPUFA blocked PAF-induced TLR4 and PAFR mRNA expression. The authors concluded that LCPUFA modulates gene expression of key factors involved in experimental NEC pathogenesis and that this effect could in part explain the protective effect of LCPUFA on neonatal NEC.

Supplementation of infant formulas with ARA and DHA favorably modulates the development of the nervous system, the retina, the auditory system and the digestive system in some animal and human trials; preliminary data also suggest a benefit on immune system development and function in the neonatal period (Bassaganya-Riera *et al.*, 2007). An investigation of the immune modulating effects of ARA and DHA when fed in combination with infant formulas ingredients to neonatal piglets following their exposure to an inactivated influenza virus vaccine was reported by Bassaganya-Riera *et al.* (2007). Neonatal piglets were weaned on Day 2 of age and distributed into 16 groups of three littermates each (designed on the basis of litter of origin, maternal concentration of antiinfluenza virus titers, initial body weight, age, and sex (data on these factors not reported). Within each group, piglets were randomly assigned to a 30-day dietary consumption of either a control infant formula, sow's milk, or an ARA/DHA-enriched formula. On Day 9, eight blocks of piglets were immunized with an inactivated influenza virus vaccine. On study Days 0, 9, 16, 23 and 30 after weaning, influenza virus-specific T-cell proliferation and phenotype of T-cell subsets in peripheral blood were measured. On study Day 28, a delayed-type hypersensitivity (DTH) reaction test using an

intradermal injection of influenza virus antigen or PBS as the negative control was administered. Injection sites were examined at 0 and 24 hours for erythema, edema, and induration. Cytokine messenger RNA expression, determined by quantitative real time reverse transcriptase-polymerase chain reaction, was done on Day 30. Animals were euthanized at Day 30 and tissues were taken for immunohistochemical evaluation of mucosal secretory IgA, RNA isolation and gene expression analyses and histologic analyses. Results showed that influenza virus-specific $CD4^+$ and $CD8^+$ T-cell *ex vivo* lymphoproliferative responses were significantly lower on Day 23 after vaccination in piglets receiving dietary ARA/DHA supplementation or sow's milk compared to piglets consuming the control formula. Thus, it appeared that the ARA/DHA enriched formula and sow milk may have suppressed influenza virus-specific T-cell responses on Day 23 after feeding. On Day 28, the DTH test was administered to measure influenza virus-specific cell-mediated immune responses *in vivo*. There was no significant increase in DTH values in vaccinated piglets fed control formula compared with the other treatment groups; thus results from cell-mediated immunity are consistent with the lower values seen in the *ex vivo* T-cell lymphoproliferative responses to influenza virus stimulation. Proliferation and effector functions of T-cells are controlled through both cell contact-dependent and soluble cytokine-dependent mechanisms. In this study, cytokine mRNA expression (*i.e.,* interleukin-10) was significantly greater in piglets fed the ARA/DHA enriched formulas or sow milk than control formula. Thus, ARA/DHA effects of antigen-specific T-cell responses may be modulated through an interleukin 10-dependent mechanism. Upon necropsy, no adverse effects associated with ARA/DHA consumption were observed with respect to formula intake, body weights, or organ weights.

Table 29. Animal studies demonstrating nutritional support from ARA/DHA supplements in the diet

Reference	Animal Model	Test Substance/Dose (% of total fatty acids)	Duration of Study	Results
Diau *et al.* (2005)	Term and preterm baboons	1. Control groups: term and preterm baboons fed commercial non-supplemented infant formula; 2. Test group 1: term baboons fed formula supplemented with 0.55% ARA and 0.30% DHA; 3. Test group 2: preterm baboons fed formula supplemented with 1.21% ARA and 0.61% DHA; Reference group: term baboons breast-fed since birth. (Source of LCPUFA not specified; % of ARA and DHA relative total dietary energy content.)	28 days (adjusted to time of normal term birth)	Dietary supplementation with a mixture of ARA/DHA increased DHA in the CNS but had little influence on ARA concentrations. Serum triglycerides were significantly ($p<0.05$) depressed in both test groups compared to controls but no other significant effects were observed. The results indicated: 1) ARA and DHA are found in high concentration throughout the primate central nervous system (CNS) particularly in gray matter such as basal ganglia; and 2) ARA levels in most CNS regions are not affected by ARA in the diet up to twice that added to current infant formulas; 3) DHA levels increased in all CNS regions examined in neonatal baboons fed supplemented diet. The findings suggested that ARA concentrations in the CNS are more tightly controlled than DHA levels which may be associated with the role of ARA as a precursor of potently bioactive eicosanoids.

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Reference	Animal Model	Test Substance/Dose (% of total fatty acids)	Duration of Study	Results
Hsieh *et al.* (2007)	Neonate term baboons	4. Control group: non-supplemented commercial infant formula; 5. Test group 1: Infant formula supplemented with 0.67% ARA and 0.33% ARA; 6. Test group 2: Infant formula supplemented with 0.67% ARA and 1.00% DHA (Source of LCPUFA not specified; concentrations based on wt/wt mg/100 kcal.)	12 weeks	No significant effects on LCPUFA administration were noted for anthropometric measures (body, weight, head circumference and crown-rump length). Relative organ weights of brain, liver, thymus, spleen heart, lungs, right kidney and pancreas were not significantly different in treated vs. control animals. In liver, RBC and plasma, ARA rose significantly ($p<0.05$) only in the moderately and highly supplemented groups relative to the control (except for liver which was significantly different only in the moderately supplemented group). No changes in ARA concentration were found in the cerebral cortex, retina, putamen, caudate and amygdala. DHA concentrations were significantly increased ($p<0.05$) in liver, heart, plasma, RBC and the CNS regions and DHA in cerebral cortex increased in the group with higher levels of DHA.

Reference	Animal Model	Test Substance/Dose (% of total fatty acids)	Duration of Study	Results
Huang *et al.* (2007)	Male piglets of normal gestational age	• Control: unsupplemented infant formula; • Test group 1: infant formula supplemented with 0.63% ARA and 0.34% DHA; • Test group 2: infant formula supplemented with 1.29% ARA and 0.70% DHA; • Test group 3: infant formula supplemented with 3.15% ARA and 1.66% DHA (Percentages based on amount of ARA or DHA/100 kJ derived from fungal and algal sources, respectively.)	28 days	Results showed increased DHA in brain, retina, liver, adipose tissue, plasma, and erythrocytes by 1.1- to 25.8-fold relative to controls. Although ARA was increased in liver, adipose tissue, plasma, and erythrocytes (*i.e.*, 1.1 to 6.0-fold higher than controls), brain and retinal ARA were refractive to dietary ARA. Plasma and erythrocyte DHA were positively associated with DHA in neural (brain and retina) and visceral (liver and adipose) tissues. However, plasma and erythrocyte ARA did not correlate with ARA in neural tissue, brain and retina; they were only significantly correlated with peripheral tissues such as liver and adipose. Because ARA concentrations were increased from dietary ARA in visceral but not neural tissues, plasma and erythrocyte ARA were not considered an indicator of ARA in neural tissues, brain or retina. Thus, elevation of blood ARA in response to dietary supplementation may be only transient due to the role of blood as a nutrient transport medium, however, neural tissue concentrations remain constant.

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Reference	Animal Model	Test Substance/Dose (% of total fatty acids)	Duration of Study	Results
Kothapalli *et al.* (2007)	Neonate term baboons	• Control: baboon neonates fed commercial non-supplemented infant formula • Test group 1: baboon neonates fed formula supplemented with 0.67%ARA; and 0.33% DHA (wt./wt.% of total fatty acids.) • Test group 2: baboon neonates fed formula supplemented with 0.67% ARA and 1.00% DHA (wt./wt.% of total fatty acids.) (Source of LCPUFA not specified.)	12 weeks	Tissue collected from the precentral gyrus of the cerebral cortex showed several novel differentially-expressed transcripts in the cerebral cortex were modulated by dietary LCPUFA. At 12 weeks of age, subtle changes in gene expression were detected in 1,108 of 54,000 probe sets, with most showing, 2-fold change. Increased expression of the mitochondrial proton carrier, UCP2; differential expression of PLA2G6, implicated in childhood neurodegeneration; and increased expression of several genes involved in neural development and visual perception were observed. Results demonstrated that LCPUFA supplementation of infant formula within the normal range of human milk induced changes in gene expression across an array of processes in neonatal baboons.

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Reference	Animal Model	Test Substance/Dose (% of total fatty acids)	Duration of Study	Results
Mollard and Weiler (2006)	Male piglets	• Control diet: unsupplemented infant formula; • Test group 1: formula supplemented with 0.5% ARA and 0.10% DHA; • Test group 2: formula supplemented with 1.0% ARA and 0.2% DHA; • Test group 3: formula supplemented with 2.0% ARA and 0.4% DHA. ARA was derived from fungal oil and DHA was from algal oil; percentages based on g of ARA or DHA/100 g of total fatty acids.	15 days	Dietary ARA/DHA did not alter femur growth or mineral content when added in amounts (0.5:0.1 g/100 g fat) similar to that observed in human milk. Higher amounts of ARA:DHA at 1.0:0.2 g/100 g fat , but not at ARA:DHA of 2.0:0.4 g/100 g fat, significantly elevated calcium ($p<0.05$) and reduced zinc, but had no effect on magnesium or phosphorus in the femur. No significant effects on calcium absorption or urinary mineral excretion were seen and ARA:DHA supplementation in this animal model did not affect mineral accretion.

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Reference	Animal Model	Test Substance/Dose (% of total fatty acids)	Duration of Study	Results
Hsieh *et al.* (2008)	Baboon term neonates	• Control: baboon neonates fed commercial non-supplemented infant formula • Test group 1:baboon neonates fed formula supplemented with 0.67%ARA; and 0.33% DHA • Test group 2: baboon neonates fed formula supplemented with 0.67% ARA and 1.00% DHA (Source of LCPUFA and basis of dietary percentages not specified.)	12 weeks	Blood chemistry results at 6 and 12 weeks showed that serum triglyceride levels were significantly lower ($p<0.05$) for both groups of LCPUFA fed neonates compared with controls. Calcium was significantly higher ($p<0.05$) in the low DHA test group compared with controls and the high DHA test group. However, all clinical chemistry parameters were within published ranges for baboons of this age group. No other significant effects of LCPUFA administration were detected in other clinical chemistry parameter or in any of the white cell parameters measured in total blood counts performed at 2, 4, 8, 10, 12 weeks. Results from this study support a lack of adverse effects from ARA/DHA on clinical chemistry or blood white cell parameters.
Lu *et al.* (2007)	Neonatal Sprague-Dawley rats	• Control group: non-supplemented commercial infant formula; • Test group 1: ARA (fungal oil) 0.69% and DHA(algal oil) 0.54%; • Test group 2: ARA 0.71% and DHA 0.54% (from egg phospholipid); • Test group 3: 0.50% DHA (algal oil) and 0% ARA (ARA/DHA percentages based on total fatty acids.)	48 to 72 hr until clinical signs occurred.	Compared to controls, LCPUFA supplementation reduced the incidence of necrotizing colitis (NEC) and inhibited intestinal platelet activating factor receptor (PAFR) and toll-like receptor 4 (TLR4) gene expression. (PAFR and TLR4 are both important receptors in the pathogenesis of epithelial cell NEC.) The authors concluded that LCPUFA modulates gene expression of key factors involved in experimental NEC pathogenesis and that this effect could in part explain the protective effect of LCPUFA on neonatal NEC.

Reference	Animal Model	Test Substance/Dose (% of total fatty acids)	Duration of Study	Results
Hess *et al.* (2008)	Neonatal suckling piglets	• Control group: liquid diet not supplemented with LCPUFA; • Test group 1: 0.50% ARA; • Test group 2: 2.5% ARA; • Test group 3: 5.0% ARA; • Test group 4: 5.0% EPA (eicosapentaenoic acid); and • Reference group: sow milk (ARA was derived from fungal oil; source of EPA not specified; all percentages were based on total fatty acids; w/w%.)	16 days	Piglets on all treatments grew rapidly and daily body weight gains were unaffected by dietary content of LCPUFA. Clinical hematology measurements were unaffected by treatment. Small intestinal morphology (ileal villus height and area) did not differ among tested groups. Fatty acid analysis of intestinal mucosa showed a dose-response relationship between dietary ARA concentration and ARA content of mucosal phospholipids, with an enrichment plateau at 8 days of feeding. By Day 8, compared to controls, ARA concentrations in jejunal mucosa were enriched by 47, 272, and 428%, at levels of ARA supplementation of 0.5, 2.5 and 5%, respectively. Maximal degree of enrichment in the ileum exceeded that in the jejunum. Congruent with the concentration of ARA measured in sow milk, ARA enrichment of mucosal phospholipid from the 0.5% ARA formula-fed pigs was similar to that of the sow milk controls. Thus, elevated levels of dietary ARA are well tolerated in a neonatal piglet model and produce an increase in ARA content of intestinal mucosal phospholipids.

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Reference	Animal Model	Test Substance/Dose (% of total fatty acids)	Duration of Study	Results
Bassaganya-Riera *et al.* (2007)	Neonatal piglets	• Control: unsupplemented infant diet formula; • Test group: infant formula supplemented with 0.63% ARA (fungal oil) and 0.34% DHA (algal oil). Reference group: sow milk (Dietary percentages based in terms of total fatty acids.)	30 days	Influenza virus-specific $CD4^+$ and $CD8^+$ T- cell *ex vivo* lymphoproliferative responses were significantly lower on day 23 after influenza virus vaccination of piglets receiving dietary ARA/DHA supplementation or sow's milk compared to piglets consuming the control formula. On day 28, a delayed type hypersensitivity (DTH) test showed no significant increase in DTH values in vaccinated piglets fed control formula compared with the other treatment groups. Results from cell-mediated immunity were consistent with the results of the ex vivo T cell lymphoproliferative responses to influenza virus stimulation. Cytokine mRNA expression (ie, interleukin-10) was significantly greater ($p<0.05$) in piglets fed the ARA/DHA enriched formulas or sow milk than control formula. Thus, ARA/DHA modulated antigen-specific T cell responses may be modulated through an interleukin 10-dependent mechanism. No adverse effects associated with ARA/DHA consumption were observed with respect to formula intake, body weights, or organ weights.

