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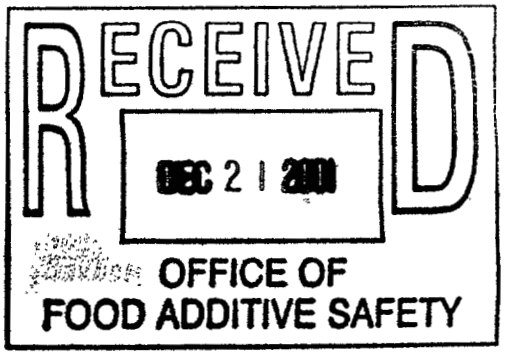
December 18, 2001



Linda Kahl, Ph.D.
Office of Premarket Approval (HFS-215)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street SW
Washington, D.C. 20204

Re: GRAS Exemption Claim for Docosahexaenoic Acid Rich Oil Derived from Tuna (DHA-rich oil) and Arachidonic Acid Rich Oil Derived from *Mortierella alpina* Peyronel 1S-4 (*M. alpina*) (AA-rich oil; SUNTGA40S) as Sources of DHA and AA in Term and Preterm Infant Formulas

Dear Dr. Kahl:

We wish to notify you that Ross Products Division, Abbott Laboratories, has determined that Docosahexaenoic Acid Rich Oil derived from tuna (DHA-rich oil; Mochida International Co., LTD, Tokyo, Japan) and Arachidonic Acid Rich Oil derived from *Mortierella alpina* Peyronel 1S-4 (*M. alpina*) (AA-rich oil; SUNTGA40S; Suntory LTD, Osaka, Japan) are GRAS as sources of DHA and AA in Term and Preterm Infant Formulas.

Determination of the GRAS status of AA-rich oil and DHA-rich oil has been made through the deliberation of experts, Dr. Vasilios Frankos (ENVIRON International Corporation), Dr. Sheila Innis (University of British Columbia), Dr. Michael Georgieff (University of Minnesota), Dr. M.W. Anders (University of Rochester Medical Center), Dr. Sanford Miller (Georgetown Center for Food and Nutrition Policy), and Dr. Howard Sprecher (The Ohio State University). The Expert Panel reviewed all the available data on the short-term and long-term toxicity of AA-rich oil and DHA-rich oil, human exposure to AA and DHA through human milk, and human clinical safety studies evaluating safety of administered intakes of AA-rich oil and DHA-rich oil and have concluded that:

There is no evidence in the available information on AA-rich oil and DHA-rich oil that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when it is used at levels that are now current or that might reasonably be expected from the proposed applications. AA-rich oil and DHA-rich oil are GRAS for use as ingredients in term and preterm infant formulas, as proposed by Ross Products Division, Abbott Laboratories.

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www.environcorp.com

It is the opinion of the Expert Panel that other qualified and competent scientists reviewing the same publicly available data would reach the same scientific conclusion. Therefore, AA-rich oil and DHA-rich oil, to be used as ingredients in term and preterm infant formulas, are GRAS, when used to supplement preterm and term infant formula at target mean levels of: 0.40% and 0.25% , respectively for Similac® Special Care®; 0.40 % and 0.15%, respectively, for Similac NeoSure® and; 0.40% and 0.15%, respectively, for Similac® With Iron.

In accordance with the criteria set forth in the GRAS notification proposed regulation found at 62 Fed. Reg. 18938, 18961 (1997), Ross Products Division, Abbott Laboratories, submits the following information as part of its GRAS exemption claim.

Name and Address of Notifier

Ross Products Division
Abbott Laboratories
625 Cleveland Avenue
Columbus, Ohio 43215-1724

Name of GRAS Substances

Docosahexaenoic Acid (DHA) Rich Oil Derived From Tuna (DHA-rich oil)

Arachidonic Acid (AA) Rich Oil Derived From *Mortierella alpina* Peyronel 1S-4 (*M. alpina*) (AA-rich oil; SUNTGA40S)

Intended Use

AA-rich oil and DHA-rich oil are intended to be added to preterm infant formulas (Similac® Special Care® and Similac NeoSure®) and term infant formula (Similac® With Iron). Similac® Special Care® (SSC) is an exempt infant formula used in the hospital management of premature infants. Similac NeoSure® is an exempt infant formula used in the post-discharge management of premature infants. Similac® With Iron (SWI) is a non-exempt infant formula used for the routine feeding of term infants. Levels of addition of the oils to formulas will result in target mean levels of AA and DHA (g/100g fatty acids) as follows: SSC (0.40% and 0.25%, respectively); Similac NeoSure® (0.40% and 0.15%, respectively); SWI (0.40% and 0.15%, respectively).

Basis for GRAS Determination

AA-rich oil and DHA-rich oil have been determined to be GRAS ingredients on the basis of scientific procedures.

Availability of Data

The data and information that serve as the basis for this GRAS notification, including information in appendices, will be sent to FDA upon request or are available for the FDA's review and copying at reasonable times at the office of Claire Kruger, Ph.D., Principal, ENVIRON International Corporation, 4350 North Fairfax Drive, Suite 300, Arlington, VA. 22203, telephone: (703) 516-2309, facsimile: (703) 516-2393.

GRAS Exemption Claim

The uses of AA-rich oil and DHA-rich oil as sources of AA and DHA in term (Similac® With Iron) and preterm (Similac® Special Care® and Similac NeoSure®) infant formulas, will result in target mean levels of AA and DHA (g/100g fatty acids) as follows: SSC (0.40% and 0.25%, respectively); Similac NeoSure® (0.40% and 0.15%, respectively); SWI (0.40% and 0.15%, respectively). Because AA-rich oil and DHA-rich oil are GRAS for the intended use, they are excluded from the definition of a food additive, and thus may be marketed for this use without the need to promulgate a specific food additive regulation under 21 CFR.

We enclose an original and two copies of this notification for your review. If you have any questions, please contact me at the above phone number and address.

Sincerely,

Claire L. Kruger, Ph.D., D.A.B.T.
Principal
ENVIRON Corporation

Enclosures

GRAS DETERMINATION FOR DOCOSAHEXAENOIC ACID RICH OIL DERIVED FROM TUNA AND ARACHIDONIC ACID RICH OIL DERIVED FROM *MORTIERELLA ALPINA*

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OFFICE OF
FOOD ADDITIVE SAFETY

Prepared for

Ross Products Division
Abbott Laboratories
Columbus, Ohio

Prepared by

ENVIRON International Corporation
Arlington, Virginia

December 12, 2001

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Attachment 3	Review of Plasma Cholesterol
Attachment 4	Human Studies

I. GRAS EXEMPTION CLAIM

A. Name and Address of Notifier

Ross Products Division
Abbott Laboratories
625 Cleveland Avenue
Columbus, Ohio 43215-1724

B. Name of GRAS Substances

Docosahexaenoic Acid (DHA) Rich Oil Derived From Tuna (DHA-rich oil)

Arachidonic Acid (AA) Rich Oil Derived From *Mortierella alpina* Peyronel 1S-4 (*M. alpina*) (AA-rich oil; SUNTGA40S)

C. Intended Use

AA-rich oil (SUNTGA40S) and DHA-rich oil are intended to be added to preterm infant formulas (Similac® Special Care® and Similac NeoSure®) and term infant formula (Similac® With Iron). Similac® Special Care® (SSC) is an exempt infant formula used in the hospital management of premature infants. Similac NeoSure® is an exempt infant formula used in the post-discharge management of premature infants. Similac® With Iron (SWI) is a non-exempt infant formula used for the routine feeding of term infants. Levels of addition of the oils to formulas will result in target levels of AA and DHA (g/100g fatty acids) as follows: SSC (0.40% and 0.25%, respectively); Similac NeoSure® (0.40% and 0.15%, respectively); SWI (0.40% and 0.15%, respectively).

D. Basis for GRAS Determination

1. Introduction

In this document, we demonstrate that intake of AA-rich oil derived from *M. alpina* (AA-rich oil) and DHA-rich oil derived from tuna (DHA-rich oil), resulting from its intended use in preterm and term infant formulas, is safe, and is also Generally Recognized As Safe ("GRAS"), under the Federal Food, Drug, and Cosmetic Act (FDCA or the Act). To accomplish this, first, the safety of intake of AA-rich oil and DHA-rich oil under the intended conditions of use is established. Then, this intake level is determined to be GRAS by demonstrating that the safety of AA-rich oil and DHA-rich oil for the intended use is generally recognized by experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food.

The regulatory framework for establishing whether a substance is GRAS in accordance with Section 201(s) of the FDCA is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: (1) through

scientific procedures (§170.30(b)); or (2) through experience based on common use in food in the case of a substance used in food prior to January 1, 1958 (§170.30(c)). A scientific procedures GRAS determination under §170.30(b) requires the same quantity and quality of scientific evidence as is required to obtain approval of the substance as a food additive.