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3. Studies in Human Infants

a) Introduction

Rapid accretion of LCPUFA takes place in an infant's nervous tissue during pre- and early postnatal life when studies have suggested that infants may not synthesize sufficient amounts of LCPUFA from their precursors to cover growth periods with high demand (O'Connor *et al.* 2001). ARA is a key constituent of all cell membranes in the body and it is especially abundant in neural tissues (Hadders-Algra *et al.*, 2007; Heird and Lapillonne, 2005). It is also a precursor of eicosanoids, which have an important role as structural components of nervous tissue and in cellular signal transfer (Gibson and Makrides, 2001; Heird and Lapillonne, 2005; Eilander *et al.*, 2007) and is also a precursor for prostaglandins, thromboxanes, and leukotrienes, which have important roles in immunological function and other physiologic processes (Mandal *et al.*, 2008). In addition, supplementation of DHA in infant formulas without supplemental ARA may reduce circulating ARA in preterm and term infants ingesting these formulas (Hoffman *et al.*, 2000) and has been associated in some studies with a negative effect on infant growth (Carlson *et al.*, 1996; Ryan *et al.*, 1999; Koletzko *et al.*, 2008).

Effects of supplementation of term infant formula with ARA and DHA on cognitive and visual parameters have shown mixed results in the majority of meta-analyses. However, the effects of ARA/DHA supplementation may be subtle and difficult to discern in tests with infants. Addition of a dietary source of ARA and DHA may support the optimal nutritional status of preterm and term infants. Summaries of the parameters from clinical studies relevant to demonstrating safety and nutritional support of ARA supplementation of infant formula are presented in this section (in chronological order) and summarized in Table 28.

b) Clinical study summaries

Several human clinical studies that were summarized earlier to provide information on selected safety endpoints after infant exposure to ARA-rich oil (Section IV.A.2.) also included endpoints that support the nutritional sufficiency of ARA-supplementation of infant formula. Those studies include Fang *et al.* (2005), Groh-Wargo *et al.* (2005), Clandinin *et al.* (2005), Birch *et al.* (2005), Hoffman *et al.* (2006), Pastor *et al.* (2006), Siahanidou *et al.* (2007), Burks *et al.* (2008), Field *et al.* (2008), Henriksen *et al.* (2008), Hoffman *et al.* (2008),

Makrides *et al.* (2005), Simmer *et al.* (2008a,b), and Rosenfeld *et al.* (2009). The details of the methodology from these studies is found in Section IV.A.2 and will not be repeated in this section.

A double-blind, randomized, comparative study in larger (> 2000 g body weight) preterm infants of gestational ages 30-37 weeks and in full feeding status was conducted to evaluate the effect of 6 months feeding with formula supplemented with ARA and DHA (Fang *et al.*, 2005). At four and six months after study entry, visual acuity analysis was performed using steady-state visual evoked potentials (VEP) and preferential looking tests with Lea grating acuity cards and Hiding Heidi low contrast "FACE" cards. A cognitive examination was done using Bayley Mental Development Index (MDI) and Physical Development Index (PDI) scores at 6 months and 1 year after entering the study. Supplementation of formula with ARA and DHA resulted in a beneficial effect on neurodevelopment as determined in this study by an improvement in the scores of the PDI and MDI (by repeated measures ANOVA), however, the study period was short and the case number was small. There was no difference in visual acuity in supplemented infants in comparison to control infants.

Clandinin *et al.* (2005) conducted a prospective, randomized, double blinded study of 361 preterm infants (≤ 35 weeks postmenstrual age (PMA)) randomized across three formula groups (1) Control, no supplementation; (2) ARA from fungal oil and algal-DHA (DHA from algal oil,); and (3) ARA from fungal oil and fish-DHA (DHA from fish oil). Term infants breast-fed ≥4 months (n=105) were a reference group. General development was assessed using the Bayley Scales of Infant Development II MDI and PDI, administered to all infants at 118 weeks PMA (18 months after term). Mean Bayley MDI and PDI scores of the breast-fed term infants at 118 weeks PMA (18 months after term) were near the reference norm of 100 and statistically significantly higher than those for any preterm group. The ARA-DHA supplemented formula groups had higher MDI and PDI scores than did the control (unsupplemented) group, thus suggesting better developmental outcomes.

Bouwstra *et al.* (2005) evaluated potential effects of supplementation of commercial infant diets with 0.45% ARA plus 0.30% DHA on neurological developmental

outcome at 18 months in a prospective, double-blind, randomized control study of 474 healthy term infants who were either breast-fed (n=159), given commercial diet formula (control; n=169) or ARA/DHA supplemented formula for two months (n=146). The groups of infants evaluated had previously demonstrated a beneficial effect of LCPUFA intake at three months of age. At the age of 18 months, neurodevelopmental condition was assessed by the age-specific neurological examination of Hempel and the Bayley scales. The groups did not show statistically significant differences in clinical neurological condition, neurological optimality score, fluency score, and the psychomotor and mental development indices. Multivariate analysis confirmed that there was no effect of type of feeding on neurological condition. The authors concluded that subtle effects of both breast feeding and ARA/DHA supplementation may be difficult to detect at 18 months of age; no adverse effects of supplementation were reported.

Morale *et al.* (2005) determined the relationship between duration of dietary supply of LCPUFA (contained in infant formula at levels of 0.72% ARA and 0.36% DHA) and visual acuity at 52 weeks of age. Data from 243 healthy term infants who participated in four randomized clinical trials of LCPUFA supplementation of infant formula at a single research center were combined. The primary outcome was visual acuity at 52 weeks of age as measured by swept sweep VEP. Data from two reference groups of breast-fed healthy term infants who were tested concurrently with the clinical trials were included. Taken together, the four randomized clinical trials and two breast-fed reference groups yielded 12 groups of infants with varying source and durations of LCPUFA dietary supply. Results indicated that a longer duration of LCPUFA supply was associated with better mean acuity at 52 weeks of age. The relationship between duration of dietary LCPUFA supply and sweep VEP acuity at 52 weeks was similar whether the LCPUFA were provided via formula containing 0.72% ARA and 0.36% DHA or human milk. Duration of breast feeding was associated with individual infants' sweep VEP acuity outcomes at 52 weeks ($r=-0.286$; $p<0.005$). The duration of LCPUFA supply during infancy has a similar relationship to sweep VEP acuity at 52 weeks in breast-fed infants regardless of birth order. The authors concluded there was a continued benefit from a supply of ARA and DHA from LCPUFA in infants through 52 weeks of age, suggesting that the brain may not have sufficient stores of LCPUFA from an early postnatal supply to support the optimal maturation of the visual cortex.

Birch *et al.* (2005) evaluated LCPUFA administration using amounts typical for human milk (ARA 0.72% and DHA 0.36% of total fatty acids) in a double-masked, randomized controlled clinical trial of 103 infants by using sweep VEP acuity as the primary outcome. VEP acuity in the LCPUFA supplemented group was significantly better than that in the non-supplemented control group at all time points measured. Red blood cell concentration of ARA was 15-18% higher in the LCPUFA supplemented group than in the control group. Red blood cell DHA concentrations in the LCPUFA group were 215% higher than in the control group by 39 weeks. Both increases were statistically significant. LCPUFA supplementation of term infant formula during the first year of life yielded differences in visual function and total red blood cell lipid composition in this study.

Hoffman *et al.* (2006) conducted a randomized, double-blind, prospective trial to assess the effects of two different formula levels of LCPUFA on blood phospholipid ARA and DHA in term infants at 120 days of age. The study evaluated the effects of different levels of ARA, ALA, DHA and LA in two commercially available infant formulas on the percentage of DHA and other LCPUFA in infant RBCs and plasma phospholipids. Compared with infants fed a lower LCPUFA formula (21 mg ARA, 8 mg DHA, 110 mg alpha-linolenic (ALA), and 1,000 mg linoleic acids (LA) per 100 kcal), the those who consumed formula with a higher level of LCPUFA (34 mg ARA, 17 mg DHA, 85 mg ALA, and 860 mg LA per 100 kcal) had significantly greater percentages of fatty acids as DHA in RBC phosphatidylethanolamine, RBC phophatidylcholine, total RBC and plasma phospholipids. Results showed that greater amounts of dietary ALA do not produce as great an increase in DHA in blood lipids as reformed dietary DHA. In effect, even though elongation and desaturation of dietary ALA was occurring in infants fed the lower LCPUFA formula, the conversion was not adequate to yield blood DHA percentages comparable with those of higher LCPUFA formula. A similar response to LCPUFA supplementation was observed with omega-6 fatty acids. Compared with infants in the higher LCPUFA formula group, infants receiving lower LCPUFA formula had higher percentages of LA and ARA precursors 18:3n6 and 20:3n6 in all four blood lipid fractions. However, blood ARA content was lower in infants fed the lower LCPUFA formula compared to the higher LCPUFA formula. These results indicated that conversion of precursors does not compensate for lower intakes of preformed ARA and DHA from the infant formula. This suggests that *in vivo*

formation from precursor fatty acids may not provide adequate ARA and DHA for incorporation into neural or retinal tissues.

Although Autistic Disorder (AD) is associated with several congenital conditions, the cause for most cases is unknown. A case-control study using data from the Autism Internet Research Survey, (an online parental survey conducted from February to April 2005 with results for 861 children with 150 cases and 38 controls in the age range of ≥2yrs and ≤4 yrs with AD), was used to investigate whether breast feeding or the use of infant formula supplemented with ARA and DHA is protective for AD (Schultz *et al.*, 2006). Four years was chosen as the cutoff for the study since ARA supplementation in infant formula would not have been available in the US for older children at the time of the study was conducted. The results of this study indicated that absence of breast feeding, when compared to breast feeding for ≥6 months, was significantly associated with an increase in the odds of having autistic disorder. In addition, in comparison to infants that received exclusive breast feeding, use of infant formula without ARA and DHA supplementation was associated with a significant increase in the odds of autistic disorder in all cases investigated. The authors concluded that children who were not breast-fed or were fed infant formula without ARA/DHA supplementation were significantly more likely to have autistic disorder. Children with AD were also significantly less likely to have been breast-fed or receive formula supplemented with ARA/DHA than children who developed normally. This study relied on self-reported data regarding the diagnosis of AD, was not a random sampling of the population and was based on a small group of children.

The findings from a multi-center, prospective, open-label, 12-month observational study, which assessed the effects of infant formula supplemented with 0.64% ARA and 0.32% DHA/100 kcal compared with control formulas (containing either low levels of ARA + DHA or no ARA + DHA), on the incidence of respiratory illnesses in 1342 term infants were reported by Pastor *et al.* (2006). Among the infants, a significantly higher incidence of bronchiolitis was observed at five, seven and nine months in control infants fed either non-supplemented formula or with lower levels of ARA/DHA in the first year of life, compared to those who consumed supplemented infant formula. The authors concluded that ARA/DHA supplementation of infant

formula may have a positive impact in reducing respiratory tract diseases such as bronchiolitis/bronchitis, upper airway infection and rhinitis.

Sala-Vila *et al.* (2006) investigated whether the chemical form of LCPUFA affects their incorporation into erythrocyte membrane phospholipids. In this study, full term infants were fed breast milk or supplemented infant formula containing ARA together with DHA derived from egg phospholipids or from low eicosapentaenoic acid fish oil and fungal triglycerides from birth to three months of age. Blood samples were collected from each infant at birth (cord blood) and at three months. The fatty acid composition of erythrocyte phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, before and after administration of the experimental diet, was quantified. At three months, infants given supplemented formula showed lower concentrations of DHA (in phosphatidylcholine and in phosphatidylethanolamine) and ARA (in phosphatidylcholine) than infants receiving breast milk. The incorporation patterns of ARA into membrane phospholipids differed. There was a decrease in phophatidylcholine and maintenance in phophatidylethanolamine, suggesting that ARA accrues mainly in phosphatidylcholine in the membranes of developing tissues. The concentrations of ARA in the erythrocyte phophatidylcholine of all formula-fed infants (irrespective of source) were significantly lower than those in the human milk group. Because breast milk had higher levels of ARA than those in the formula used in this study, the authors proposed that the incorporation of the ARA into erythrocyte phospholipids depended mainly on the lipid concentration of the diet received rather than the chemical form in which it was delivered. Similar conclusions were reached regarding DHA incorporation into membrane phospholipids.

Forty-two infants with classical phenylketonuria (PKU) entered a prospective, double-blind, randomized study to investigate the effects on biochemical and physiological outcomes of a phenylalanine-free infant formula containing ARA and DHA (Agostoni *et al.*, 2006). Infants with PKU, who were otherwise healthy and of normal birth weight were randomized to either control (standard phenylalanine-free infant formula) or test formula (phenylalanine-free infant formula with added ARA and DHA); infants began feeding with the formula to which they had been randomized at entry, which was within days of their diagnosis.

Infants were followed until they reached one year of age or upon discontinuation of the formula. Between entry and 20 weeks of age, median DHA levels in erythrocyte membrane phospholipids decreased by 15% in the LCPUFA supplemented group and by 61% in the control group. At 20 weeks of age, median ARA levels had also decreased from baseline by 21% in the control group, remaining almost unchanged in the supplemented group. At one year, there was no difference in the between-group decline in ARA levels from baseline. There was no evidence that formula assignment had an effect on either the cognitive or psychomotor development of the children. A dietary supply of LCPUFA in infants with PKU prevents the decline in these fatty acid levels in comparison to a diet supplying minimal sources of LCPUFA.

In order to investigate the effect of ARA and DHA on long term visual and cognitive outcomes, Birch *et al.* (2007) reported outcomes from four-year-old children who, for the first 17 weeks of their lives, were assigned to one of three diets in a single-center, double-blind randomized clinical trial (conducted 2003-2005). Study diets included formula supplemented with only DHA, formula supplemented with ARA plus DHA, or no supplementation. In addition, a group of breast-fed infants who had been concurrently enrolled in the study served as a reference standard. Fifty-two of 79 healthy term infants on the study diets who participated in the 2003-2005 clinical trial were available for follow-up in this study. Twenty six of the children had consumed unsupplemented infant formula and the other 26 consumed the same formula, supplemented with 0.72% ARA and 0.35% DHA (% total fatty acids). In addition, 32 of 40 breast-fed infants from the reference group of this clinical trial were available for comparison. The children were evaluated for the outcome measures of visual activity (visual evoked potential; VEP) and the Wechsler Preschool and Primary Scale of intelligence-Revised. At four years of age, the children who had consumed the control formula as infants had poorer visual activity than those who were breast-fed for an average of 43 weeks or those who had been fed formula containing ARA/DHA during the first 17 weeks of life. The formula-supplemented group had visual acuity and verbal IQ scores that did not differ significantly from breast-fed children at four years of age. The control formula group had poorer verbal IQ scores than the breast-fed group when assessed at four years. The result of this trial suggested that ARA/DHA supplementation of infant formula for at least the first four months after birth supports visual activity and IQ maturation similar to that of breast-fed infants.

Infants participating in a longitudinal study investigating dietary effects on the development of psychophysiological function were selected to evaluate the effect of LCPUFA-supplemented formula. Outcomes assessed included auditory sensory and discrimination responses (Jing *et al.*, 2007). In this study, 41 healthy full term infants were fed breast milk or milk formula supplemented with ARA/DHA and effects on speech processing were evaluated by recording event-related potentials (ERPs) to two synthesized consonant-vowel stimuli at three and six months of age. ERP recording is a noninvasive method to detect neural responses to speech sounds in infants and is believed to reflect neural activities at different brain levels. Behavioral assessment was also obtained. The results of this study showed similar auditory sensory and discrimination responses in breast-fed infants and those fed LCPUFA-supplemented formula during the first six months of life. The neurophysiologic correlates of these processes were similar and correlated positively with the behavioral scores of Bayley Index of Infant Development. Therefore, the absence of significant differences in the processing of speech sounds between breast-fed and LCPUFA-supplemented infants supports an indication that LCPUFA in infant formula may contribute to auditory function development.

Carnielli *et al.* (2007) quantified endogenous LCPUFA synthesis by preterm infants in the Netherlands who had been fed infant formula containing ARA and DHA. Twenty-two preterm infants were randomly assigned to either the group with no supplementary LCPUFA or an LCPUFA group. The [^{13}C] content of major selected plasma fatty acids was measured by using gas chromatography –isotope ratio mass spectrometry of plasma samples collected at birth and at age one, three and seven months. Dietary LCPUFA groups had higher [^{13}C] content than did the endogenously synthesized LCPUFA derived from linolenic and α-linolenic acids. Absolute endogenous LCPUFA synthesis and the percentage of LCPUFA endogenous synthesis relative to dietary intake were calculated. Endogenous synthesis (expressed as percentage and absolute value) of ARA and DHA decreased significantly over time in both groups. ARA and DHA in plasma phospholipids of infants fed the no-LCPUFA formula were significantly lower than those in infants fed the formula containing ARA and DHA.

Neonatal immune development is influenced throughout gestation by maternal immunity as well as after birth through consumption of breast milk. Human milk contains

quantities of LCPUFA as well as their precursors and animal studies have demonstrated that these LCPUFA modulate T cell functions such as the ability to proliferate and produce cytokines in response to mitogen or antigen stimulation (Field *et al.*, 2008). The importance of consuming ARA and DHA on immune function in healthy term infants fed either a standard term formula (n =14) or the same formula supplemented with the 0.34% ARA and 0.20% DHA (w/w% of total fatty acids; n=16) was studied by Field *et al.* (2008) (as discussed previously in Section 5.3.2). Peripheral blood collected at study entry and after four weeks of treatment was used to measure immune cell response (the rate of [^{3}H]thymidine uptake and cytokine production after stimulation with phytohaemagglutinin (PHA). Blood samples from a group of 16 infants that had been exclusively fed human milk since birth were used for comparison of immune cell distributions. Compared with cells from infants receiving only human milk (HM), the rate of [^{3}H] thymidine uptake in response to PHA, but not IL-2 production, was lower for formula +ARA/DHA supplement fed infants ($P < 0.05$). Compared with HM-fed infants, formula-fed infants (without added ARA/DHA) produced more TNF-alpha (unstimulated) and had a fewer CD3+CD44+ cells before stimulation and fewer CD11c+ cells post-stimulation ($P < 0.05$). However, the formula + ARA/DHA fed infants had an immune cell distribution (higher percentage CD3+CD44+ and CD4+CD28+ cells) and cytokine profile (lower production of TNF-alpha post-stimulation) that did not differ from that found in HM fed infants. These results indicated that commercial formula supplemented with ARA + DHA produced a immune cell profile in infants that was more similar to that in infants fed human milk than in those fed unsupplemented formula.

Burks *et al.* (2008) reported results from two studies comparing the effect on growth in healthy infants of a new amino acid-based formula (AAF) and an extensively hydrolyzed formula (EHF), both with ARA and DHA supplementation at levels similar to those in human milk worldwide. In addition, the hypoallergenicity of this new AAF in infants and children with confirmed cow's milk allergy was evaluated. The first study demonstrated that this formula was safe and supported growth in healthy, term infants. In the second study, 29 of the 32 subjects completed both challenges and no allergic reaction was seen in any of the 32 subjects. The results provided 95% confidence that at least 90% of infants and children allergic to cow's

milk would have no reaction to the new AAF. The authors concluded that the new AAF with ARA and DHA at levels similar to those in human milk worldwide is hypoallergenic.

Hoffman *et al.* (2008) conducted a randomized, double blinded, controlled parallel group study in healthy term infants from 14 to 120 days of age that received either: (1) control, soy-based formula without supplementation or (2) soy-based formula supplemented with at least 34 mg ARA/100 kcal and 17 mg DHA/100 kcal. Compared with the control group, percentages of the fatty acids ARA and DHA in total RBC and plasma phospholipids were significantly higher in infants in the ARA + DHA group at 120 days of age. Results suggest that feeding healthy term infants soy-based formula supplemented with and ARA DHA from single cell oil sources at concentrations similar to human milk significantly increased circulating levels of ARA and DHA when compared with the control group. Soy-based infant formula supplemented with ARA and DHA increased ARA and DHA status similar to milk-based formulas with similar levels of added ARA and DHA.

Simmer *et al.* (2008a) reviewed fifteen randomized trials to assess the safety and benefit of feeding formula supplemented with LCPUFA on preterm infants. Benefit was assessed by measures of visual acuity and intellectual development. Visual acuity over the first year was measured by Teller or Lea acuity cards in eight studies, by VEP in six studies and by ERG in two studies. Of the fifteen studies reviewed, ten reported results on visual acuity; three of these trials were of DHA only supplementation. Most studies found no significant differences in any visual assessment between supplemented and control infants (one study with n-3 and n-6 LCPUFA supplementation noted that VEP acuity was more mature at six months post-term in a subgroup of supplemented infants). Seven of the trials reviewed assessed neurodevelopment (three trials used DHA only supplementation); most of these trials used Bayley Scales of Infant Development (BSID) at 12 to 24 months post-term. Three out of seven studies (two using n-3 and n-6 LCPUFA supplementation and one with DHA-only supplementation) reported some benefit of LCPUFA in different populations of supplemented infants at different postnatal ages. Meta-analysis of BSID of four studies at 12 months and three studies at 18 months post-term showed no significant effect of supplementation on neurodevelopment. Two studies using DHA only supplementation demonstrated lower novelty preferences (possibly predictive of lower

intelligence) in the supplemented compared with the control group. In summary, no clear long-term benefit on neurodevelopment was demonstrated in the clinical trials reviewed or by pooling the data from the clinical trials. Study authors noted, however, that overall, methodology varied considerably between studies, making a summary of the combined data difficult. Some of the disparity between findings may have been due to different combinations of LCPUFA in the supplement and different concentrations of essential fatty acids in the control formula

The effects of ARA/DHA supplementation were reported by Henriksen *et al.* (2008) for a randomized, double blinded, placebo-controlled study of preterm infants (n=141; birth weights <1500 g). Supplementation led to increased plasma concentrations of ARA and DHA in the early neonatal period. At the 6-month follow-up evaluation, the intervention (ARA/DHA) group performed better on the problem-solving sub score, compared with the control group. There was no difference in responses to novel images among the groups. The authors concluded that supplementation with ARA/DHA for preterm infants fed human milk in the early neonatal period was associated with better recognition memory and higher problem-solving scores at 6 months.

Simmer *et al.* (2008b) identified fourteen randomized trials to assess the safety and benefit of feeding formula supplemented with LCPUFA on term infants. The main outcomes to assess benefit were visual acuity and neurodevelopmental growth. Visual acuity was measured at various stages throughout the first three years of life by nine studies. Visual evoked potential was used to assess visual acuity in five studies. The remaining four studies used Teller visual acuity cards. The results were inconsistent. Three studies reported beneficial effect of LCPUFA supplementation on visual acuity while the remaining six did not. Neurodevelopmental outcome was measured at different ages throughout the first two years by eleven studies. Bayley scales of infant development (BSID) were used in eight studies. Only one showed a beneficial effect of LCPUFA supplementation on BSID scales. Pooled meta-analysis of the data also did not show any statistically significant benefit of LCPUFA supplementation on either mental or psychomotor developmental index of BSID. One study reported better novelty preference measured by Fagan Infant test at nine months in supplemented infants compared with controls. Another study reported better problem solving at 10 months for infants given ARA/DHA

supplementation. One study used Brunet and Lezine developmental test to assess the developmental quotient and did not find beneficial effects of LCPUFA supplementation. The authors concluded that results of most of the well conducted RCTS have not shown beneficial effects of LCPUFA supplementation of formula milk on the visual or neurodevelopmental outcomes of infants born at term. Only one group of researchers has shown some beneficial effects on VEP acuity, while 2 groups of researchers have shown some beneficial effect on mental development. Supplementation of infant formula to attain increased mental development was not supported by the current analysis.

4. Summary of Physiological Effects

Although infant dietary requirements for lipids have traditionally been considered in terms of energy metabolism, various studies have demonstrated that some lipids, particularly LCPUFA also play an important role in the brain, especially during development. Supplementation of infant formula with ARA and DHA on cognitive and visual parameters has shown mixed results with some studies showing a positive effect on visual and mental development. However, the effects of ARA/DHA supplementation may be subtle and difficult to discern in tests with infants. Differences in study design may also contribute to these disparate findings; this includes differences in amount of LCPUFA supplementation, functional outcomes and sample size.

Neural and retinal tissues are rich in ARA and DHA and these fatty acids are important in developing brain and visual cortex of infants during a rapid stage of development. ARA is a key constituent of all cell membranes in the body and it is especially abundant in neural tissues (Hadders-Algra *et al.*, 2007; Heird and Lapillonne, 2005). It is also a precursor of eicosanoids, which have an important role as structural components of nervous tissue and in cellular signal transfer (Gibson and Makrides, 2001; Heird and Lapillonne, 2005; Eilander *et al.*, 2007) and is a precursor for prostaglandins, thromboxanes, and leukotrienes, which have important roles in immunological function and other physiologic processes (Mandal *et al.*, 2008). ARA and DHA supplementation of infant formula has been shown to be well tolerated by infants, to support normal growth and development, and to have nutritional benefits.

C. TOXICOLOGY STUDIES

1. Introduction

Numerous animal safety studies have been conducted over a period of more than a decade on ARA-rich oils derived from *M. alpina*. Submissions of these data along with production process and final product specification information and corroborative information from studies of growth and tolerance in infant populations have been reviewed by scientific agencies and regulatory bodies in the EU, Australia/New Zealand and the United States. The conclusion reached is that these commercially available ARA-rich oils, meeting appropriate food grade specifications, provide a safe ARA source for addition to infant formula. These sources of refined ARA-rich oil have been the subject of animal and *in vitro* studies similar to those conducted for RAO, which is intended for addition to preterm and term infant formula resulting in an ARA level of 0.4% and 0.75% of total fat content, respectively. This level of infant formula supplementation is GRAS (GRN41, GRN80, GRN94) in the US (FDA, 2001a, 2001b, 2006) and has also been authorized by the EC (authorized by the European Commission under EC 258/97 (Commission Decision 2008/968/EC)). A critical part of the safety evaluation is bridging new ingredient information to existing information on chemically similar/essentially equivalent ingredients identified in the published literature. Thus, safety studies reported in this section confirm that RAO's safety profile matches that of other refined ARA-rich oils reported in the scientific literature.

The following section summarizes the safety studies of RAO sponsored by Cargill and includes three genotoxicity assays and a 90-day rat feeding study with an *in utero* exposure phase. As described previously, RAO is chemically similar/essentially equivalent to other commercially available ARA-rich oils. Results from the toxicology studies of RAO as summarized below are consistent with those from toxicology studies on other ARA-rich oils. This information corroborates the safety of RAO, another safe and well-tolerated source of ARA-rich oil.[7]

[7] Summaries of recent publications on safety of ARA-rich oils published by Lina *et al.* (2006) and Nisha *et al.* (2009) are included in section 8.2.

2. Summary of safety studies of RAO

The safety of a refined ARA-rich oil (RAO) was evaluated for reverse mutation, chromosome aberration and gene mutation, and in a 90-day Wistar rat feeding study with *in utero* exposure. The results of the genotoxicity assays were all negative. The *in utero* phase of the 90-day study involved dietary exposure to 0.5%, 1.5% and 5% RAO and two controls diets, a standard feed low-fat diet and a high-fat diet supplemented with 5% corn oil. This exposure covered four weeks prior to mating, through mating, gestation and lactation until offspring (F_1) weaning. A subsequent 90-day feeding study in the F_1 rats evaluated the same test and control diets. Statistically significant effects were seen for selected histopathology, clinical chemistry and organ weight endpoints; however, other than increased absolute and relative monocytes seen in both sexes of high-dose rats, the observations were not attributed to treatment for one or more reasons. Results were consistent with observations seen in previous subchronic feeding studies of rats given other ARA-rich oils. At the highest dose of RAO administered in the subchronic study, approximately 3,000 mg/kg bwt/day, no adverse effects attributed to consumption of the ARA-rich oil were exhibited; this represents a daily RAO intake that is approximately 29-fold greater than the anticipated intake of 42 mg ARA/kg bwt/day (104 mg of RAO/kg bwt/day) for term infants and 45-fold greater than the intake of 27 mg ARA/kg bwt/day (67 mg of RAO/kg bwt/day) for preterm infants.