Moreover, in addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted. This so-called "common knowledge" element of a GRAS determination consists of the following two components: (1) the data and information relied upon to establish the scientific element of safety must be generally available; and (2) there must be a basis to conclude that a consensus exists among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a GRAS determination based on scientific procedures, are applied below in an assessment of whether AA-rich oil and DHA-rich oil, employed as ingredients in term (SWI) and preterm (SSC and Similac NeoSure®) infant formulas are GRAS. Addition of the oils to formulas will result in target levels of AA and DHA as follows: SSC (0.40% and 0.25%, respectively); Similac NeoSure® (0.40% and 0.15%, respectively); SWI (0.40% and 0.15%, respectively).

2. Safety of AA-Rich Oil and DHA-Rich Oil for the Proposed Uses

a) Traditional Approach for Evaluating the Safety of Food Additives

The regulatory criterion by which the safety of a food additive is judged is that "there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use" (21 CFR § 170.3(i)). This regulation specifies that three factors be considered in determining safety. These are:

- The probable consumption of the substance and of any substance formed in or on food because of its use;
- the cumulative effect of the substance in the diet, taking into account any chemically or pharmacologically related substance or substances in such diet;
- safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food and food ingredients, are generally recognized as appropriate.

After consideration of these factors, the FDA usually establishes an acceptable daily intake (ADI) for the additive. The ADI represents the maximum amount of the additive that can be safely consumed by humans on a daily basis for a lifetime. The FDA has specified that an ADI is usually established by application of a safety factor of at least 100 to the lowest NOAEL identified in the most sensitive animal species studied. The FDA also considers evidence that

might justify use of a different safety factor (21 CFR § 170.22). Except where evidence is submitted which justifies use of a different safety factor, a safety factor in applying animal experimentation data to man of 100 to 1 is used; that is, tolerance for the use of a human food ingredient will not exceed 1/100th of the maximum amount demonstrated to be without harm to experimental animals.

b) Approach for Evaluating the Safety of AA-Rich and DHA-Rich Oils

The approach to the safety evaluation of AA- and DHA-rich oils is modeled on the strategy developed for nutrients by the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes Food and Nutrition Board Institute of Medicine (IOM).

The Committee developed the concept of Tolerable Upper Intake Level (UL), which is the highest level of daily nutrient intake that is likely to pose no risks of adverse health effects to almost all individuals in the general population. The model that describes dietary reference intakes is shown in Figure I-1.

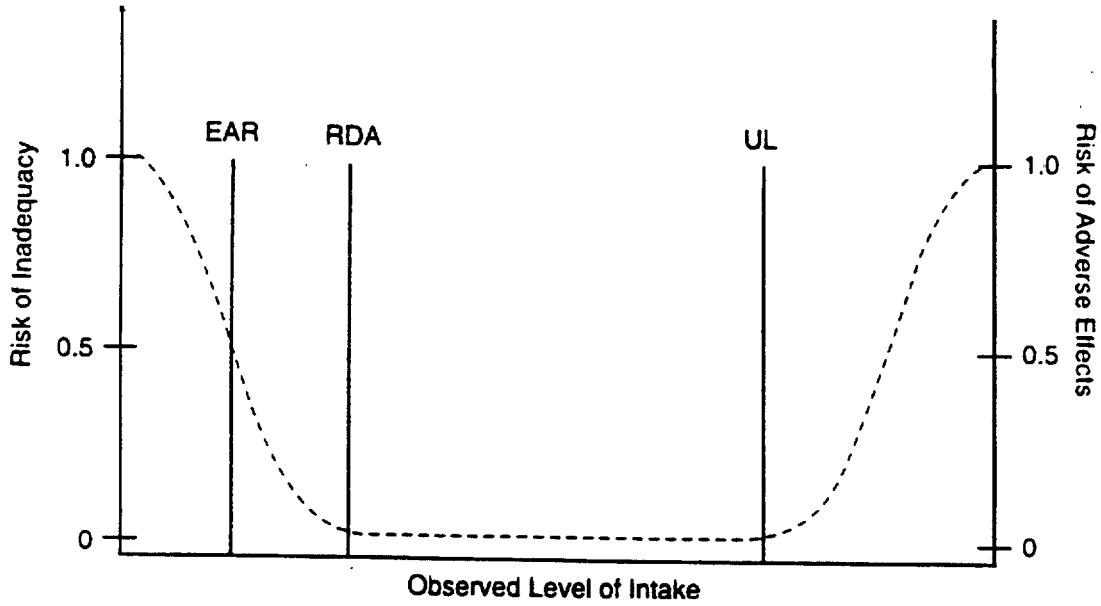

Figure I-1
Dietary Reference Intakes

This figure shows that the Estimated Average Requirement (EAR) is the intake at which the risk of inadequacy is 0.5 (50%) to an individual. The Recommended Dietary Allowance (RDA) is the intake at which the risk of inadequacy is very small-only 0.02 to 0.03 (2-3%). The Adequate Intake (AI) does not bear a consistent relationship to the EAR or the RDA because it is set without being able to estimate the average requirement. It is assumed that the AI is at or above the RDA if one could be calculated. At intakes between the RDA and the Tolerable Upper Intake Level (UL), the risks of inadequacy and of excess are both close to 0. At intakes above the UL, the risk of adverse effect may increase.

It can be seen that, unlike the situation with non-nutrients, there is a risk of inadequacy at levels of intake that are too low, with an increasing risk of adverse effects as intake levels above the UL. Like all chemical agents, it is recognized that nutrients can produce adverse health effects if intakes are excessive. Unlike the situation for assessing risk of an environmental chemical, however, nutrients are essential for human well-being and often for life itself within a certain range of intakes. An appropriate model for derivation of ULs consists of a set of scientific factors that should be considered explicitly. The risk assessment approach that is used to consider these factors is subject to two types of scientific uncertainties: (1) those related to data and (2) those associated with any inferences that are required when directly applicable data are not available. The data on nutrient toxicity are generally available from studies in human populations and, therefore, may not be subject to the same uncertainties (related to

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interspecies extrapolations) associated with the available data on nonessential chemicals.

A principal feature of the risk assessment process for noncarcinogens is the long-standing acceptance that no risk of adverse effects is expected unless a threshold dose (or intake) is exceeded. The adverse effects that may be caused by a nutrient almost certainly occur only when the threshold dose is exceeded.

The primary types of data used as background for identifying nutrient hazards in humans are human and animal studies. Six key issues that are addressed in the data evaluation are: (1) evidence of adverse effects in humans; (2) causality; (3) relevance of experimental data, including utility of animal data, route of exposure, duration of exposure; (4) mechanisms of toxic action; (5) quality and completeness of the database, and; (6) identification of distinct and highly sensitive subpopulations.

The UL is generally based on a no-observed-adverse-effect level (NOAEL) that is identified for a specific circumstance in the hazard identification and dose-response assessment steps of the risk assessment. The NOAEL is the highest intake (or experimental dose) of a nutrient at which no adverse effects have been observed in the individuals studied. The derivation of a UL from a NOAEL involves a series of choices about what factors should be used to deal with uncertainties. Uncertainty factors (UFs) are attempts to deal with gaps in data (for example, lack of data on humans or lack of adequate data demonstrating a NOAEL) and with incomplete knowledge regarding the inferences required (for example, the expected variability in response within the human population). In general, when determining a UF, interindividual variation in sensitivity, extrapolation from animal to human, extrapolation from LOAEL to NOAEL, and use of subchronic data to predict chronic data are all considered as potential sources of uncertainty.

The UL is derived by dividing the NOAEL (or LOAEL) by all the relevant UFs. The derivation of a UL involves the use of scientific judgment to select the appropriate NOAEL (or LOAEL) and UF. The characterization of the potential risk (for example, scientific judgment used in deriving a UL from a NOAEL) is provided from a nutritional risk assessment perspective. It requires explicit consideration of all choices made, both regarding the data used and the uncertainties accounted for.

c) Tolerable Upper Intake Level (UL) for AA-Rich Oil and DHA-Rich Oil

The risk assessment model designed to derive ULs for nutrients is used to derive the UL for AA-rich oil and DHA-rich oil as ingredients in infant formula. To do this, a systematic evaluation of information available on the composition of the oil sources, and an evaluation of an extensive database, consisting of both animal and human exposure and safety studies, was completed. The nature and quality of available information was critically analyzed to determine the

appropriate NOAEL and UF for derivation of the UL for AA-rich oil and DHA-rich oil in infant formulas.

Sources of AA-Rich Oil and DHA-Rich Oil

AA-rich oil (SUNTGA40S) is a product of fermentation of the fungal species *M. alpina* Peyronel 1S-4. *M. alpina* is also the source of ARASCO, an AA-rich single-cell oil that is determined to be GRAS (Letter of May 17, 2001 from FDA in response to GRN 000041). The subject of this GRAS determination is the oil SUNTGA40S.

SUNTGA40S is essentially identical to ARASCO; the major differences observed between the two oils are in the unsaponifiable matter and sterol content (information taken from GRN 00041). SUNTGA40S has a much lower amount of unsaponifiable matter (0.315, 0.30 and 0.37%) compared to Martek's ARASCO oil (1.73, 1.36, 1.69 and 1.18%). Sterol content for SUNTGA40S (0.24, 0.27 and 0.34%) is also much lower than Martek's ARASCO oil (1.5%). The manufacturing process for SUNTGA40S removes more of these impurities than the process for manufacturing ARASCO.