3. Safety studies of RAO

Potential genotoxicity of RAO was investigated *via* (1) a bacterial reverse mutation assay in *Salmonella typhimurium* (Tester strains TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (Tester strain WP2*uvr*A), (2) a gene mutation study at the TK locus in mouse lymphoma cells, and (3) a chromosome aberration test in CHO cells. A 90-day feeding study with preceding *in utero* exposure was conducted following the same protocol and using the same strain of Wistar rat reported in the studies of a refined ARA-rich oils published by Hempenius *et al.* (2000) and Lina *et al.* (2006). In the present study, parent (F_0) animals were fed test or control diets over 11-12 weeks, starting four weeks prior to mating, throughout mating, gestation and lactation until weaning of the F_1 offspring. A 90-day feeding study, which complied with OECD testing guidelines (OECD, 1998a) and EEC Directive 2001/59/EEC (EC, 2001) followed in the

F_1 rats. All studies were conducted in accordance with Good Laboratory Practice (GLP) standards (OECD, 1998b).

RAO samples 60403385-1 through 60403385-35 of lot 060331, manufactured as described in section 2.0, were used in all studies. The constituent fatty acids and their respective percentages in the test substance are presented in Table 30. For comparison purposes, included in Table 30 are the ranges of fatty acid distribution measured in nine separate lots of RAO, including lot 060331, mean values and standard deviations associated with those ranges, and the fatty acid distributions reported in the scientific literature for other refined ARA-rich oils that are currently both Generally Recognized as Safe and Authorized under European Directives as novel foods for addition to infant formula.

RAO contained 43.31% ARA (C20:4n-6), 0.27% eicosapentaenoic acid (EPA; C20:5n-3) and no DHA (C22:6n-3). The test material was stored in a freezer (<-18° C) under nitrogen. The reference substance (corn oil), lot numbers 406617M and 407368M, was obtained from Oliehoorn, Zwaag, The Netherlands and stored in a refrigerator (2–10° C).

4. Genotoxicity

The potential genotoxicity of RAO was evaluated *via* three different genotoxicity tests: (1) a bacterial reverse mutation assay, *i.e.*, Ames assay, in four *Salmonella typhimurium* tester strains and one *Escherichia coli* tester strain (TNO, 2007a), (2) a chromosome aberration assay in cultured Chinese hamster ovary (CHO) cells (TNO, 2007b), and (3) a gene mutation assay with mouse lymphoma L5178Y cells at the thymidine kinase (TK) locus (TNO, 2007c). Each study was conducted in the presence and absence of S9 metabolic activation, which was prepared from homogenates of male Wistar rat livers following intraperitoneal (*i.p.*) dosing (500 mg/kg bwt) of Aroclor 1254 (20% w/v in soybean oil) sourced from Monsanto Company, St. Louis MO, USA. All three studies were conducted during the Spring and Summer of 2007 at the laboratories of TNO Quality of Life in Zeist, The Netherlands. These studies all met the acceptance criteria for validity because positive control substances induced the expected genotoxic effects and the results for the negative control substances were within historical ranges.

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Table 30. Constituent fatty acids and respective percentages in RAO lot 060331, nine lots of RAO manufactured over a 28-month period, and reported in the scientific literature for other ARA-rich oils

		Analysis of nine lots of RAO		Fatty acid distributions reported for other ARA-rich oils.	
	Lot 060331* (Area %)	Range (Area %)	Avg. (SD) (Area %)	ARA-rich oil #1[Ψ] (Area %)	ARA-rich oil #2[Σ] (Area %)
Myristic (C14:0)	0.41	0.28-0.41	0.34 (0.04)	0.4-2.4	0.37(0.03)
Palmitic (C16:0)	7.45	5.87-7.45	6.69 (0.58)	7.8-16.4	12.1(0.87)
Palmitoleic (C16:1n-7)	0.19	0.19-0.29	0.22 (0.03)	0.1-1.8	0.09(0.02)
Stearic (C18:0)	5.72	5.26-5.89	5.58 (0.25)	10.3-11.4	7.38(0.45)
Oleic (C18:1n-9)	9.27	5.48-9.27	7.48 (1.50)	11.7-14.0	5.81(0.51)
Linoleic (C18:2n-6)	5.90	4.69-9.81	7.21 (1.66)	6.3-7.1	9.09(0.79)
γ-Linolenic (C18:3n-6)	2.45	2.16-2.45	2.33 (0.10)	3.4-4.0	2.32(0.03)
Arachidic (C20:0)	0.89	0.80-0.89	0.83 (0.03)	0.9-1	0.79(0.04)
Eicosenoic (C20:1n-9)	1.43	0.24-0.45	0.34 (0.09)	0.4-0.5	0.40(0.04)
Eicosadienoic (C20:2n-6)	0.45	0.41-0.51	0.44 (0.03)	NR	0.72(0.10)
Eicosatrienoic (C20:3n-6)	3.81	3.54-4.06	3.78 (0.15)	2.8-4.3	3.9(0.05)
Arachidonic (C20:4n-6)	43.31	41.81-43.66	42.91 (0.66)	38.6-51.4	43.5(2.98)
Eicosapentaenoic (C20:5n-3)	0.27	0.08-0.40	0.15 (0.11)	0-0.1	0.13(0.02)
Behenic (C22:0)	3.23	3.18-3.42	3.27 (0.10)	1.5-1.8	2.98(0.08)
Lignoceric (C24:0)	9.41	9.41-11.98	10.50 (0.82)	1.3-1.5	8.15(0.19)
Hexacosanoic (C26:0)	1.11	1.11-1.72	1.40 (0.19)	NR	0.09(0.01)

* Lot of RAO used in the testing described herein; ** the nine lots of RAO included Lot 060331; Ψ = Arterburn *et al.* (2000a, 2000b), Boswell *et al.* (1996) and Hempenius *et al.* (2000); Σ = EFSA (2008); SD = standard deviation.

a) Bacterial reverse mutation

1) Materials

Reagent and control chemicals used in the Ames assay included nicotinamide adenine dinucleotide phosphate, disodium salt (NADP; Roche Diagnostics, Woerden, The Netherlands); D-glucose-6-phosphate, disodium salt (G-6-P); 9-aminoacridine (9-AA); N-ethyl-N-nitrosurea (ENU); dimethylsulfoxide (DMSO); Mitomycin C and benzo(a)pyrene (BAP) (all from Sigma Chemical Company, St. Louis MO, USA); 2-nitrofluorene (2-NF); 2-aminoanthracene (2-AA) and sodium azide (NaZ) (all from Aldrich

Chemical Company, Brussels, Belgium). The tester strains used in this assay were provided by the National Institute of Public Health, Bilthoven, The Netherlands.

2) Experimental design

The standard plate incorporation method with histidine-requiring *S. typhimurium* tester strains TA 1535, TA 1537, TA 98 and TA 100; and tryptophan-requiring *E. coli* tester strainWP2 *uvr*A, was used in this test of RAO. The positive control mutagens used for each tester strain are shown in Table 31.

Table 31. Reference mutagens and concentrations used for each tester strain in the bacterial reverse mutation assay of RAO

Tester strain	With S9 mix; concentration	Without S9 mix; concentration
TA 1535	2-AA; 2 µg/plate	NaZ; 1 µg/plate
TA 1537	BAP; 4 µg/plate	9-AA; 80 µg/plate
TA 98	2-AA; 2 µg/plate	2-NF; 2 µg/plate
TA 100	2-AA; 2 µg/plate	NaZ; 1 µg/plate
WP *uvr*A	2-AA; 80 µg/plate	ENU; 100 µg/plate
2-AA = 2-aminoanthracene; BAP = benzo(a)pyrene; ENU = N-ethyl-N-nitrosurea; 2-NF = 2-nitrofluorene; NaZ = sodium azide; S9 mix = metabolically activated liver fraction from Aroclor 1254-induced male Wistar rats.		

The negative control substance was DMSO, which also served as the dilution solvent for the RAO concentrations tested in this assay. Those test concentrations were 0, 62, 185, 556, 1667 and 5000 µg/plate. Because the results from previous Ames assays of very similar refined ARA-rich oils have been published (Arterburn *et al.*, 2000b), it was not necessary to conduct a preliminary range-finding investigation in this study. The study was conducted in accordance with OECD Guideline 471 (OECD, 1997a), which is based on the detailed descriptions described previously by Ames *et al.* (1975) and Maron and Ames (1983).

b. Chromosome aberration

1) Materials

Tissue culture media and other substances used in this assay included Ham's F-12 with Glutamax-1 (Invitrogen), fetal calf serum (BioWhittaker), phosphate buffered saline (PBS), penicillin/streptomycin (Life Technologies B.V., Breda, The Netherlands), NADP and G-6-P (Roche Diagnostics, Almere, The Netherlands), glacial acetic acid, Giemsa stain, DMSO (Merck-Darmstadt, Germany), methanol (Biosolve), Mitomycin C and Demecolcine (Sigma-Aldrich Chemie GmbH, Germany) and cyclophosphamide (Asta Werke, Bielefeld, Germany). CHO cells were obtained from The University of Leiden, The Netherlands.

2) Experimental design

This study was conducted following OECD Guideline 473 (OECD, 1997b). Two separate chromosome aberration tests were conducted, each in the presence and absence of S9 metabolic activation; duplicate cultures were used in each test. DMSO served as both the diluent for RAO and the negative control substance. The positive control substances were Mitomycin C for testing in the absence of S9; and cyclophosphamide for testing in the presence of S9. The RAO concentrations and positive controls employed in Tests 1 and 2 of this investigation are shown in Table 32.

In both tests, three concentrations of RAO, plus negative and positive controls, were selected for evaluation of structural chromosome aberrations. (If possible, the highest concentration selected for evaluation should exhibit a clear indication of cytotoxicity and in this testing, the mitotic index, *i.e.*, the percentage of cells in mitosis, was used as that cytotoxicity measure.) For each selected concentration, the potential effects on chromosome aberration were evaluated in 200 well-spread metaphases, each with 20-22 centromeres, which were examined microscopically for chromatid-type aberrations (*e.g.*, gaps, breaks, fragments, interchanges) and chromosome-type aberrations (*e.g.*, gaps, breaks, minutes, rings, dicentrics) according to the criteria specified in OECD (1997b).

Table 32. Concentrations/conditions used in tests 1 and 2 of the chromosome aberration assay of RAO

Test 1		Test 2	
+ S9 mix (μg/ml)	- S9 mix (μg/ml)	+ S9 mix (μg/ml)	- S9 mix (μg/ml)
0*	0	0	0*
2	2	39	5
3.9	3.9	78	10
7.8	7.8	156	20
15.6	15.6	3135	39
31.3	31.3	625	78
62.5	62.5	1250	156
125	125	2500	313
250	250	5000	625
500	500	--	1250
1000	1000	--	2500
2500	2500	--	5000
5000	5000	--	--
5**	5***	5**	0.05***

* the negative control was dimethyl sulfoxide (DMSO); ** positive control (Cyclophosphamide); *** positive control (Mitomycin C); S9 mix = metabolically activated liver fraction from Aroclor 1254-induced male Wistar rats.

In Test 1, the treatment and harvesting times were four and 18 hours, respectively. In RAO treatments both with and without S9, 125 μg/ml (as a non-precipitating concentration) and 2500 and 5000 μg/ml (as precipitating concentrations) were selected for further examination. Cells treated without S9 showed cytotoxicity at 5000 μg/ml and slight cytotoxicity at 2500 μg/ml; no cytotoxicity was observed in cells treated with S9.

In Test 2, the treatment time for cells in the presence of S9 was four hours while treatment time for cells in the absence of S9 was 18 hours; the harvesting time for all cells was 18 hours. In treatments both with and without S9, 156 μg/ml (as a non-precipitating concentration) and 2500 and 5000 μg/ml (as precipitating concentrations) were selected for further examination. In cells treated without S9, slight cytotoxicity was noted only at 2500 μg/ml while in cells treated with S9, only slight cytotoxicity was noted at 2500 and 5000 μg/ml.

A test substance is considered to be negative in the chromosomal aberration test if it produces neither a dose-related increase in the number of structural chromosomal aberrations nor a reproducible positive response at any of the test points.

5. Gene mutation

a) Materials

Culture medium and other substances used in this assay included RPMI 1640 medium (with HEPES and L-glutamine) (BioWhitacker, Verviers, Belgium), penicillin, streptomycin, sodium pyruvate and horse serum (Gibco BRL, Paisley, Scotland); NADP, methyl methanesulfonate (Aldrich, Milwaukee WI, USA), dimethylsulfoxide (DMSO), trifluorothymidine (TFT), 3-methylholanthrene (MCA) and G-6-P. The L5178Y mouse lymphoma cells used in this assay were sourced from the MRC Cell Mutation Unit, University of Sussex, UK.

b) Experimental design

This study was conducted in compliance with OECD Guideline 476 (OECD, 1997c) and EC Directive 2000/32/EC (EC, 2000). Approximately one week before treatment, L5178Y cells were generated from frozen stock and cultured in a humidified incubator (37° C, 5% CO_2). For the cytotoxicity and gene mutation aspects of this assay, approximately 3,000,000 and 5,000,000 cells were used *per* culture (- and + S9), respectively. Cytotoxicity of RAO was determined by measurement of (1) the initial cell yield, (2) the relative suspension growth (RSG) and, (3) the relative total growth (RTG).[8]

After treatments, cells were checked for visible aberrant effects; cell viability was checked at the higher RAO concentrations. A positive mutagenic response was considered when the induced mutant frequency (*i.e.*, the mutant frequency induced by RAO minus the mutant frequency induced by the negative control) was >126 mutants/1,000,000 clonable cells; an equivocal response was >88 mutants *per* 1,000,000 clonable cells.

[8] Initial cell yield is the ratio of cells remaining after treatment to cells treated with the vehicle control (*i.e.*, DMSO). The RSG is a measure of the cumulative cell growth rate after 24 and 48 hours of treatment compared to untreated controls; and the RTG is the product of the initial cell yield, the RSG and the colony-forming ability (*i.e.*, the cloning efficiency) after 48 hours of treatment compared to negative controls. The RTG is a measure of cytotoxicity that occurs in all phases of the study.

L5178Y cells were treated in duplicate for four and 24 hours, with and without metabolic activation, respectively, at RAO test concentrations of 0, 429, 858, 1715, 3500 and 5000 µg/ml. The test substance would have been considered mutagenic in the gene mutation test at the TK locus if a concentration-related increase in mutant frequency was noted or if a reproducible positive response was observed for at least one test substance concentration. Conversely, the test substance would not have been considered mutagenic in this test if neither a dose-related increase in mutant frequency nor a reproducible positive response was seen at any test concentration. Statistical procedures were not performed for this test.

6. Subchronic toxicity with *in utero* exposure

a) Animals and maintenance

Parental (F_0) male and female Wistar Outbred (Crl:WIWU) rats (45 males and 85 females) were obtained from Charles River Deutschland, Sulzfeld, Germany. After acclimation and confirmation of satisfactory health status, commencement of treatment began (premating period). At the time, F_0 males were approximately 10 weeks old and F_0 females were about 11 weeks old; the age difference being deliberate, to avoid brother-sister matings. Premating body weights did not exceed ± 20% of the mean weight for each sex. Offspring (F_1) rats were randomly selected from the F_0 litters on post-partum Day 21. The rats were housed under conventional conditions, in macrolon cages with sterilized wood shavings (Lignocel) as bedding material and shreds of paper as environmental enrichment (Enviro-dri, Lillico, Betchworth, England). During the premating period, the parent rats were housed maximally four *per* cage, separated by sex. During the mating period, two females were caged with one male from the same group until they mated. During gestation and lactation, the dams were housed individually. In the period between weaning and the start of the subchronic study, the F_1 rats were housed one to five *per* cage until each cage contained five males or five females *per* group. In the 90-day feeding study, same sex F_1 rats were housed in groups of five. Animal housing conditions were conventional; room temperature was targeted at 22 -24 ° C and relative humidity at 30– 70%; air changes ≥ 10 *per* hour and 12 hours *per* day of fluorescent light exposure. The animals were fed a commercial rodent diet (Rat & Mouse No.3 diet; RM3), SDS Special Diets Services, Witham, England). Diet and domestic tap water were provided *ad libitum*. In all study phases, feed consumption was

measured by weighing the cage feeders and water consumption was measured by weighing the cage water bottles.

b) Experimental design

This study was conducted in compliance with OECD Guideline 408 (OECD, 1998a) with the design consisting of two phases: (1) an *in utero* exposure phase in which F_0 parents were fed the test or control diets starting 4 weeks prior to mating, throughout mating, gestation and lactation until weaning of the F_1 rats; and (2) a traditional 90-day feeding study in which selected F_1 offspring consumed the same diets as did the F_0 generation rats (TNO, 2008). The study included five exposure groups composed of two control groups (a low-fat reference control group fed the RM3 standard diet and a high-fat control group fed the RM3 diet supplemented with 5 % corn oil) and three test groups that consumed diets containing 0.5%, 1.5% and 5% (wt/wt) RAO. The 0.5% and 1.5% test diets were adjusted with corn oil to 5% added fat. (The percentages of RM3 feed, corn oil and RAO in the two control and three test diets appear in Table 33 and the results from the analysis of four lots of the RM3 diet used in the studies described herein, plus calculated values provided by the feed supplier for parameters not evaluated by lot analysis, appear in Table 34.

Table 33. Relative constituents of standard rat feed, corn oil and RAO fed to exposure groups in the 90-day study

	Composition *per* kg of diet				
Diet constituent	Control (low-fat)	Control (high-fat)	RAO (low-dose)	RAO (mid-dose)	RAO (high-dose)
RM3 feed (g)	1000	950	950	950	950
Corn oil (g)	0	50	45	35	0
RAO (g)	0	0	5	15	50

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Table 34. Analysis of RMB3 feed

Results of RM3 feed batch analyses conducted by the feed supplier (4 batches analyzed)					
Nutrient	Avg. % / SD (n=4)	Nutrient	Avg. % / SD (n=4)	Contaminant*	Avg. (mg/kg) / SD (n=4)
Moisture	9.7 / 0.53	Calcium	1.18 / 0.066	Fluoride	14.25 / 3.1
Crude fat	5.0 / 0.19	Phosphorous	0.77 / 0.021	Nitrate (as $NaNO_3$)	19 / 6.38
Crude protein	23.0 / 0.61	Sodium	0.29 / 0.044	Nitrite (as $NaNO_2$)	0.275 / NA[a]
Crude fiber	4.4 / 0.49	Chloride	0.049 / 0.056	Lead	0.38 / 0.148[b]
Ash	6.6 / 0.26	Potassium	0.90 / 0.029	Arsenic	0.73 / 0.075
Vitamin C	Not reported	Magnesium	0.22 / 0.005	Cadmium	0.112 / 0.032
Vitamin E	0.008 / 0.001	Iron	0.025 / 0.001	Mercury	0.005 / 0[c]
Vitamin A (IU/gm)	13.45 / 0.044	Copper	0.002 / <0.000	Selenium	0.375 / 0.078
		Manganese	0.009 / 0.001	Total viable organisms/gm	900 / NA[d]
		Zinc	0.006 / 0.001	Fungal units	70 / 60.6
Calculated nutrients in RM3 feed provided by the feed supplier					
Nutrient	%	Energy	(mJ/kg)	Fatty acids (%)	
Crude fat	3.86	Gross energy	15.2	*Saturated*	
Crude protein	20.21	Digestible energy	12.42	Lauric; C12:0 (0.05)	Palmitic; C16:0 (0.36)
Total dietary fiber	15.43	Metabolizable energy	11.36	Myristic; C14:0 (0.20)	Stearic; C18:0 (0.09)
Pectin	1.43	AFE	13.90	*Monounsaturated*	
Hemicellulose	9.20	(% AFE in oil)	(11.50)	Myristoleic; C14:1 (0.01)	Oleic; C18:1 (1.03)
Cellulose	3.93	(% AFE in protein)	(26.93)	Palmitoleic; C16:1 (0.13)	
Lignin	1.50	(% AFE in carbohydrate)	(61.57)	*Polyunsaturated*	
Starch	33.92			Linoleic; C18:2n6 (1.15)	Arachidonic; C20:4n6 (0.22)
Sugar	5.75			Linolenic; C18:3n3 (0.17)	Clupanodonic; C22:5n3 (0.04)

* Contaminants not detected in any of the four analyses included aflatoxins, PCB, DDT, Dieldrin, Lindane, Heptachlor, Malathion, mesophilic spores, *Salmonella sp.*, *Entero bacteriaceae*, *Escherichia coli*, and antibiotic activity; a = nitrite (as $NaNO_2$) was detected in only one of the four batches tested; b = lead was detected in two of the four batches tested; c = mercury was detected in two of the four batches tested; d = total viable organisms/gm were detected in one of the four batches tested; AFE = Atwater fuel energy; Avg. = average; SD = Standard Deviation; IU = International Units.

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Fresh batches of the test and control diets were prepared approximately once every month, sealed in airtight plastic containers under a nitrogen blanket to avoid oxidation, and stored in a freezer (<-18° C). Twice a week, selected containers were thawed and the contents were used to replace the feed in the animal feeders. The constituents and homogeneous distribution of the test substances in the diet, and the stability in the animal room (4 days) or freezer (6 weeks) were confirmed by quantitative determination using gas chromatography with flame ionization detection after chloroform/methanol extraction, saponification and derivatization.

c) Parental (F_0) rats

Sixteen females and eight males were allocated randomly to each exposure group of F_0 rats, numbers that were deemed sufficient to result in at least 10 litters *per* group. After a pre-mating period of 4 weeks, in which the F_0 rats were fed their respective test or control diets, the females were mated (two females with one male from the same group). Vaginal smears were made daily and the day on which sperm was observed in the smears was considered gestation Day 0. The administration of test or control diets was continued throughout gestation and lactation. The morning after birth was considered post partum Day 1. On post partum Day 4, litters of more than eight pups were adjusted by randomly eliminating surplus pups to obtain four males and four females *per* litter. At post partum Day 21, the F_1 litters were weaned. All surviving female parent rats were killed under CO_2/O_2 anesthesia shortly after pup weaning; F_0 males were killed and discarded without necropsy shortly after mating. The female F_0 rats were subjected to a complete macroscopic examination and the number of implantation sites in the uterus was determined.

The following examinations and/or measurements were made during premating, mating, gestation and lactation:

Clinical observations. General condition and behavior of all animals were checked daily.

Body weight. The body weight of each animal was recorded at baseline and once every week thereafter. Mated females were weighed on gestation Days 0, 7, 14 and 21. Females that gave birth were weighed on post partum Days 1, 4, 14 and 21.

Feed consumption. During the premating period, feed consumption of both males and females was measured *per* cage once *per* week. Feed consumption by females was recorded during pregnancy at the end of successive periods (Days 0–7, 7–14 and 14–21), and during lactation, at the end of Days 1–4, 4–7 and 7–14; feed consumption was not measured during mating. The intake of test substance *per* kg body weight was calculated from the respective dietary concentration, feed consumption and body weight. (Water consumption was not recorded for F_0 rats.)

Reproductive data and indices. For the assessment of reproductive performance, mating, fertility, fecundity, gestation, live births and viability indices were calculated, and the sex ratio of pups was determined. For each litter, the litter size (dead and live pups), number of male and female pups, and number of pups with external abnormalities were determined on post-partum Days 1, 4, 7, 14 and 21. The litters were weighed on post-partum Days 1, 4 (before and after culling), 7 and 14. At post-partum Day 21 (*i.e.*, weaning), all pups were weighed individually.

d) Offspring (F_1) rats

At weaning, F_1 rats were randomly selected from the F_0 litters (10 rats/sex/group, each selected from a different litter). During the period between weaning and the start of the 90-day feeding study, feeding of the respective test or control diets to the selected F_1 rats was continued. The following observations were made:

Clinical observations. The general condition and behavior of all animals were checked daily. Neurobehavioral testing, comprising detailed clinical observations outside the cage, was conducted on all animals once weekly. In addition, behavioral endpoints (functional observational battery) and motor activity assessments were performed during the last week of the treatment period (Moser *et al.*, 1997). Ophthalmoscopic observations, made using an ophthalmoscope after induction of mydriasis by a solution of atropine sulfate, occurred during the first week of the feeding study in all rats of all groups, and during the last week of the study in all rats of both the high-fat control group and the 5% RAO test group. Because no treatment-related ocular changes were observed in those latter two groups, eye examinations were not extended to the animals in other groups.

Body weight. The body weight of each animal was recorded at initiation of the 90-day feeding study and once *per* week thereafter.

Feed and water consumption. The quantity of feed consumed was measured *per* cage, over successive periods of 3 or 4 days. The intake of test substance *per* kg body weight was calculated from the nominal dietary concentration, the feed consumption and the body weight. Water consumption was measured during 5-day periods in study Weeks 1, 6 and 12.

Hematology and clinical chemistry. EDTA or citrate (for determination of prothrombin time and activated prothrombin time) was used as an anticoagulant in blood collected from the abdominal aorta of all rats at necropsy. The rats were fasted overnight but water was freely available. Samples were examined for hemoglobin, packed cell volume, red blood cell count, reticulocytes, total white blood cell count, prothrombin time, activated partial thromboplastin time, thrombocyte count (Advia 120 Hematology Analyzer) and differential white blood cell count (microscopic examination), and the indices, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), were calculated. Clinical chemistry analyses were performed on plasma samples derived from heparinized blood at necropsy. The measurements comprised alkaline phosphatase activity (AP), aspartate aminotransferase activity (AST), alanine aminotransferase activity (ALT), *gamma*-glutamyl transferase (GGT), total protein, albumin, ratio albumin to globulin, urea, creatinine, glucose, total bilirubin, total chlolesterol, triglycerides, phospholipids, calcium, sodium, potassium, chloride and inorganic phosphate (Olympus AU-400 analyzer).

Renal concentration test and urinalysis. On Days 86-87 of the study, rats were deprived of water for 24 hours. During the final 16 of those 24 hours, rats were also deprived of feed while urine was collected from stainless steel metabolism cages (one rat *per* cage) and analyzed for volume, density (using a refractometer), appearance, pH, glucose, occult blood, ketones, protein, bilirubin, urobilinogen (Combur test strips) and microscopy of the sediment.

Necropsy. Overnight-fasted rats (with access to water) were killed under CO_2/O_2 anesthesia on Days 92-93 (males) and Days 93-94 (females) and subjected to an immediate complete macroscopic examination with organ weights measured as soon as possible after

dissection to avoid drying. The weights of the adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, pituitary, prostate, testes, thymus, thyroid and uterus were recorded and related to the final body weight. Specimens from those organs and from the following tissues were preserved in neutral 4% formalin, embedded in paraffin wax, sectioned at 5 μm and stained with hematoxylin and eosin for histopathology: aorta, axillary lymph nodes, cecum, colon, eyes, gut-associated lymphoid tissue (GALT, including Peyer's patches), lungs, mammary gland (females), mesenteric lymph nodes, esophagus, pancreas, parathyroid, parotid salivary glands, rectum, seminal vesicles (with coagulating glands), skeletal muscle (thigh), sciatic nerve, skin, small intestine (duodenum, jejunum and ileum), spinal cord (three levels, retained in vertebral column), sternum with bone marrow, stomach (glandular and non-glandular), sublingual and submaxillary salivary glands, trachea/bronchi, urinary bladder and vagina, plus any other tissue(s) showing gross lesions. Histopathological examinations were performed on all specimens collected from rats in the high-fat control group and the 5% RAO test group. Kidneys, liver and gross lesions from all rats in all exposure groups were also examined microscopically.

e) Statistical analysis

Means ± standard error of the mean (SEM) were calculated. Two separate statistical analyses were conducted: the test groups were first compared with the high-fat control group, and then all groups were compared with the low-fat control group. Analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests (Dunnett, 1955) were used for evaluating data on body weights, feed consumption, hematology, clinical chemistry, urinalysis and organ weights. Before the ANOVA, Bartlett (Bartlett, 1937) and Shapiro-Wilk tests (Shapiro and Wilk, 1965) were run for homogeneity of variance, and normality of data distribution, respectively. If variances were not homogeneous or data not normally distributed, the data were stepwise log- or rank-transformed and ANOVA was conducted on the transformed data. If the ANOVA yielded a significant effect ($P<0.05$), intergroup comparisons were made with the control group by Dunnett's multiple comparison test. Fisher's exact probability test (Fisher, 1970) was used for the evaluation of data on reproductive performance (the numbers of mated and pregnant females, females with live born pups, females surviving delivery, females with stillborn pups or lost litters, live born and stillborn pups, pups lost at various stages, pups surviving 21 days), and for the evaluation of histopathological changes. Kruskal–Wallis non-parametric ANOVA (Kruskal and

Wallis, 1952) followed by Mann–Whitney U-tests (Mann and Whitney, 1947) were used for evaluating precoital time, duration of gestation and litter size, reticulocytes, relative differential white blood cell counts, and semi-quantitative urinary parameters. Functional observational battery and motor activity results were evaluated by ANOVA followed by Dunnett's multiple comparison tests (continuous data); by Kruskal–Wallis non-parametric ANOVA followed by multiple comparison tests or by Pearson X^2 (Chernoff and Lehmann, 1954).