Some of the studies presented in the GRAS determination were conducted on SUNTGA25. SUNTGA40S is a more pure form of AA-rich oil than SUNTGA25. Specifications for SUNTGA40S oil are set at appropriate levels to ensure that there are no unacceptable levels of free fatty acids, unsaponifiable material, heavy metals, peroxide, PCB or pesticides.

M. alpina Peyronel 1S-4 is not pathogenic and does not produce any toxins of concern. A mycotoxin screen indicates no mycotoxin contamination. Results of toxicity tests which have been conducted on SUNTGA25, unsaponifiable matter of SUNTGA25 and the fungus culture, have indicated that *M. alpina* Peyronel 1S-4 does not produce any toxins of concern. A review of the changes introduced into the production process to manufacture SUNTGA40S confirm that the changes do not introduce new impurities or alter the composition of the oil in a qualitative manner compared to SUNTGA25. A final product composition comparison indicates that the major change is an increase in the AA content (from approximately 25% in SUNTGA25 to approximately 40% in SUNTGA40S), a reduction in the unsaponifiable matter and reduction in the sterol, 24,25-methylenecholest-5en-3β-ol.

The biological significance of exposure to the novel sterol produced by *M. alpina* Peyronel 1S-4 was evaluated by examining its potential for estrogenic activity. The SUNTGA40S product was evaluated for estrogenic activity in three *in vitro* studies. Results of these studies demonstrate that SUNTGA40S does not bind *in vitro* to the estrogen receptor. The total unsaponifiable components and cyclopropanesterol, by-products in SUNTGA40S, were able to weakly displace [^{3}H]estradiol from the estrogen receptor. Therefore, estrogenic and anti-estrogenic activity of SUNTGA40S and total unsaponifiable components was

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studied *in vitro*. Neither test article functioned as an agonist or antagonist of human estrogen receptor in yeast.

The source for DHA-rich oil is tuna. The oil is manufactured by standard oil processing techniques; mixed tocopherols or ascorbyl palmitate and monoglycerides are added to help preserve quality. Specifications for DHA-rich oil are set at appropriate levels to ensure that there are no unacceptable levels of free fatty acids, unsaponifiable material, heavy metals, peroxide, PCB, dioxin or pesticides. In addition, the specification for an upper limit of EPA content and a target for DHA/EPA ratio is included to ensure that EPA levels introduced into the final infant formula from fish oil supplementation will not produce untoward effects. The GRAS affirmation of menhaden oil (21 CFR §184.1472) specifies that at levels of intake of EPA plus DHA less than 3000 mg/person/day (50 mg/kg/day for a 60 kg person), there is no significant risk for increased bleeding time beyond the normal range. A calculation of the mean DHA plus EPA intake by infants from formulas supplemented with DHA-rich oil as proposed in this GRAS document, is 26 mg/kg/day for preterm infants from SSC, 13 mg/kg/day from Similac NeoSure® and 10 mg/kg/day from SWI. In addition, clinical studies in infants of AA-rich and DHA-rich oil supplemented formula indicated that there is no concern for an adverse effect on hematologic parameters as a result of product ingestion. A study conducted in neonatal piglets using DHA-rich oil corroborated a lack of adverse effect on hematologic parameters at levels of ingestion at least 7-fold over estimated human infant ingestion (Ross Products Division 2001).

Currently, DHA-rich oil from tuna is used in infant formulas, yogurt, bread and fish sausage in Japan and in infant formulas, bread and fish sausage in Korea.

Adult Rat Toxicology Studies of AA-Rich Oil (SUNTGA25; SUNTGA40S) and DHA-Rich Oil (derived from tuna)

SUNTGA25 and SUNTGA40S (AA-rich oils derived from *M. alpina*) have been tested in acute, subacute, and subchronic assays conducted in adult rats and in mutagenicity assays. No mutagenicity was seen in an Ames/Salmonella assay with SUNTGA25. No chromosome aberrations were seen in an assay from a Chinese hamster fibroblast cell line with SUNTGA25.

Two subchronic adult rat toxicity studies have been conducted on AA-rich oils produced by Suntory, one with SUNTGA25 and one with SUNTGA40S. SUNTGA25 was tested at dose levels of 600, 1200 and 3000 mg/kg for 13 weeks (Suntory 1992b). There were no consistent treatment related adverse effects seen with SUNTGA25 administration, with the exception of effects in the liver. At the mid- and high dose levels of SUNTGA25 there was a statistically significant increase in relative liver weight and an apparent increase in the incidence of vacuolar degeneration (fatty change). The increase in vacuolar degeneration was slight for the high dose groups compared to controls. There was no correlation

between the histopathologic findings and the increase in liver weight seen at the mid- and high doses. Sudan III staining was minimal to slight in all dose groups, including control. Although fatty change may have contributed slightly to the liver weight increase seen, the data was contradictory and inconclusive.

A subchronic study (13 weeks) of adult rats was conducted with SUNTGA40S and DHA-rich oil (Suntory 1997). SUNTGA40S was tested in three dose groups at mean dose levels of 312 (0.5% of diet), 626 (1% of diet) and 1255 (2% of diet) mg/kg/day. An additional group tested SUNTGA40S + DHA-rich oil (1% of diet for each oil; 2% total) at a dose of 644 mg/kg/day for each oil (total of 1,287 mg/kg/day AA-rich oil + DHA-rich oil). In all dose groups given AA-rich oil, males, but not females, showed a statistically significant increase in activated partial thromboplastin time (APTT) compared to control. All values were within historical control range, however, and not associated with an adverse health consequence to the animals. No other treatment-related adverse findings were seen at any dose level of SUNTGA40S. Statistically significantly increased APTT and PT levels were seen in males but not females from the AA-rich + DHA-rich oil group compared to control. The value for PT was within historical control range, however the APTT value was slightly higher than historical control (30.1 ± 4.7 compared to the maximum historical value of 29.6 seconds). The finding of significantly increased APTT and PT levels are confounded in the AA-rich + DHA-rich oil test group by the presence of DHA and EPA in the basal diet. It is also possible that a mild vitamin K deficiency produced by the basal diet may have exacerbated effects of the fish oil in the AA-rich + DHA-rich oil test group. In addition, because this finding was only seen in males, who received a lower dose of DHA + EPA on a mg/kg bodyweight basis than females, it is unclear that the increased PT and APTT can be related solely to test article administration. The finding was not associated with an adverse health consequence to the animals. (The finding in this rat study of increased APTT and PT was not reproduced in a neonatal piglet study of AA-rich and DHA-rich oils). No other adverse dose or test article related findings in clinical chemistry, hematology, organ weights, gross pathology or histopathology were seen.

Based on the results of this study, administration of AA-rich oil as 2% by weight of the diet and administration of 1% DHA-rich oil in combination with 1% AA-rich oil in the diet (by weight), did not produce test article related effects associated with adverse health consequences in adult rats.

Adult Rat Toxicology Studies of AA-Rich Oils and DHA-Rich Oils from Other Sources

In order to evaluate the safety of AA-rich oil derived from *M. alpina* (alone or with a DHA source), additional rat toxicology studies that were conducted using other AA containing oils from this fungal source were examined. The studies differed in some important aspects with regard to study design. Dose levels, negative controls used, positive or vehicle controls used, and percent of total fat in the diet are not standardized among studies. In studies of this type,

where the test material is a source not only of nutrition, but also may have a bioactive effect, there is a high degree of difficulty associated with interpreting results produced at high levels of exposure. The physiologic and nutritional impact of high levels of such a test article added to the diet can be difficult to distinguish from a toxicological manifestation of exposure. An added confounding factor is derived from the addition of other oils to some test material groups in order to standardize fat intake. The added oils themselves have the potential to produce nutritional disturbances at high levels of addition. Another difficulty with the study designs is defining the appropriate comparison group. Some studies used a standard rat chow control, however, this will not give an adequate comparison in terms of assessing the contribution of the fat intake on the results. Although some studies utilized a group fed a high fat diet as a point of comparison for test groups, the result of this comparison, is for all intents and purposes, comparing one test group against another test group.

Interpretation of results from animal studies conducted with an AA containing oil, alone or in combination with a DHA containing oil, is not a straightforward matter, nevertheless, it is possible to analyze the patterns of target organ effect, dose-response and gender specificity indicated across studies to form conclusions regarding the safety of *M. alpina* as a source of oil for AA. In response to GRN 000007, FDA highlighted five areas of concern from an initial review of animal toxicology data of ARASCO and DHASCO. These concerns were: potential for test article related effects on liver, spleen, kidney, hematologic parameters and reproductive parameters (Letter from FDA in response to GRAS Notice No. GRN 000007).

These issues were subsequently addressed in GRN 000041, Martek's Notification for ARASCO and DHASCO. FDA cited in their May 17, 2001 response to GRN 000041 the conclusions upon which Martek based their GRAS determination of ARASCO and DHASCO. In Martek's view, these polyunsaturated fatty acids (PUFAs) pose unique testing problems because it can be difficult to distinguish whether an observed effect related to the test material is a normal physiological response to the high dietary load of that particular macronutrient, a dietary deficiency that is related to the presence of a large amount of test material in the diet or a toxicological effect. In Martek's view, statistically significant effects, including increased relative liver weight, increased relative spleen weight and a change in some blood chemistries reported in some of the animal toxicology studies are not adverse toxicological findings related to ARASCO and DHASCO as sources of AA and DHA, respectively. Based on the information provided by Martek, FDA has no questions at this time regarding Martek's conclusion that ARASCO and DHASCO are GRAS sources of AA and DHA, respectively for term infant formula (May 17, 2001 response to GRN 000041).