7. Results

a) Genotoxicity studies

RAO was negative in all three genotoxicity assays, each which was conducted in the presence and absence of S9 metabolic activation. In the Ames assay, RAO did not produce a dose-related increase in mean revertants and did not produce a positive response at any of the test concentrations (TNO, 2007a). In the chromosome aberration assay, RAO induced neither a dose-related increase in the number of structural chromosomal aberrations nor a reproducible positive response at any of the test concentrations (TNO, 2007b). Finally, in the mouse lymphoma assay, RAO produced no relevant increases in mutant frequency at any of the test concentrations (TNO, 2007c).

b) Subchronic toxicity with *in utero* exposure

1) Intake of the test substance

Except during lactation when intake by dams doubled, the overall RAO intakes by the F_0 and F_1 rats were approximately 0.3, 0.9 and 3 g/kg bwt/day in the low-, mid- and high-dose group, respectively Table 35.

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Table 35. Mean RAO intakes by males and females in each 90-day study exposure group

	Test groups (% RAO)					
	0.5		1.5		5.0	
Generation / Study phase	Mean RAO intakes during each study phase (mg/kg/day)					
	Males	Females	Males	Females	Males	Females
F_0 / Pre-mating	260	300	790	870	2590	2960
F_0/ Gestation	--	280	--	840	--	2960
F_0/ Lactation	--	670	--	1940	--	6250
F_1 / Sub-chronic study	295	315	879	927	3095	3244

2) Results obtained in f_0 rats and observations in pups

Clinical observations, growth and feed intake. No treatment-related clinical signs were observed during the premating, gestation and lactation periods. Gross examination of the F_0 females at necropsy did not reveal any test substance effects on maternal organs and tissues. There were no consistent treatment-related effects on body weights and body weight gain in F_0 rats during the premating, gestation and lactation periods. There were no significant differences in feed intake between the treatment groups and the high-fat control group. Feed intake in the low-fat control group was statistically significantly higher (overall about 12%) than in all high-fat groups during the premating and the gestation period; this effect is ascribed to the higher caloric density of the high-fat diets.

Reproduction indices. There were no treatment-related differences in fertility and reproductive performance among the groups. Indices for mating, female fecundity, female fertility, male fertility, gestation, birth and viability, as well as precoital and gestation times were similar among the groups (Table 36). One stillborn pup was observed in one litter of the mid-dose group. There were no dams with all stillborn pups and no statistically significant effect was observed on post implantation loss. In the 5% RAO test group, the mean number of pups delivered and the mean number of live pups on Day 1 and on Day 4 (preculling) were statistically significantly lower than in the low-fat controls and high-fat controls. This change was primarily due to a high incidence of small litters (≤6 pups in 5 of 16 dams). Two of the dams with the lowest number of implantations were mated by the same male. Because one male was responsible for the two smallest litters, the low number of implantations in these females may be

a chance finding as a consequence of poor sperm quality in one male, which could not be confirmed because the male F_0 rats were not necropsied thereby disallowing testicular histopathology to be conducted. However, because no pathological findings were noted in the reproductive organs (including uterus, testes and epididymides) in the F_1 generation and no other impacts on fertility were observed, the low number of implantations in the high-dose group of the F_0 generation is considered random. In the mid-dose group, the delivered pup numbers were comparable to those in both the low-fat- and high-fat controls but because one pup was stillborn, live pup numbers on Days 1 and 4 were significantly lower compared to the high-fat control group only. The number of delivered pups was not significantly affected, the difference was only slight/within normal range, and not statistically significant compared to the low-fat controls. Therefore, this finding is also considered random.

Observations in pups. There were no relevant differences among the groups in general condition and body weights of the pups, or in viability, sex ratio or number of pups *per* litter (Table 36). On post-partum Days 7-14, pup weight and weight gain were statistically significantly higher in all high-fat groups (test groups and high-fat controls) than in low-fat controls; these significant differences were not seen on post-partum Days 14-21. Overall, some increases in pup weights were noted during lactation, but they were inconsistent, and, moreover, increased pup weights are not considered to represent an adverse effect. Because these differences were not dose-related, they were not ascribed to treatment.

Table 36. Reproductive outcomes from the 90-day study of RAO$^{\pi}$ (values in parentheses are standard deviations)

	Control groups		Test groups (% RAO)		
Parameter	Low-fat	High-fat	0.5	1.5	5.0
No. of females mated	16	16	16	16	16
No. of males that sired	7	7	8	8	8
No. of females pregnant	14	14	16	15	15
Mean pre-coital time (days)	2.3 (0.3)	2.3 (0.3)	3.1 (0.3)	2.5 (0.3)	2.9 (0.4)
Live birth index (%)	100	100	100	99	100
Mean live pups/litter at birth	10.9 (0.4)	10.9 (0.3)	10.2 (0.5)	9.9 (0.3)*, u	7.9 (0.8)**, Δ, u
Mean live pups/litter, Day 4, preculling	10.9 (0.4)	10.9 (0.3)	10.1 (0.5)	9.8 (0.3)*, u	7.9 (0.8)**, Δ, u
Mean litter size, Day 21	8.0 (0.0)	7.9 (0.1)	7.9 (0.1)	8.0 (0.0)	6.7 (0.5)
Viability index, Days 4-21 (%)	100	99	100	100	100
Sex ratio, Day 1 (% males)	55	46	53	52	49
Sex ratio, Day 21 (% males)	48	49	53	51	49
Post-Implantation loss (%)	6.1 (1.7)	12.1 (7.0)	10.9 (2.9)	10.9 (3.5)	14.1 (4.5)
Pups delivered (total)	10.9 (0.4)	10.9 (0.3)	10.2 (0.5)	10.0 (0.4)	7.9 (0.8)**, Δ, u
Mean pup wt, Day 1 (g)	5.1 (0.1)	4.9 (0.2)	5.4 (0.2)	5.1 (0.1)	5.3 (0.3)
Mean pup wt, Day 14 (g)	25.8 (0.5)	27.5 (0.6)	29.2 (0.7)ΔΔ, a/d	27.8 (0.4)	28.3 (0.9)Δ, a/d
Mean male pup wt, Day 21 (g)	43.8 (1.1)	46.2 (1.0)	47.7 (1.3)	46.7 (0.6)	47.0 (1.5)
Mean female pup wt, Day 21 (g)	41.8 (0.8)	44.4 (0.9)	47.2 (1.0)ΔΔΔ, a/d	45.0 (0.7)	44.5 (1.5)
Mean pup wt, Day 21	42.8 (0.9)	45.3 (0.9)	47.3 (0.9)ΔΔ, a/d	45.9 (0.7)	46.1 (1.5)

π = values in parentheses are standard deviations; a/d = ANOVA plus Dunnett's test (2-sided); u = Kruskal-Wallis & Mann-Whitney U test; wt = weight; statistical significances indicated by asterisk(s) (*) are compared to high-fat controls; statistical significances identified by delta(s) (Δ) are compared to low-fat controls; * = P<0.05; ** = P<0.01; Δ = P<0.05; ΔΔ = P<0.01; ΔΔΔ = P<0.001.

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3) Results obtained in F_1 rats in the subchronic study.

Intake of the test substance. Due to the decrease in feed intake *per* kg body weight with increasing age of the rats, the intake of test substance *per* kg body weight gradually decreased in all groups. The overall intake of RAO in the F_1-animals was approximately 0.3, 0.9 and 3.1 g/kg bwt/day in males and 0.3, 0.9 and 3.2 g/kg bwt/day in females of the low-, mid- and high-dose group, respectively (Table 35).

Clinical observations and mortality. There were no treatment-related clinical signs. One low-dose female was killed in moribund condition on Day 30 for reasons unrelated to treatment. Weekly arena testing and the results of the neurobehavioral observations and motor activity assessment did not indicate any neurotoxic potential of the test substance. Ophthalmoscopic examination did not reveal any treatment-related changes.

Growth, feed intake and water consumption. There were no consistent differences in body weights between the test groups and the low-fat control group or the high-fat control group. Feed intake tended to be lower in all high-fat groups (including the high-fat controls) than in low-fat controls. This effect is ascribed to the higher caloric density of the high-fat diets. Compared to the low-fat control group, water consumption tended to be decreased in the high-fat groups (including the high-fat corn oil controls) in females.

Hematology. The results of the hematology evaluation are presented in Table 37. There were no statistically significant differences in red blood cell counts, MCV, MCH, MCHC and reticulocytes and clotting potential between the test groups and the low-fat controls. Compared to the high-fat controls, hemoglobin was significantly decreased in mid- and high-dose males ($P<0.05$ and $P<0.01$, respectively), packed cell volume (PCV) was decreased in high-dose males ($P<0.05$), thrombocytes were increased in mid-dose males ($P<0.01$). There were no effects on females for hemoglobin, PCV and thrombocytes; however, prothrombin time was increased in high-dose females ($P<0.01$), yet not in any male exposure group. These changes did not differ significantly from the low-fat controls, were well within the range of historical control data and are considered to be a physiological variation without toxicological relevance. Total white blood cell count was increased in low-dose females ($P<0.05$) compared to low-fat controls and in high-dose females compared to both the low-fat controls and high-fat oil controls (both

P<0.01); and in high-dose males compared to the high-fat controls only (P<0.01). Percent lymphocytes were decreased in high-dose males (P<0.01) compared to the low-fat controls. There were no female effects on percent lymphocytes or male effects on absolute lymphocytes, but in females, absolute lymphocyte values were significantly increased in the low- and high-dose groups compared to the low-fat controls (both P<0.01). Percentage and absolute neutrophils were increased in high-dose males compared to the low-fat controls (both P<0.01); absolute neutrophils were also increased when compared to high-fat controls (P<0.01). There were no statistically significant effects in any female exposure group for percent or absolute neutrophils. Absolute basophils were increased in high-dose females compared to both control groups (P<0.01). There were no significant effects on percent basophils in females and no effects on percent or absolute basophils in males. Similarly, there were no effects on percent or absolute eosinophils in either sex. In each sex of high-dose rats, statistically significant increases were seen in both percent and absolute monocytes. For percent monocytes in males: P<0.05 compared to the high-fat controls and P<0.01 compared to low-fat controls; in females: P<0.01 compared to the high-fat controls and P<0.05 compared to low-fat controls. For absolute monocytes in males: P<0.01 compared to the high-fat controls and P<0.05 compared to low-fat controls; in females: P<0.01 compared to both control groups.

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Table 37. Measured hematological parameters following various RAO doses for 90 days in male and female rats[π]

	Control groups [A, B]		Test groups (% RAO) [A, B]		
Parameter	Low-fat	High-fat	0.5	1.5	5.0
Males					
Red blood cells (10^{12}/l)	9.51 (0.17)	9.41 (0.52)	9.40 (0.36)	9.22 (0.32)	9.19 (0.4)
Hemoglobin (mM/l)	9.81 (0.39)	10.00 (0.35)	9.92 (0.19)	9.67 (0.28)*	9.57 (0.28)**
Packed cell volume (l/l)	0.480 (0.016)	0.491 (0.027)	0.490 (0.018)	0.472 (0.020)	0.463 (0.023)*
MCV (fl)	50.47 (1.2)	52.18 (1.6)	52.11 (2.0)	51.19 (1.2)	50.45 (2.2)
MCH (fM/l)	1.03 (0.03)	1.07 (0.05)	1.05 (0.04)	1.05 (0.02)	1.04 (0.04)
MCHC (mM/l)	20.45 (0.20)	20.38 (0.58)	20.28 (0.51)	20.49 (0.33)	20.66 (0.53)
Reticulocytes (%)	1.94 (0.19)	1.87 (0.29)	1.92 (0.27)	2.06 (0.35)	2.21 (0.28)
Thrombocytes (10^9/l)	1060 (74)	980 (170)	1030 (190)	1170 (100)**	1130 (140)
Prothrombin time (Sec)	29.12 (0.96)	28.80 (2.40)	28.08 (2.94)	28.00 (0.97)	28.89 (1.02)
WBC count	10.23 (2.76)	8.90 (2.54)	10.66 (2.25)	10.86 (2.16)	12.92 (2.86)**
Lymphocytes (%)	87.17 (2.71)	83.91 (5.72)	87.54 (3.48)	86.29 (4.16)	79.65 (4.86)ΔΔ
Lymphocytes (Abslte)	8.93 (2.52)	7.54 (2.48)	9.39 (2.29)	9.39 (2.04)	10.26 (2.08)
Neutrophils (%)	8.71 (2.82)	11.75 (5.49)	8.38 (3.01)	9.34 (3.61)	15.62 (4.66)ΔΔ
Neutrophils (Abslte)	0.88 (0.33)	0.99 (0.49)	0.85 (0.25)	1.02 (0.44)	2.06 (0.89)**, ΔΔ
Eosinophils (%)	1.09 (0.32)	1.26 (0.44)	1.35 (0.66)	1.38 (0.60)	1.11 (0.23)
Eosinophils (Abslte)	0. 12 (0.06)	0.10 (0.00)	0.14 (0.05)	0.14 (0.05)	0.12 (0.04)
Basophils (%)	1.63 (0.33)	1.66 (0.28)	1.28 (0.29)	1.39 (0.35)	1.43 (0.41)
Basophils (Abslte)	0.17 (0.07)	0.16 (0.05)	0.13 (0.05)	0.15 (0.07)	0.18 (0.06)
Monocytes (%)	1.38 (0.27)	1.46 (0.41)	1.41 (0.44)	1.61 (0.63)	2.17 (0.81)*, ΔΔ
Monocytes (Abslte)	0.15 (0.07)	0.13 (0.07)	0.16 (0.05)	0.18 (0.06)	0.27 (0.12)**, Δ
Females					
Red blood cells (10^{12}/l)	8.83 (0.36)	8.83 (0.41)	8.66 (0.27)	8.66 (0.37)	8.89 (0.34)
Hemoglobin (mM/l)	9.83 (0.36)	9.89 (0.31)	9.70 (0.42)	9.70 (0.31)	9.76 (0.24)