An independent examination, conducted as part of this GRAS evaluation of AA-containing and DHA-containing oils, determined that repeat dose adult rat

studies of other AA-containing oils derived from *M. alpina* alone or in combination with a DHA-containing oil, show increased liver and spleen weights at high doses of the oils.

It was clear from a review of all studies conducted using an AA-containing oil from *M. alpina* (alone or with a DHA source), that after 13 weeks, liver weights were increased at levels of addition of test oil ≥ 3000 mg/kg/day (alone or in combination with a source of DHA). The liver weight increases seen at very high dose levels of AA containing oil alone or in combination with DHA-containing oil (≥3000 mg/kg/day for 13 weeks) were not associated with corroborative evidence from clinical chemistry or histopathology to indicate that this finding is a serious irreversible toxic event. There was no evidence of hepatocellular hyperplasia, extensive inflammatory infiltrates, necrosis or neoplasia. The effects seen on liver weight are similar to those seen with the administration of other fatty acids at high dose levels, particularly fish oils (Rustan et al. 1992; Skuladottir et al. 1994; Murphy et al. 1997; Yaqoob et al. 1995; Mohan et al. 1991; De Craemer et al. 1994; Yamazaki et al. 1987). The liver histopathology findings in all studies of AA-containing oil (alone or in combination with a source of DHA-containing oil) are minimal to slight and would be considered to be a reversible change. Hepatic vacuolation was seen not only in the test groups, but frequently in the high fat control animals. There was no evidence from histopathologic examination that fat accumulation accounted for the entire increase seen in liver weight. The periportal cytoplasmic alteration found in response to AA oil administration was determined from histopathologic analysis to be consistent with increased glycogen storage. Although it can not be completely excluded, evidence from a follow-up study strongly suggests that the liver effects seen at doses up to 5000 mg/kg of AA-rich oil were not due to peroxisome proliferation. There does not appear to be any consistent treatment related effect on liver at doses levels ≤3000 mg/kg, suggesting that this is a threshold for the response.

In rat studies conducted with AA-containing oil (alone or with a source of DHA) increased spleen weight was seen after 13 weeks of administration at high doses of test article intake. This effect has also been seen in rat studies of menhaden oil administration (Yaqoob et al. 1995), supporting the view that increased spleen weight at high levels of ingestion of PUFAs in animal studies is a general response to high PUFA intake and not necessarily a toxic phenomenon associated with a particular source.

An evaluation of the clinical chemistry and histopathology data from the rat studies conducted using AA-containing oil and DHA-containing oil indicates that although spleen weight increases are noted, there is no corroborative data suggesting a toxic or irreversible effect. Routine hematology and clinical chemistry parameters related to the immune system did not reveal evidence of an immunotoxic effect. It is postulated that spleen weight increase is an adaptive response in these animals to high doses of PUFAs. PUFAs may have profound

biological activity within the immune system. It is also clear that there is a threshold for this response and a moderate amount of (n-6) and (n-3) PUFA are necessary for a competent immune response. In animal studies, no consistent effect on spleen weight is seen at doses ≤1700 mg/kg /day for 13 weeks.

Neonatal Piglet Studies of AA-Rich Oil and DHA-Rich Oil

Data presented from an analysis of adult rat studies of AA-rich oil, with and without a source of DHA-rich oil indicates that at doses lower than 1700 mg/kg/day, no adverse effects are expected in that species. It is not possible, however, to extrapolate the results from adult rat studies to human infants. Adult rats, for example, are poor models for evaluating the consequences of high fat intakes. An adult rat diet is typically 5% fat by weight (2.7% fat as percent of caloric value) as opposed to an infant diet where 50% of the calories are derived from fat. The differences between adult animals and neonatal animals, such as in GI absorption, make it impossible to predict if a neonate would be more sensitive to the effects of oil ingestion or, if they are more sensitive, by how much.

It is critical, therefore, to the safety evaluation of AA-rich oil (SUNTGA40S) and DHA-rich oil (derived from tuna), as ingredients in infant formulas, to evaluate them in an animal model that can be used for extrapolation to the human infant. A protocol for a neonatal piglet study was carefully designed utilizing the information gleaned from previous work in the adult rat model as well as available information on bioactive ingredients. The objective of the study was to assess any unique susceptibility of a neonatal animal, a surrogate model for human neonate, to possible adverse effects of AA-rich or DHA-rich oils.

The newborn piglet is a commonly used model for the study of human developmental gastrointestinal function (Moughan 1992; Miller 1987), and long chain polyunsaturated acid metabolism (Chiang 1989). Responsiveness of organs such as liver, brain and bone to long chain polyunsaturated acid feeding has been reported (Arbuckle 1991; de la Presa-Owens 1998; Weiler 2000). Swine milk is similar to human milk in its fat and fatty acid content (Rioux 1997; Arbuckle 1991; Weiler 2000) and the animals grow very rapidly in the first three weeks of life. The rapid growth of all organs in the piglet, which grow faster than those of the human infant, may increase the sensitivity of the piglet to potential adverse effects of AA-rich or DHA-rich oils. The newborn piglet provides the opportunity to observe the interaction between high nutrient requirements and relative metabolic immaturity in a situation analogous to the human infant.

Piglets delivered at term gestation were chosen as the appropriate animal model for this study. There is no current practical premature piglet animal model. Delivering animals even a few days prior to term results in respiratory immaturity that makes extrauterine survival difficult in the absence of specialized respiratory support (personal communication, Peggy Borum, Ph.D.). Other potential complications with the premature model include the need for medications (including antibiotics) related to a very immature immune system. The term

piglets used in this study were provided with maternal colostrum for three days so that exogenous immunologic support (e.g., immunoglobulin) will not be required. This is provided by allowing the piglet to suckle after term delivery. An alternative, postnatal injection of gamma globulin, was rejected due to potential masking effects that this might have on any adverse effects of the test articles (personal communication, FDA meeting December 19, 2000).

An analysis of results from the adult rat toxicology studies of several AA containing oils from *M. alpina*, with and without a source of DHA, indicates that at doses of these long-chain polyunsaturated fatty acids below 1700 mg/kg/day, no adverse effects are expected in that species. Increased liver and spleen weights seen at higher doses in the adult rat model may be the result of nutritional imbalance, pharmacologic activity or impurity profile. The margin of safety between the NOAELs determined from these adult rat studies and the calculated human exposure is on the order of 10-fold on a body weight basis and is appropriate for this type of ingredient.

Unlike traditional food additives, bioactive ingredients (such as AA and DHA), similar to nutrients, typically do not have a large margin of safety between the recommended level of intake and the Tolerable Upper Intake Level (UL). A summary of the calculated margins of safety (UL/Recommended Dietary Allowance (RDI) or Adequate Intake (AI)) for several nutrients indicates that for these ingredients, typical margins of safety lie between 2 and 10. This contrasts with the higher margins, i.e., 100-fold, typically generated for traditional food additives.

It was therefore reasonable to choose dose levels for the piglet study that provided an exaggeration of expected human intake within the margins of safety established for nutrients, and by extrapolation, for other bioactive ingredients.

Dose levels were chosen on the basis of an exaggeration of the estimated mean intake of AA and DHA by infants consuming formula containing target levels of AA-rich oil and DHA-rich oil. This exaggeration was intended to test tolerance of the neonatal piglet to levels of consumption of AA, DHA and AA + DHA that are, respectively: 5-fold, 5-fold and 3 fold + 3-fold higher than the estimated intakes of infants to these long-chain polyunsaturated fatty acids. The highest estimated mean intake of AA and DHA by infants was found in the preterm infant clinical trial of Similac® Special Care®. At term corrected age, these infants consumed on average approximately 32 mg/kg/day AA and approximately 20 mg/kg/day DHA. (The lowest intakes of AA and DHA were 12 and 3 mg/kg/day, respectively, at 12-months in the term infant study of Supplemented Similac® with Iron).

At a 5-fold exaggeration of the estimated mean AA intake, the target AA dose for piglets was 160 mg/kg/day. At a 5-fold exaggeration of the estimated mean DHA intake, the target DHA dose for piglets was 100 mg/kg/day. At a 3-

fold exaggeration of the estimated mean AA + DHA intakes, the target AA + DHA dose for piglets was 96 + 60 mg/kg/day, respectively (total 156 mg/kg/day AA + DHA).

In the neonatal piglet study, actual mean intake of AA was 250 mg/kg/day, an 8-fold exaggeration of estimated human infant AA intake for preterm infants ingesting SSC at term corrected age (estimated from Study AG38). The exaggeration exposure to AA was 21-fold for term infants consuming SWI at 12 months in the term infant study (Study AF92). For DHA, actual mean intake was 136 mg/kg/day, 7-fold exaggeration of estimated human infant DHA intake for preterm infants ingesting SSC at term corrected age (estimated from Study AG38). The exaggeration of exposure to DHA was 45-fold for term infants consuming SWI at 12 months in the term infant study (Study AF92). Actual mean intake of AA + DHA was 236 mg/kg/day, a 4-fold exaggeration of human infant AA + DHA intake for preterm infants ingesting SSC at term corrected age (estimated from Study AG38). Exaggeration of exposure for term infants consuming SWI at 12 months in the term infant Study (AF92) is 16-fold.

Administration of AA-rich oil, DHA-rich oil and AA-rich oil + DHA-rich oil to neonatal piglets did not produce adverse effects resulting in adverse health consequences to the animals under the conditions of this study. Mean intake of AA-rich oil, DHA-rich oil and AA-rich + DHA-rich oils (mg/kg/day) for male piglets was 618, 653 and 365 + 387, respectively; for female piglets, mean intake (mg/kg/day) was 623, 691 and 388 + 412, respectively. There were no test article related effects that would result in adverse health consequences on clinical signs, body weights, food consumption, clinical chemistry and hematology, organ weights or gross and histopathology. The doses given resulted in exaggerations of exposure to AA, DHA and AA + DHA of at least 8-fold, 7-fold and 4-fold over human preterm infant exposure, respectively, which are greater than the intended target margins of safety for this study. The safety of ingestion of AA-rich and DHA-rich oils was determined in the neonatal piglet model at doses that establish an appropriate margin of safety consistent with that established for many nutrients. Results from this study corroborate the findings from the clinical trials conducted by Ross Products. It is reasonable to assume that no adverse effects would occur from ingestion of AA-rich and DHA-rich oils at the intended levels of exposure.

Corroborative evidence for a lack of adverse effect on liver weight in the neonatal piglet model is derived from a study reported by de la Presa-Owens et al. (1998). In this study, neonatal piglets were fed AA-rich oil (SUNTGA25) or DHA-rich oil (derived from tuna) using formulas containing 0.8% AA or 0.3% DHA. There was no significant increase in absolute or relative liver weights in the AA-rich oil supplemented formula-fed group compared to control formula fed piglets. A significant decrease in relative liver weight was seen in the group given DHA-rich oil supplemented formula compared to formula fed control. There were no significant differences in absolute or relative liver weights in the

AA- or DHA-rich oil supplemented formula groups compared to the sow milk reference group.

Clinical Studies of AA-Rich Oil and DHA-Rich Oil

There are several lines of evidence that were considered to determine the appropriate level of supplementation of infant formula with AA and DHA-rich oils to provide a source of AA and DHA for infants. It is known that fetal requirements of AA and DHA are high due to the incorporation of these fatty acids into the retina and CNS. Intrauterine and postnatal estimates of AA and DHA accretion give an estimate of the amount of AA and DHA which may need to be supplied to the infants either directly or through conversion of their precursors. Additionally, biochemical and functional measurements can be used to estimate fatty acid requirements. One can compare plasma and RBC phospholipid AA and DHA levels in infants fed human milk to infants fed formula with or without supplemental AA and DHA as a measure of the biologic availability. It is assumed that the n-3 and n-6 fatty acid content of human milk of all well-nourished mothers meets or exceeds the n-3 and n-6 fatty acid requirement of the infant for normal physiological and biochemical development. In addition, the range of mean AA/DHA ratios calculated from reported mean values of AA and DHA in human milk from U.S. and European women is 0.43 to 6.00. This represents the normal range of AA/DHA ratios in human milk to which infants are exposed. It is therefore reasonable to examine the levels of AA and DHA found in human milk as a guide to the appropriate levels of AA and DHA supplementation for infant formula. The results of formula supplementation with AA and DHA can be compared from a functional perspective by evaluating the outcome of AA and DHA supplementation on visual acuity and mental development. Results of these analyses determined that supplementation of infant formula with AA- and DHA-rich oils should produce the following mean levels of AA and DHA in infant formula: SSC (0.40% and 0.25%, respectively); Similac NeoSure® (0.40% and 0.15%, respectively); SWI (0.40% and 0.15%, respectively). The target ratio of AA/DHA for SSC is 1.6, the target AA/DHA ratio for Similac NeoSure® and SWI is 2.7.

In order to confirm the safety of the level of supplementation, clinical trials of AA and DHA supplemented infant formula were conducted in the target populations (preterm and term infants). The addition of a mean level of 0.43% AA (range 0.42– 0.45%) and 0.27% DHA (range 0.24 – 0.32%) to SSC, a preterm infant formula provided a mean ± SD intake of 134 ± 35 and 106 ± 28 mg/kg/day SUNTGA25 and DHA-rich oils, respectively to the infants in Study AG38. (The calculated mean equivalent intake for AA from SUNTGA40S would be 84 mg/kg/day). (Note: The AG38 study looked at SSC ingestion from first feeds to term CA, however, SSC is marketed as an in-hospital feeding formula (i.e. to hospital discharge).)

The addition of a mean level of 0.43% AA (range 0.41-0.44%) and 0.16% DHA (range 0.15 – 0.18%) in Similac Neosure®, a preterm formula for use after

hospital discharge provided an estimated mean ± SD intake of 114 ± 46 and 49 ± 20 mg/kg/day SUNTGA25 and DHA-rich oils, respectively, to the infants in Study AG38. (The calculated mean intake for SUNTGA40S would be 71 mg/kg/day). (Note: Similac Neosure® is marketed as a post-discharge formula for preterm infants.)

The addition of a mean level of 0.46% AA (range 0.42 – 0.48%) and 0.13% DHA (range 0.11 – 0.15%) in SWI, a term infant formula provided an estimated mean ± SD intake of 108 ± 16 and 39 ± 6 mg/kg/day SUNTGA425 and DHA-rich oils, respectively in Study AF92. (The calculated mean equivalent intake for AA from SUNTGA40S would be 67 mg/kg/day).

Addition of AA and DHA-rich oils to the formulas at these levels was not found to have any adverse effect on infant's growth, multiple indices of development, morbidity, incidence of serious and/or adverse events, antibiotic use, clinical chemistries, or antioxidant status as compared to infants fed the unsupplemented formulas. Additionally, the supplemented formulas were well tolerated and the infants had normal stool characteristics as compared to infants fed the control formulas. The AA + DHA supplemented Similac NeoSure® formula was associated with significantly higher plasma cholesterol levels as compared to infants fed the unsupplemented formula. However, despite the statistically significant difference, the mean cholesterol levels of the infants consuming the supplemented formula were within normal reference ranges for breast fed infants. The higher cholesterol levels are not associated with an adverse clinical effect. Finally, the AA + DHA supplemented preterm formulas were found to have beneficial effects on measures of visual and cognitive development.

An independent External Advisory Panel was convened to evaluate the issue of mortality and sudden infant death syndrome (SIDS) in Ross-sponsored long-chain polyunsaturated fatty acids (LCP) studies as well as other published studies of LCP-supplemented infant formula. The GRAS Expert Panel accepts the findings of the External Advisory Panel. A thorough evaluation of data from the AG38 study and assessment of infant mortality (including deaths reported as SIDS) in all Ross-sponsored studies, as well as published studies conducted by others, do not provide evidence of an increased risk of SIDS or infant mortality associated with addition of AA and DHA to formulas for preterm or full-term infants from the fungal and tuna oil sources as studied in 1-year feeding trials (AG38 and AF92).

Given the absence of any adverse outcomes from either Clinical Trial AG38/AH95, the study of preterm infants, or Clinical Trial AF92, the study of term infants, it can be concluded that intake of AA and DHA-rich oils from infant formulas, at levels and ratios used in these studies, is safe and well tolerated by infants.

Conclusion

In conclusion, a UL can be derived for ingestion of AA-rich oil *from M. alpina* and DHA-rich oil from tuna. An examination of the sources of these oils indicates no reason for concern for toxigenicity or pathogenicity. The specifications are appropriate and batch analyses indicate no contamination with impurities of concern. Animal toxicology data verify that the oils do not produce any untoward toxicity. Effects seen on liver and spleen enlargement in adult rat toxicology studies are similar to those produced at high levels of intake by other sources of PUFAs and is a consequence of the biological activity of PUFAs with ingestion at much higher than physiologic levels. The effects are subject to a threshold, below which no adverse treatment related effects were seen. This threshold occurred in rat toxicity studies at 3000 mg/kg/day and 1700 mg/kg/day for effects seen on liver and spleen, respectively.

Administration of AA-rich oil, DHA-rich oil and a combination of AA-rich oil + DHA-rich oil to neonatal piglets did not produce any adverse test article related effects under the conditions of this study. There were no adverse test article related effects on clinical signs, body weights, food consumption, clinical chemistry and hematology, organ weights or gross and histopathology. Actual mean intake (males and females combined) of AA was approximately 250 mg/kg/day, at least an 8-fold exaggeration of estimated human infant AA intake. Actual mean intake (males and females combined) of DHA was approximately 136 mg/kg/day, at least a 7-fold exaggeration of estimated human infant DHA intake. Actual mean intake (males and females combined) of AA + DHA was approximately 236 mg/kg/day, at least a 4-fold exaggeration of estimated human infant AA + DHA intake.