	Control groups [A, B]		Test groups (% RAO) [A, B]		
Parameter	Low-fat	High-fat	0.5	1.5	5.0
Packed cell volume (l/l)	0.470 (0.020)	0.468 (0.016)	0.463 (0.018)	0.459 (0.016)	0.459 (0.011)
MCV (fl)	53.26 (1.6)	53.06 (2.2)	53.52 (1.7)	53.02 (1.2)	51.64 (2.0)
MCH (fM/l)	1.11 (0.04)	1.12 (0.05)	1.12 (0.04)	1.12 (0.03)	1.10 (0.04)
MCHC (mM/l)	20.92 (0.37)	21.12 (0.28)	20.91(0.37)	21.15 (0.25)	21.28 (0.39)
Reticulocytes (%)	2.06 (0.50)	2.14 (0.43)	2.22 (0.52)	2.12 (0.39)	2.20 (0.42)
Thrombocytes (10^9/l)	1030 (96)	980 (68)	950 (140)	1020 (100)	1020 (110)
Prothrombin time (Sec)	27.38 (1.41)	26.42 (0.98)	27.28 (1263)	27.35 (1.50)	28.20 (1.07)**
WBC count	5.49 (1.38)	6.02 (0.99)	7.56 (1.76)Δ	6.81 (1.05)	8.44 (2.42)**, ΔΔ
Lymphocytes (%)	85.67 (3.57)	88.09 (2.94)	88.72 (2.66)	86.85 (4.25)	82.26 (7.95)
Lymphocytes (Abslte)	4.70 (1.26)	5.31 (0.97)	6.72 (1.60)ΔΔ	5.95 (1.07)	6.86 (1.85)ΔΔ
Neutrophils (%)	10.96 (3.99)	8.33 (2.74)	7.38 (2.16)	9.37 (3.95)	12.68 (7.61)
Neutrophils (Abslte)	0.60 (0.22)	.49 (0.15)	0.57 (0.19)	0.63 (0.22)	1.13 (0.80)
Eosinophils (%)	1.02 (0.36)	1.25 (0.60)	1.38 (0.47)	1.22 (0.41)	1.30 (0.52)
Eosinophils (Abslte)	0. 06 (0.05)	0.07 (0.07)	0.10 (0.05)	0.09 (0.03)	0.11 (0.03)
Basophils (%)	1.31 (0.50)	1.34 (0.42)	1.46 (0.29)	1.34 (0.25)	1.65 (0.45)
Basophils (Abslte)	0.06 (0.05)	0.09 (0.03)	0.01 (0.00)	0.09 (0.03)	0.14 (0.05)**, ΔΔ
Monocytes (%)	1.06 (0.61)	0.98 (0.35)	1.09 (0.32)	1.19 (0.32)	2.09 (0.86)**,Δ
Monocytes (Abslte)	0.04 (0.05)	0.07 (0.05)	0.09 (0.03)	0.10 (0.00)	0.18 (0.14)**, ΔΔ

π = data are mean values with standard deviations in parentheses; Statistical significances identified by asterisk (*) are compared to high-fat controls; Statistical significance identified by delta (Δ) are compared to low-fat controls; * = P<0.05; ** = P<0.01; Δ = P<0.05; ΔΔ = P<0.01; Abslte = absolute; A = All tests for statistical significance were determined *via* One-way Analysis of Variance; B = data in parentheses represents the standard deviation; MCV = Mean corpuscular volume; MCH = Mean corpuscular hemoglobin; MCHC = Mean corpuscular hemoglobin concentration; Sec = seconds; WBC = white blood cell.

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Clinical chemistry. The clinical chemistry results are presented in Table 38.

Compared to the low-fat and high-fat controls, statistically significant decreases in cholesterol concentrations were noted in both sexes of the high-dose group, P<0.01 except in males compared to low-fat controls where it was P<0.05. In both sexes, plasma phospholipids significantly decreased in the high-dose groups compared to the high-fat controls (P<0.01 for males; P<0.05 for females) and low-fat controls (P<0.01 for both sexes); and in the mid-dose females (P<0.05 compared to high-fat controls; P<0.01 compared to low-fat controls). Statistically significant decreases were also seen in triglycerides but only in males (P<0.01 compared to high-fat controls; P<0.05 compared to low-fat controls). Urea concentrations were increased in high-dose males compared to both the low-fat controls and high-fat controls (both P<0.01). In high-dose females, urea concentration was significantly increased compared to the high-fat controls only (P<0.01). When compared to the high-fat controls, statistically significant increases were seen for inorganic phosphate in the high-dose groups of both sexes (P<0.01 for males, P<0.05 for females). Statistically significant changes were also seen for the activities of alkaline phosphatase (increased in high-dose males compared to the low-fat controls, P<0.01) and aspartate aminotransferase (decreased in high-dose females compared to the low-fat controls, P<0.05) and for plasma potassium (decreased in both high-fat control females compared to low-fat controls, P<0.01; and high-dose females compared to low-fat controls, P<0.05).

Urinalysis. The renal concentration test showed a decrease in urinary density in high-dose females compared to both control groups. This finding was only slight and ascribed to physiological variation. Semi-quantitative (dipstick) urinary observations and microscopy of the urinary sediment did not reveal any relevant changes.

Organ weights. Four statistically significant increases were seen in absolute organ weights compared to high-fat controls, one in mid-dose males (liver, P<0.05), three in high-dose females (adrenals, liver and spleen; all P<0.01) (Table 39).

Table 38. Clinical chemistry parameters following various RAO doses for 90 days in male and female rats[π]

	Control groups [A, B]		Test groups (% RAO) [A, B]		
Parameter	Low-fat	High-fat	0.5	1.5	5.0
Males					
Cholesterol (mM/l)	1.85 (0.24)	1.94 (0.28)	2.11 (0.35)	2.33 (0.32)	1.53 (0.31)**, Δ
Triglycerides (mM/l)	0.87 (0.25)	0.93 (0.19)	0.86 (0.22)	0.95 (0.21)	0.63 (0.09)**, Δ
Phospholipids (mM/l)	1.52 (0.13)	1.55 (0.17)	1.57 (0.19)	1.59 (0.18)	1.11 (0.16)**, ΔΔ
Urea (mM/l)	6.17 (0.96)	6.17 (0.88)	5.85 (1.61)	6.25 (0.89)	8.01 (1.34)**, ΔΔ
AP (U/l)	83.6 (18.8)	95.8 (8.5)	91.9 (17.6)	91.0 (12.8)	108.3 (16.8)ΔΔ
AST (U/l)	68.5 (6.4)	66.0 (7.5)	68.2 (8.8)	68.4 (10.1)	70.8 (6.3)
PO_4 (mM/l)	2.90 (0.11)	2.59 (0.40)	2.72 (0.32)	2.76 (0.30)	3.06 (0.18)**
Potassium (mM/l)	5.76 (0.37)	5.40 (0.54)	5.52 (0.36)	5.58 (0.56)	5.84 (0.75)
Females					
Cholesterol (mM/l)	2.27 (0.27)	2.25 (0.26)	2.09 (0.26)	2.07 (0.39)	1.74 (0.26)**, ΔΔ
Triglycerides (mM/l)	0.74 (0.14)	0.82 (0.20)	0.70 (0.13)	0.78 (0.25)	0.99 (0.40)
Phospholipids (mM/l)	2.16 (0.25)	2.08 (0.17)	1.91 (0.21)	1.81 (0.26)*,ΔΔ	1.48 (0.20)*,ΔΔ
Urea (mM/l)	7.26 (0.91)	6.46 (1.21)	7.21 (1.13)	7.77 (2.54)	8.60 (1.43)**
AP (U/l)	68.3 (21.2)	66.0 (13.7)	75.7 (11.5)	80.5 (27.8)	71.3 (7.6)
AST (U/l)	73.2 (7.5)	69.4 (25.6)	65.6 (5.9)	67.6 (7.7)	62.1 (4.9)Δ
PO_4 (mM/l)	2.21 (0.47)	2.16 (0.29)	2.40 (0.41)	2.42 (0.21)	2.52 (0.20)*
Potassium (mM/l)	6.39 (0.69)	5.68 (0.37)ΔΔ	5.84 (0.30)	5.80 (0.78)	5.59 (0.63)Δ

π = data are mean values with standard deviations in parentheses; Statistical significances identified by asterisk (*) are compared to high-fat, corn oil controls; Statistical significance identified by delta (Δ) are compared to low-fat controls; * = P<0.05; ** = P<0.01; Δ = P<0.05; ΔΔ = P<0.01; A = All tests for statistical significance were determined *via* One-way Analysis of Variance; B = data in parentheses represents the standard deviation; AP = alkaline phosphatase; AST = aspartate aminotransferase; PO_4 = inorganic phosphate.

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Table 39. Terminal body weights (g) and absolute organ weights (g) following various RAO doses for 90 days in male and female rats[π]

	Control groups [A, B]		Test groups (% RAO) [A, B]		
	Low-fat	High-fat	0.5	1.5	5.0
Males					
Terminal body weight	371 (28)	380 (33)	374 (36)	403 (29)	373 (20)
Adrenals	0.046 (0.006)	0.046 (0.008)	0.046 (0.008)	0.048 (0.006)	0.049 (0.010)
Brain	1.91 (0.05)	1.93 (0.11)	1.92 (0.12)	1.99 (0.09)	1.98 (0.06)
Heart	1.99 (0.14)	1.20 (0.11)	1.15 (0.11)	1.26 (0.14)	1.15 (0.07)
Kidneys	2.18 (0.16)	2.07 (0.12)	2.13 (0.21)	2.44 (0.64)	2.16 (0.17)
Liver	10.14 (0.72)	9.74 (1.08)	9.74 (1.36)	10.97 (0.81)*	10.73 (0.57)
Spleen	0.70 (0.08)	0.68 (0.07)	0.65 (0.08)	0.76 (0.09)	0.76 (0.07)
Thymus	0.36 (0.09)	0.37 (0.07)	0.40 (0.12)	0.40 (0.13)	0.35 (0.04)
Thyroid	0.020 (0.006)	0.022 (0.005)	0.021 (0.005)	0.025 (0.004)	0.023 (0.007)
Pituitary	0.012 (0.002)	0.011 (0.003)	0.011 (0.002)	0.011 (0.003)	0.012 (0.003)
Testes	3.19 (0.41)	3.26 (0.14)	3.25 (0.36)	3.43 (0.24)	3.32 (0.33)
Epididymides	1.26 (0.14)	1.35 (0.15)	1.26 (0.11)	1.38 (0.18)	1.27 (0.14)
Prostate	0.93 (0.19)	0.89 (0.21)	0.92 (0.12)	0.97 (0.24)	1.00 (0.19)
Females					
Terminal body weight	220 (9)	211 (9)	215 (19)	211 (12)	211 (14)
Adrenals	0.058 (0.008)	0.051 (0.009)	0.059 (0.008)	0.057 (0.003)	0.062 (0.006)**
Brain	1.75 (0.07)	1.77 (0.06)	1.78 (0.05)	1.77 (0.04)	1.81 (0.05)
Heart	0.77 (0.06)	0.73 (0.06)	0.73 (0.06)	0.72 (0.05)	0.72 (0.07)
Kidneys	1.32 (0.1)	1.27 (0.10)	1.35 (0.11)	1.32 (0.19)	1.27 (0.08)
Liver	5.45 (0.54)	5.00 (0.37)	5.22 (0.50)	5.10 (0.71)	5.80 (0.58)**
Spleen	0.45 (0.04)	0.41 (0.03)	0.46 (0.05)	0.44 (0.06)	0.51 (0.07)**
Thymus	0.27 (0.05)	0.27 (0.04)	0.28 (0.05)	0.30 (0.04)	0.29 (0.05)

	Control groups [A, B]		Test groups (% RAO) [A, B]		
	Low-fat	High-fat	0.5	1.5	5.0
Thyroid	0.016 (0.004)	0.016 (0.003)	0.019 (0.004)	0.020 (0.004)	0.019 (0.003)
Pituitary	0.014 (0.003)	0.015 (0.002)	0.013 (0.004)	0.013 (004)	0.014 (0.004)
Ovaries	0.072 (0.008)	0.068 (0.008)	0.070 (0.015)	0. 070 (0.007)	0.070 (0.012)
Uterus	0.74 (0.36)	0.53 (0.1)	0.53 (0.13)	0.82 (0.32)	0.80 (0.30)

π = data are mean values with standard deviations in parentheses; Statistical significances identified by asterisk (*) are compared to high-fat, corn oil controls; * = P<0.05; ** = P<0.01; A = All tests for statistical significance were determined *via* One-way Analysis of Variance; B = data in parentheses represents Standard Deviation.

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For relative organ weights (*i.e.*, organ to body weight ratios; Table 40), additional statistically significant differences were observed and for relative male liver weight, they included both increases and decreases. Compared to low-fat controls, relative liver weights were decreased significantly in the high-fat group (P<0.01) and the low-dose group (P<0.05) - both likely incidental - but were significantly increased in the high-dose group (also P<0.05). When compared to high-fat controls, a significant relative male liver weight increase was seen at the mid-dose (P<0.05) and the high-dose (P<0.01); however, the mid-dose finding is unlikely to be treatment-related because it was nearly identical to the relative liver weight for low-fat control males; a significant increase in relative male spleen weight was also noted in the high-dose group (P<0.01). In high-dose females, statistically significant increases were seen for relative adrenal, liver and spleen weights compared to high-fat controls (all P<0.01). When compared to low-fat controls, statistically significant female high-dose relative liver and spleen weight increases were also observed (P<0.05 for liver and P<0.01 for spleen). No other statistically significant relative organ weight changes occurred.