Because the test articles contain bioactive components, it is most appropriate to derive a UL from clinical trials in the target population. Addition of AA-rich oils and DHA-rich oils to infant formulas in large studies of preterm and term infants indicated no adverse effects on infant's growth, development, morbidity, incidence of serious and or adverse events, antibiotic use, clinical chemistries, or antioxidant status as compared to infants fed the unsupplemented formulas. Additionally, the supplemented formulas were well tolerated and the infants had normal stool characteristics as compared to infants fed the control formulas. A review of other clinical trials reported in the literature of AA-rich oil derived from *M. alpina* corroborate the safety of ingestion of oil from this source. The NOAEL for ingestion of AA and DHA-rich oils was derived from appropriately conducted, well-documented clinical trials in the target populations of concern. Corroboration of the data comes from other published clinical infant trials on AA and DHA oil supplemented formulas. There is adequate demonstration of the threshold for adverse effects from animal toxicology data. Therefore, no UF will be applied to the NOAEL from the clinical trials in order to derive the UL for AA-rich oils and DHA-rich oils.

The UL for AA and DHA (supplied as SUNTGA40S and DHA-rich oil, respectively), added to SSC, a preterm formula for use from birth to hospital discharge, is at the mean level of addition of 0.43% (range 0.42 – 0.45%) and 0.27% (range 0.24 – 0.32%), total fatty acids, respectively. (This provides a mean ± SD intake of 134 ± 35 and 106 ± 28 mg/kg/day SUNTGA25 and DHA-rich oil, from tuna respectively. The calculated mean equivalent intake for SUNTGA40S would be 84 mg/kg/day). The target ratio of AA/DHA is 1.6, based on target levels of AA and DHA addition to the formula.

The UL for AA and DHA (supplied as SUNTGA40S and DHA-rich oil, respectively) added to Similac NeoSure®, a preterm formula for use from hospital discharge, is at the mean level of addition of 0.43% (range 0.41 – 0.44%) and 0.16% (range 0.15 – 0.18%) total fatty acids, respectively. (This provides a mean ± SD intake of 114 ± 46 and 49 ± 20 mg/kg/day SUNTGA25 and DHA-rich oil, from tuna respectively. The calculated mean equivalent intake for SUNTGA40S would be 71 mg/kg/day). The target ratio of AA/DHA is 2.7, based on target levels of AA and DHA addition to the formula.

The UL for AA and DHA (supplied as SUNTGA40S and DHA-rich oil, respectively) added to SWI, a term infant formula, is at the mean level of addition of 0.46% (range 0.42 – 0.48%) and 0.13% (range 0.11– 0.15%), total fatty acids respectively. (This provides a mean ± SD intake of 108 ± 16 and 39 ± 6 mg/kg/day SUNTGA25 and DHA-rich oil, from tuna respectively. The calculated mean equivalent intake for SUNTGA40S would be 67 mg/kg/day). The target ratio of AA/DHA is 2.7, based on target levels of AA and DHA addition to the formula.

d) Estimated Daily Intake (EDI) of AA-Rich Oil and DHA-Rich Oil from Proposed Uses

The estimated daily intakes (EDIs) of AA and DHA-rich oils from supplemented SSC, Similac NeoSure®, and SWI formulas are derived from the calculations of intake in the clinical trials using these supplemented formulas. It should be noted, however, that the intake derived for AA-rich oil is based on the use in the clinical trials of SUNTGA25, a fungal oil that is approximately 25% AA by weight. The AA-rich oil that is the subject of this GRAS document is SUNTGA40S. It is more concentrated than SUNTGA25 with approximately 40% AA by weight. Given the higher concentration of AA in SUNTGA40S, as compared to SUNTGA25, less oil will need to be added to the infant formula in order to provide the same amount of AA. The estimated mean intake of SUNTGA40S will be reduced by 37.5% compared to the intake values derived from these clinical trials from the use of SUNTGA25.

The EDI for AA-rich and DHA-rich oils and AA, DHA and EPA from the clinical trails is presented in the Tables I-1, I-2 and I-3.

TABLE I-1
Estimated Intake of AA-rich oil, DHA-rich oil, and AA, DHA and EPA Fatty Acid Intake by Preterm Infants Consuming Similac® Special Care® Formula Supplemented with 0.43% AA and 0.27% DHA *
Exclusive Formula Feeders**

		AA-rich oil	DHA-rich oil	AA	DHA	EPA
Time	N	mg/kg/d Mean ± SD		mg/kg/d Mean ± SD		
In-hospital	34	86.8 ± 12.5	68.5 ± 9.9	21.0 ± 3.0	13.1 ± 1.9	3.7 ± 0.5
Term	28	134.1 ± 35.4	105.9 ± 27.9	32.4 ± 8.5	20.3 ± 5.3	5.8 ± 1.5

* AA provided by AA-rich oil; DHA provided by DHA-rich oil: intakes based upon oil content of formulas, mean fatty acid content of the oils, and formula intake data collected from in-hospital records and 3-day diet records collected prior to each of the term CA visit in Ross Clinical Trial AG38.
** Exclusive Formula Feeders are defined as those infants receiving less than 100 mL human milk/kg of birth weight for the duration of the initial hospital stay and receiving study formula more than 80% of the times fed daily to term CA.

TABLE I-2
Estimated Intake of AA-rich oil, DHA-rich oil, and AA, DHA and EPA Fatty Acid Intake by Preterm Infants Consuming Similac NeoSure® Formula Supplemented with 0.43% AA and 0.16% DHA *
Exclusive Formula Feeders**

		AA-rich oil	DHA-rich oil	AA	DHA	EPA
Month CA	N	mg/kg/d Mean ± SD		mg/kg/d Mean ± SD		
2	22	114.3 ± 45.9	49.0 ± 19.7	27.5 ± 11.0	10.1 ± 4.0	2.7 ± 1.1
4	23	113.7 ± 47.0	48.8 ± 20.1	27.3 ± 11.3	10.0 ± 4.1	2.7 ± 1.1
6	20	97.1 ± 36.3	41.6 ± 15.6	23.3 ± 8.7	8.5 ± 3.2	2.3 ± 0.9
9	18	82.6 ± 47.1	35.4 ± 20.2	19.8 ± 11.3	7.3 ± 4.1	2.0 ± 1.1
12	14	69.9 ± 40.7	30.0 ± 17.4	16.8 ± 9.8	6.2 ± 3.6	1.7 ± 1.0

* AA provided by AA-rich oil; DHA provided by DHA-rich oil: intakes based upon oil content of formulas, mean fatty acid content of the oils, and formula intake data collected from 3-day diet records collected prior to study visits in Ross Clinical Trial AG38.
** Exclusive Formula Feeders are defined as those infants receiving less than 100 mL human milk/kg of birth weight for the duration of the initial hospital stay and receiving study formula more than 80% of the times fed daily to term CA.

TABLE I-3 Estimated Intake of AA-rich oil, DHA-rich oil, and AA, DHA and EPA Fatty Acid Intake by Term Infants Consuming Similac® with Iron Formula Supplemented with 0.46% AA and 0.13% DHA *						
		AA-rich oil	DHA-rich oil	AA	DHA	EPA
Visit (month)	N	mg/kg/d Mean ± SD		mg/kg/d Mean ± SD		
1	69	105.3 ± 13.4	38.0 ± 4.8	25.7 ± 3.3	7.15 ± 0.91	1.89 ± 0.24
2	63	107.8 ± 16.3	38.9 ± 5.9	26.3 ± 4.0	7.31 ± 1.10	1.94 ± 0.29
4	60	94.5 ± 16.6	34.1 ± 6.0	23.1 ± 4.1	6.41 ± 1.13	1.70 ± 0.30
6	59	75.7 ± 16.5	27.3 ± 5.9	18.5 ± 4.0	5.14 ± 1.12	1.36 ± 0.30
9	59	60.2 ± 14.0	21.7 ± 5.1	14.7 ± 3.4	4.08 ± 0.95	1.08 ± 0.25
12	54	48.2 ± 17.9	17.4 ± 6.5	11.8 ± 4.4	3.27 ± 1.21	0.87 ± 0.32
* AA provided by AA-rich oil; DHA provided by DHA-rich oil: intakes based upon oil content of formulas, mean fatty acid content of oils, and formula intakes reported in 3-day diet records collected in Ross Clinical Trial AF92						

e) Safety Evaluation Following Chronic and Acute Intake of AA-Rich Oil and DHA-Rich Oil

Evaluation of the safety of AA-rich oil and DHA-rich oil is accomplished through a review of the extensive database on the safety of both oils, including history of human exposure to AA and DHA through human milk, an analysis of the safety of the sources of both oils, a critical review of the compositional analysis of AA-rich oil and DHA-rich oil, animal and human studies of AA-rich oil and DHA-rich oil, and a derivation of a UL for exposure to these fatty acids. A comparison of the EDI of AA-rich oil and DHA-rich oil is made to the UL for the oils to confirm safety of exposure under the intended conditions of use.

Consumption of AA-rich oil and DHA-rich oil is determined to be safe when used to provide the following target levels of addition of AA and DHA to infant formulas: SSC (0.40% and 0.25%, respectively); Similac NeoSure® (0.40% and 0.15%, respectively); SWI (0.40% and 0.45%, respectively).

The safety of ingestion of these target levels of AA and DHA supplied by SUNTGA40S and DHA-rich oil, respectively, is determined by the UL derived from clinical trials of these oils added to preterm and term infant formulas. The UL's derived from these clinical trials are as follows:

The UL for AA and DHA (supplied as SUNTGA40S and DHA-rich oil, respectively), added to SSC, a preterm infant formula, is at the mean level of addition of 0.43% (range 0.42 – 0.45%) and 0.27% (range 0.24 – 0.32%), total fatty acids, respectively. (This provides a mean ± SD intake of 134 ± 35 and 106 ± 28 mg/kg/day SUNTGA25 and DHA-rich oil, from tuna respectively. The calculated mean equivalent intake for SUNTGA40S would be 84 mg/kg/day). The target ratio of AA/DHA is 1.6, based on target levels of AA and DHA addition to the formula.

The UL for AA and DHA (supplied as SUNTAGA40S and DHA-rich oil, respectively) added to Similac NeoSure®, a post-discharge preterm formula for use from hospital discharge, is at the mean level of addition of 0.43% (range 0.41 – 0.44%) and 0.16% (range 0.15 – 0.18%) total fatty acids, respectively. (This provides a mean ± SD intake of 114 ± 46 and 49 ± 20 mg/kg/day SUNTGA25 and DHA-rich oil, from tuna respectively. The calculated mean equivalent intake for SUNTGA40S would be 71 mg/kg/day). The target ratio of AA/DHA is 2.7, based on target levels of AA and DHA addition to the formula.

The UL for AA and DHA (supplied as SUNTGA40S and DHA-rich oil, respectively) added to SWI, a term infant formula for use from term CA, is at the mean level addition of 0.46% (range 0.42 – 0.48%) and 0.13% (range 0.11–0.15%), total fatty acids respectively. (This provides a mean ± SD intake of 108 ± 16 and 39 ± 6 mg/kg/day SUNTGA25 and DHA-rich oil, from tuna respectively. The calculated mean equivalent intake for SUNTGA40S would be 67 mg/kg/day). The target ratio of AA/DHA is 2.7, based on target levels of AA and DHA addition to the formula.

Therefore, AA-rich oil and DHA-rich oil, are GRAS when used together to supplement the following preterm and term infant formula at target AA and DHA levels of: 0.40 % and 0.25% , respectively for SSC (at a target ratio of AA/DHA of 1.6); 0.40% and 0.15%, respectively, for Similac NeoSure® (at a target ratio of AA/DHA of 2.7) and; 0.40% and 0.15%, respectively, for SWI (at a target ratio of AA/DHA of 2.7).

3. General Recognition of the Safety of AA-Rich Oil and DHA-Rich Oil

This document presents the information used to determine the GRAS status of AA-rich oil and DHA-rich oil for use as ingredients in term and preterm infant formula. This determination of safety is based upon a critical review of both toxicological and clinical safety studies as well as supporting information derived from a history of exposure to AA and DHA and a critical review of the compositional analysis of both oils. The sources of the reviewed information included pertinent studies and literature provided by Ross Products Division or identified in literature searches conducted through online bibliographic retrieval systems, including Medline and Dialog.

In the previous section, the proposed uses of AA-rich oil and DHA-rich oil as ingredients in term and preterm infant formulas were determined to be safe through scientific procedures. Under 21 CFR §170.30, general recognition of this safety requires that the scientific data and information upon which the determination of safety rests must ordinarily be published, but may be corroborated by unpublished studies and other data and information. The scientific data and information on which the safety determination of AA-rich oil and DHA-rich oil are based are available in the published literature or are otherwise publicly available to experts qualified by training and experience to evaluate the safety of food and food additives. Thus, the data reviewed meet the scientific procedure element required for all GRAS determinations.

Determination of the GRAS status of AA-rich oil and DHA-rich oil as a food ingredient has been made through the deliberation of experts, Dr. Vasilios Frankos (ENVIRON International Corporation), Dr. Sheila Innis (University of British Columbia), Dr. Michael Georgieff (University of Minnesota), Dr. M.W. Anders (University of Rochester Medical Center), Dr. Sanford Miller (Georgetown Center for Food and Nutrition Policy), Dr. Howard Sprecher (The Ohio State University), scientists qualified by training and experience to evaluate the safety of food ingredients. These experts have carefully reviewed all the available data on the short-term and long-term toxicity of AA-rich oil and DHA-rich oil, human exposure to AA and DHA through human milk, and human clinical safety studies evaluating safety of administered intakes of AA-rich oil and DHA-rich oil and have concluded that:

> There is no evidence in the available information on AA-rich oil and DHA-rich oil that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when it is used at levels that are now current or that might reasonably be expected from the proposed applications. AA-rich oil and DHA-rich oil are GRAS for use together as ingredients in term and preterm infant formulas, as proposed by Ross Products Division.

It is their opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same scientific conclusion. Therefore, AA-rich oil and DHA-rich oil, to be used as ingredients in term and preterm infant formulas, are GRAS, when used together to supplement preterm and term infant formula at target mean concentrations of: 0.40% and 0.25% , respectively for SSC (at a target ratio of AA/DHA of 1.6); 0.40 % and 0.15%, respectively, for Similac NeoSure® (at a target ratio of AA/DHA of 2.7) and; 0.40% and 0.15%, respectively, for SWI (at a target ratio of AA/DHA of 2.7).

Because AA-rich oil and DHA-rich oil are GRAS for the intended use, they are excluded from the definition of a food additive, and thus may be marketed for this use without the need to promulgate a specific food additive regulation under 21 CFR.

E. Availability of Information

The data and information that serve as the basis for this GRAS notification, including information in appendices, will be sent to FDA upon request or are available for the FDA'sreview and copying at reasonable times at the office of Claire Kruger, Ph.D., Principal, ENVIRON International Corporation, 4350 North Fairfax Drive, Suite 300, Arlington, VA. 22203, telephone: (703) 516-2309, facsimile: (703) 516-2393.

II. DESCRIPTION OF SUBSTANCE

A. Identity of AA-Rich Oil

1. Common or Trade Name

Arachidonic Acid (AA) Rich Oil Derived From *Mortierella alpina* Peyronel 1S-4, AA-rich oil, SUNTGA40S.

(SUNTGA25 is an AA-rich oil that contains approximately 25% AA. It is a less pure form of the AA-rich oil, SUNTGA40S (approximately 40% AA) that is the subject of this GRAS Notification. SUNTGA25 is not the subject of this GRAS Notification, however, its composition will be presented because it was used in some of the animal and clinical studies to evaluate the safety of AA-rich oil produced by Suntory derived from *Mortierella alpina* Peyronel 1S-4.)

2. Source

SUNTGA40S is an AA-rich oil derived from the fermentation of the fungal species *Mortierella alpina* Peyronel 1S-4 (AKU No. 3998; the AKU Culture Collection was established in 1960 at the Laboratory of Applied Microbiology, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan. The AKU Culture Collection has a wide variety of non-pathogenic microbes, covering bacteria, yeasts, molds and basidiomycetes. AKU now has over 500 microbial cultures available.).

(SUNTGA25 is an AA-rich oil derived from the fermentation of the fungal species *Mortierella alpina* Peyronel 1S-4 AKU No. 3998).

All growth media used for the fermentation process are U.S. food grade. The main fermentation reaction is stopped when the AA content reaches above 40%. A fermentation batch would be rejected when it does not meet the specifications - i.e., incomplete culture medium sterilization, out of spec pH, out of spec temperature of the culture medium, contamination of the medium or insufficiency of the fatty acid composition at the end of the cultivation. Contamination checks are carried out in both the seed and main cultures at the end of the cultivation.

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Following are the taxonomic characteristics of *Mortierella alpina* Peyronel 1S-4:

Cultural characteristics

- On Czapek Dox agar: At 25° C., in four days, colonies are 40-46 mm in diameter with a moderate amount of aerial mycelium; sporulation is observed. In seven days, colonies are 70-75 mm in diameter with much aerial mycelium and sporulation is rich.
- On 2% Malt extract agar: Colonies are 55-58 mm in diameter at four days at 25° C. without aerial mycelium and no sporulation.
- On Potato Dextrose Agar: At 25° C., in four days, colonies are 35-40 mm in diameter with a great deal of aerial mycelium and no sporulation. In seven days, colonies are 69-75 mm in diameter with aerial mycelium and sporulation is poor.

Microscopic characteristics

Sporangiophores arising mostly from substrate mycelia, simple, with a widened base, unbranched, 63-120 μm long. Sporangia are 8.8-12.5 μm in diameter containing numerous sporangiospores, leaving a more or less prominent columella on dehiscence; sporangia with one or a few sporangiospore were also observed. Sporangiospores are ellipsoidal, smooth on the surface, 4-4.5 x 2 μm.

3. Specifications

The product specifications for AA-rich oil are given below in Table II-1.