Pathology. Macroscopic examination at necropsy and microscopic examination of organs and tissues did not reveal any treatment-related findings of toxicological relevance. The only finding that reached the level of statistical significance was an increased incidence of hyaline droplet nephropathy in the kidneys of high-dose males compared to high-fat controls. The incidence of hyaline droplet nephropathy in high-dose males and low-fat controls was, however, comparable. Therefore this finding was not considered to be related to the ingestion of the test substance. All other histopathological changes observed were common findings in rats of this strain and age, and their incidences were comparable among all groups.

Table 40. Relative organ weights (g/kg body weight) following various RAO doses for 90 days in male and female rats[π]

	Control groups [A, B]		Test groups (% RAO) [A, B]		
	Low-fat	High-fat	0.5	1.5	5.0
Males					
Adrenals	0.125 (0.016)	0.120 (0.019)	0.124 (0.016)	0.120 (0.016)	0.132 (0.024)
Brain	5.17 (0.39)	5.12 (0.21)	5.17 (0.33)	4.95 (0.22)	5.32 (0.29)
Heart	3.23(0.21)	3.12 (0.11)	3.07 (0.13)	3.12 (0.29)	3.09 (0.24)
Kidneys	5.88 (0.32)	5.45 (0.36)	5.71 (0.33)	6.09 (1.72)	5.80 (0.35)
Liver	27.38 (0.88)	25.63 (1.12)ΔΔ	25.98 (1.72)Δ	27.26 (0.97)*	28.81 (0.93)**, Δ
Spleen	1.88 (0.18)	1.79 (0.18)	1.75 (0.15)	1.88 (0.18)	2.05 (0.15)**
Thymus	0.97 (0.2)	0.97 (0.22)	1.05 (0.28)	1.00 (0.33)	0.96 (0.12)
Thyroid	0.054 (0.014)	0.059 (0.013)	0.057 (0.013)	0.063 (0.010)	0.061 (0.018)
Pituitary	0.032 (0.004)	0.029 (0.011)	0.029 (0.003)	0.029 (0.006)	0.033 (0.007)
Testes	8.66 (1.28)	8.64 (0.67)	8.77 (1.29)	8.53 (0.61)	8.90 (0.72)
Epididymides	3.42 (0.45)	3.56 (0.35)	3.38 (0.27)	3.42 (0.39)	3.42 (0.32)
Prostate	2.54 (0.63)	2.34 (0.5)	2.47 (0.32)	2.40 (0.54)	2.69 (0.47)
Females					
Adrenals	0.263 (0.03)	0.240 (0.041)	0.276 (0.050)	0.270 (0.017)	0.293 (0.032)**
Brain	7.96 (0.40)	8.37 (0.43)	8.32 (0.56)	8.40 (0.55)	8.62 (0.51)
Heart	3.45 (0.28)	3.48 (0.32)	3.42 (0.34)	3.40 (0.33)	3.40 (0.3)
Kidneys	6.01 (0.39)	6.01 (0.46)	6.30 (0.44)	6.26 (0.59)	6.04 (0.18)
Liver	24.76 (0.52)	23.69 (1.89)	24.30 (1.86)	24.17(3.11)	27.50 (1.63)**, Δ
Spleen	2.03 (0.19)	1.97 (0.14)	2.13 (0.19)	2.11 (0.31)	2.40 (0.27)**, ΔΔ
Thymus	1.23 (0.23)	1.30 (0.18)	1.32 (0.25)	1.40 (0.21)	1.38 (0.22)
Thyroid	0.074 (0.016)	0.078 (0.014)	0.087 (0.016)	0.094 (0.022)	0.089 (0.011)
Pituitary	0.062 (0.013)	0.068 (0.010)	0.059 (0.016)	0.064 (0.184)	0.066 (0.018)
Ovaries	0.328 (0.026)	0.320 (0.04)	0.324 (0.041)	0.334 (0.049)	0.334 (0.065)
Uterus	3.33 (1.51)	2.51 (0.5)	2.45 (0.59)	3.90 (1.55)	3.81 (1.47)

π = data are mean values with standard deviations in parentheses; Statistical significances identified by asterisk (*) are compared to high-fat, corn oil controls; * = P<0.05; ** = P<0.01; A = All tests for statistical significance were determined *via* One-way Analysis of Variance; B = data in parentheses represents Standard Deviation.

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8. Discussion

The safety of refined ARA-rich fungal oils has been the subject of many different studies, all conducted according to Good Laboratory Practices (GLP) and internationally-recognized testing guidelines (*e.g.*, EPA, EC, JMHW, OECD). Boswell *et al.* (1996) reported a NOAEL of 2500 mg/kg bwt/day from a four-week gavage study conducted in male and female Sprague-Dawley (SD) rats and Hempenius *et al.* (1997) reported negative outcomes from four genotoxicity studies of a refined ARA-rich oil, (1) bacterial reverse mutation, (2) gene mutation in mouse lymphoma cells, (3) chromosome aberration in CHO cells, and (4) chromosome damage in mouse micronuclei, all conducted with and without metabolic activation. With the exception of a mouse micronucleus test, identical genotoxicity results were reported later by Arterburn *et al.* (2000b); those authors did not report findings from a mouse micronucleus test. Hempenius *et al.* (1997) also reported the results of an acute Wistar rat toxicity study (LD_{50} = 18.2 g/kg bwt) and four-week repeated dose gavage studies of (1) a refined ARA-rich oil, and (2) a 2:1 mixture of refined ARA-rich oil and a DHA-rich, fish-derived oil. Both four-week studies resulted in a NOAEL for refined ARA-rich oil of 3000 mg/kg bwt/day. Wibert *et al.* (1997) reported the findings of a four-week feeding study in Sprague-Dawley rats in which males and females were fed a mixture of refined ARA-rich oil and DHA-rich algal oil that provided a 2:1 ratio of ARA to DHA; similar, if not identical to the ARA:DHA mixture used by Hempenius *et al.* (1997). In Wibert *et al.* (1997), there were no reported treatment-related adverse effects at dietary concentrations as high as 120 g/kg. Based on the reported male and female intakes of the ARA:DHA mixture, the ARA intake was calculated to have been 4600-8400 mg/kg/day for males and 5300-8800 mg/kg/day for females.

The results from a 90-day gavage study of a refined ARA-rich oil in male and female Crl:CD®BR rats (strain not further identified) were reported by Koskelo *et al.* (1997) as resulting in no signs of toxicity at approximately 1000 mg/kg/day. In addition, the results of 90-day studies (all with preceding four-week *in utero* exposure) were reported by Burns *et al.* (1999) on a refined ARA-rich oil/DHA-rich oil mixture containing 27.8% ARA; by Hempenius *et al.* (2000) on a refined ARA-rich oil containing 38.6% ARA; and by Lina *et al.* (2006) on another refined ARA-rich oil, which contained 41.5% ARA.

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In Burns *et al.* (1999), no adverse effects were reported on male and female SD rats when fed diets containing 2:1 ARA:DHA ratios in 3-fold, 11-fold and 22-fold greater concentrations than are typically found in mother's milk. Hempenius *et al.* (2000) reported feeding Wistar outbred (Crl:(WI)WU BR) rats diets containing a refined ARA-rich oil at concentrations of 3000, 15,000 and 75,000 ppm with a resulting NOAEL at the mid-dose, which was equivalent to a daily intake of approximately 970 mg/kg bwt/day. Following the same protocol and using the same rat strain as reported by Hempenius *et al.* (2000), Lina *et al.* (2006) fed a different refined ARA-rich oil at dietary concentrations of 0.5%, 1.5% and 5.0%. Results from feeding the ARA-rich oil were comparable to those reported by Hempenius *et al.* (2000). Finally, a recent publication by Nisha *et al.* (2009) addressed the safety of an ARA-rich *M. alpina* biomass (35.6% ARA) when fed to male and female Wistar rats at dietary concentrations of 0, 2500, 5000, 7500, 10,000, 20,000 and 30,000 mg/kg for 90 days. Similar to the present work and the previously reported 90-day study findings on refined ARA-rich oils, occasional statistical significance in relative organ weights and serum biochemical/hematological parameters were not supported by histopathology findings, and there were no treatment-related microscopic or macroscopic lesions.

In the present studies, RAO was examined *via* three genotoxicity assays and a 90-day feeding study with an *in utero* exposure. Protocols for these studies were the same as those used in earlier studies of refined ARA-rich oils reported by Hempenius *et al.* (1997), Arterburn *et al.* (2000a), Hempenius *et al.* (2000) and Lina *et al.* (2006).

The administration of RAO at dietary levels up to 5% was well tolerated and affected neither health nor growth of the F_0 rats, their viability, condition, body weights or pup sex ratios. Due to the higher caloric density of the high-fat diets, feed intake in all high-fat groups of the F_0 generation - high-fat controls included - tended to be lower than in low-fat controls; observations that are also identical to earlier reports. Similarly, there were no treatment-related reproductive or developmental index observations. In the 90-day feeding study, F_1 rats showed no treatment-related changes in general condition, neurobehavioral observations, ophthalmology findings, growth, urinalysis or histopathology. As in the F_0 rats, feed intake by F_1 rats tended to be lower

in high-fat groups. This was expected due to the caloric content of the high-fat diets; this result was reported by Lina *et al.* (2006) for other high-fat diet groups.

Although statistical significance was shown for several parameters, none were considered attributable to treatment because they were (1) within historical control ranges, (2) did not demonstrate a dose-response relationship, (3) were seen with comparison to only one control, and/or (4) were seen in only one gender. The only hematological effect not encompassed by this reasoning was increased monocyte numbers in both high-dose males and females. That statistically significant increase in monocytes noted in the high-dose group may have been treatment related, but no effect on monocytes was seen in the mid-dose group.

Among clinical chemistry parameters, cholesterol and phospholipids (both sexes) and triglycerides (males) were decreased in high-dose rats compared to both controls groups, while phospholipids were also decreased in mid-dose females. These plasma lipid decreases were expected and are ascribed to the lipid lowering effects of polyunsaturated fatty acids (Harris, 1989; Hempenius *et al.*, 2000; Hammond *et al.*, 2001; Merrit *et al.*, 2003; Lina *et al.*, 2006). Increased plasma urea and inorganic phosphate concentrations were observed in both sexes of the high-dose group; however, they differed significantly only from the high-fat controls (except for urea in high-dose males, which differed from both control groups). The changes may have been treatment-related but in the absence of confirmatory histopathology, the toxicological significance of these and other changes in clinical chemistry variables in the high-dose group (*e.g.*, increased alkaline phosphatase activity in males, and decreased aspartate aminotransferase activity in females) is doubtful. It can be concluded that there were no toxicologically significant adverse effects on clinical chemistry parameters

The single sex statistically significant adrenal weight increase seen in high-dose females occurred only when compared to the high-fat controls and it was not accompanied by relevant histopathological findings. Thus, this finding was also considered random and of no toxicological consequence. Similarly, the higher relative weights of the liver and spleen in high-dose males and females compared to both control groups were not associated with treatment-related histopathological lesions in these organs and in fact are consistent with previously

reported responses to the administration of high levels of polyunsaturated fatty acids. Many authors reported increased spleen weights without corroborating histopathological findings in mice and rats administered polyunsaturated fatty acids (Danse and Verschuren, 1978; McGuire *et al.*, 1997; Hempenius *et al.*, 1997, 2000; Burns *et al.*, 1999; Rabani *et al.*, 1999; Lina *et al.*, 2006). Also, increased liver weights without morphologic corroboration have been observed in studies with polyunsaturated fatty acids (Burns *et al.*, 1999; Kroes *et al.*, 2003). Therefore, these findings in the high-dose group were considered generic effects of polyunsaturated fatty acids rather than specific effects of the test substance.

The results described for this study of RAO are consistent with earlier reported findings for similar refined ARA-rich oils and corroborate the safety of RAO as an ARA source for use in infant formulas under conditions of use that have already been determined Generally Recognized as Safe by the U.S. Food and Drug Administration and approved by the European Commission under regulation 258-97 concerning novel foods and novel food ingredients.

9. Summaries of published safety testing data on ARA-rich oils and biomass published since 2004

Nisha *et al.* (2009) conducted safety evaluation studies on an ARA-rich biomass. An ARA-rich *M. alpina* biomass (35.6% ARA) was fed to male and female Wistar rats at dietary concentrations of 0, 2500, 5000, 7500, 10,000, 20,000 and 30,000 mg/kg for 90 days. The authors concluded that dietary fortification with the *M. alpina* biomass had a positive influence on growth with no overt toxic effects on the survival, feed consumption or body weight gain throughout the treatment. Statistically significant changes in liver and spleen weights, serum biochemical and hematological indices in *M. alpina* fed groups were not concomitant with pertinent histopathological changes and hence were considered toxicologically inconsequential. Increases in organ weights in response to dietary consumption of LCPUFA have been seen previously and is believed to represent a physiological adaptation to the high dietary levels of fatty acids (Lina *et al.*, 2006). No microscopic or macroscopic lesions attributable to the treatment were seen in the experimental groups.

In Lina *et al.* (2006) rats were fed SUNTGA40S as a source of ARA at dietary concentrations of 0.5%, 1.5% and 5.0%. As in the above summarized 90-day study of RAO, Lina

et al. (2006) included a 4-week pretreatment period of the F_0 parental rats and continued exposure of the dams throughout mating, gestation and lactation. No adverse test article-related effects occurred at even the highest dietary RAO concentration administered in that study at 1.5% in the diet, equal to approximately 3,000 mg RAO/kg bwt/day and noadverse effects were seen upon health, growth, fertility or reproductive performance and there was an absence of adverse effects on pup characteristics. The pups had no significant differences in general condition, neurobehavioral observations, ophthalmoscopy, growth, urinalysis or macroscopic or microscopic findings between the test groups and low-fat or high-fat dietary control groups. Macroscopic examination of parental animals at necropsy and microscopic examinations of organs and tissues did not reveal any treatment-related findings not typically found in rats of this strain and age. Some statistically significant findings were seen in hematology, clinical chemistry and spleen weight parameters. The types of changes observed were considered typical to those seen in other studies following repeated exposure to high levels of polyunsaturated fatty acids.

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