TABLE II-1
Product specification for AA-rich oil from *M. alpina*

Criteria	Specifications
Color/Clarity	Yellow clear liquid oil
Flavor	Bland, slight (or less/) green slight (or less/none) eggy, no painty flavor
Fatty acid composition[a] AA	40% minimum (relative%)
Free fatty acids[a]	0.2% max
Unsaponifiable material[a]	<1.0%
Arsenic[b]	<0.2 ppm
Lead[b]	<0.1 ppm
Mercury[b]	<0.5 ppm
Heavy metals as lead[a]	<1 ppm
Peroxide value[a]	5.0 meq/Kg max
PCB[b]	Not detected
Pesticides[b]	Not detected

[a] per batch analysis
[b] periodic analysis

4. Composition/Impurities

The distribution of AA in the triglyceride molecule for SUNTGA25 is as follows:

000036

No. 1 position: 22%
No. 2 position: 43%
No. 3 position: 35%

The distribution of AA in the triglyceride molecule for SUNTGA40S is presented in Table II-2.

TABLE II-2
Distribution of triglyceride species containing AA in SUNTGA40S
Area % (HPLC analysis)

	Lot 98071451	Lot 99072651	Lot 99111651
AAA	9.4	10.8	11.0
2A	33.5	37.6	37.8
1A	28.0	25.2	24.7
0A	8.7	7.0	7.7
Unknown	20.5	19.5	18.6

AAA = Three arachidonoil moieties contained in the triglyceride
2A = Two arachidonoil moieties contained in the triglyceride
1A =One arachidonoil moiety contained in the triglyceride
0A = No arachidonoil moieties contained in the triglyceride
Unknown = peaks in HPLC were not identified

Batches of SUNTGA25 and SUNTGA40S oil have been analyzed for fatty acids, iodine value, peroxide, p-anisidine value, free fatty acid, tocopherols, trans isomers, unsaponifiable matter, heavy metals, polymer content, pesticides, phthalates and PCBs to demonstrate product quality and compliance with specifications. Results of these analyses are presented in Tables II-3 and II-4.

TABLE II-3
Fatty acid analysis of AA-rich oil: SUNTGA25 and SUNTGA40S (wt/wt%)

Fatty acids Wt/Wt% (g/100g total fatty acids)	SUNTGA25		SUNTGA40S					
	Batch # 93122721		Batch # 94122121		Batch # 98071451		Batch # 99072651	
	BF_3 FAMES	NaOH FAMES[a]	BF_3 FAMES	NaOH FAMES[a]	BF_3 FAMES[a]	NaOH FAMES[a]	BF_3 FAMES[a]	NaOH FAMES[a]
Saturates								
10:0	ND	ND						
11:0	ND	ND						
12:0	ND	ND						
13:0	ND	ND						
ISO14:0	ND	ND						
14:0	0.4	0.45	0.6	0.55	0.45	0.4	0.4	0.4
ISO15:0	ND	ND	ND	ND				
ANT15:0	ND	ND	ND	ND				
15:0	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.2
ISO16:0	ND	ND	ND	ND				
ANT16:0	ND	ND	ND	ND				
16:0	15.1	15.75	15.1	14.3	14.45	13.25	11.85	11.3
7ME16:0			ND	ND				
ISO17:0	ND	ND	*	ND				
ANT17:0	TR	0.1	TR	TR				
17:0					0.3	0.3	0.4	0.4
17:0 + phytanic	0.3	0.3	0.3	0.2				
ISO18:0	ND	ND	**	ND				
18:0	6.1	6.0	8	7.9	7.6	7.6	7.65	7.5
19:0	ND	0.2	***	1.2				
20:0	0.6	0.6	0.7	TR	0.7	0.8	0.8	0.8
21:0		ND		ND				
22:0	1.8	1.6	2.2	2.35	2.3	2.8	2.85	3.0
23:0		0.1		TR				
24:0	3.3	3.15	5.1	5.4	5.75	7.6	8.35	9.1
Total saturates	27.8	28.3	32.1	32	31.8	32.85	32.6	32.7
Monoenes								
12:1	ND	ND	ND	ND				
14:1	ND	ND	TR	TR	ND	TR	TR	TR
15:1	ND	ND	ND	ND				
16:1ω9	ND	ND	ND	ND				
16:1ω7	0.2	0.2	0.1	0.15	0.2	0.2	0.15	0.1
16:1ω5					0.1	TR	TR	ND
16:1ω5 + ISO17:0	ND	ND	ND	ND				
17:1	0.1	0.1	ND	ND	TR	TR		
18:1ω9	13.7	13.65	6.7	6.5	6.5	6.45	6.35	6.2
18:1ω7	0.7	0.7	0.4	0.4	0.4	0.4	0.4	0.4
18:1ω5	ND	TR	ND	TR				
19:1		ND		ND				
20:1ω11	TR	ND	ND	ND				
20:1ω9	0.5	0.45	0.3	0.7	0.45	0.5	0.5	0.5
20:1ω7	ND	ND	ND	0.3				
22:1ω11+13	ND	ND	ND	ND	0.1	0.2	0.1	0.2
22:1ω9	0.2	0.2	0.1	0.1	ND	TR	ND	0.1
22:1ω7	ND	ND	ND	ND	ND	TR	ND	TR
24:1	0.1	0.1	TR	0.2				
24:1ω9					0.3	0.4	0.35	0.3
Total monoenes	15.6	15.5	7.6	8.35	7.95	8.1	7.85	7.85
Dienes								
16:2ω6							ND	TR
18:2ω5,11					ND	TR	TR	TR
18:2ω6	24.4	24.4	13	12.7	10.95	10.2	8.75	8.5
18:2 artifacts			TR	TR				
18:2ω4					ND	TR	TR	TR
20:2ω6	0.4	0.4	0.5	0.5	0.8	0.8	0.6	0.6

TABLE II-3
Fatty acid analysis of AA-rich oil: SUNTGA25 and SUNTGA40S (wt/wt%)

Fatty acids Wt/Wt% (g/100g total fatty acids)	SUNTGA25		SUNTGA40S					
	Batch # 93122721		Batch # 94122121		Batch # 98071451		Batch # 99072651	
	BF_3 FAMES	NaOH FAMES[a]	BF_3 FAMES	NaOH FAMES[a]	BF_3 FAMES[a]	NaOH FAMES[a]	BF_3 FAMES[a]	NaOH FAMES[a]
Total dienes	24.8	24.8	13.5	13.2	11.7	11.0	9.35	9.1
Trienes								
16:3ω3	ND	ND	ND	ND				
18:3ω6	1.9	1.7	2.5	1.35	2.7	2.5	2.55	2.5
18:3ω4					ND	TR	TR	TR
18:3ω3	1.9	1.9	0.8*	0.75*	0.5	0.4	0.4	0.4
18:3ω1					TR	TR	TR	TR
18:3 artifacts	0.5	0.45						
20:3ω6	2.9	2.75	3.1	3.15	3.3	3.2	3.4	3.4
20:3ω3			ND	ND				
20:305,8,11?	0.6	0.55			0.7	0.7	0.5	0.5
Unknown			0.4	0.4				
Total trienes	6.7	6.5	6.9	5.75	7.2	6.9	6.9	6.8
Tetraenes								
16:4ω1					ND	TR	TR	TR
18:4ω3	ND	ND	ND	ND	ND	TR	TR	TR
20:4ω6	23.7	23.1	38.6	39.35	40.75	40.3	42.4	42.4
20:4ω3	ND	ND	TR	0.1			0.1	0.1
22:4ω6	0.3	0.25	0.6	0.35	0.4	0.4	0.55	0.6
22:4ω3	ND		ND	ND				
Total tetraenes	24	23.35	39.2	39.75	41.2	40.7	43.1	43.1
Pentaenes								
20:5ω3	<0.1	<0.1	0.3	0.3	0.15	0.1	0.15	0.2
21:5ω3			ND	ND	0.15	0.1		
22:5ω6	ND	ND	ND	ND				
22:5ω3	ND	ND	ND	ND			ND	TR
Total pentaenes	<0.1	<0.1	0.3	0.3			0.15	0.2
Hexaenes								
22:6ω3	ND	ND	ND	TR				
Total hexaenes	ND	ND	ND	TR	ND	ND	ND	ND
Calculated Iodine value	148	149.55	173	173.45	176.45	173.4	177.2	177.4

ND = Not detected
TR = Trace
[a]average of duplicate analysis
* Artifacts before and after are 0.1% each

000039

TABLE II-4 Analysis of AA-rich oil: SUNTGA25 and SUNTGA40S				
	SUNTGA25	SUNTGA40S		
	Batch # 93122721	Batch # 94122121	Batch # 98071451	Batch # 99072651
Components				
Iodine value[a]	156	176	172.5	175
Peroxide value[a,b]	1.3	0.55	1.9	0.55
p-Anisidine value[a]	12.05	1.35	7.45	2.9
Free fatty acid[a,c]	0.04	0.065	0.025	0.025
Alpha tocopherol (μg/g)[a]	67.5	42	27.06	202.55
Gamma tocopherol (μg/g)[a]	164.5	146	117.46	121.4
delta tocopherol (μg/g)[a]	150	168	173.76	173.41
Trans isomers	ND	ND	ND	ND
Sterol (mg/g)	5.4	0.9	1.1	2.1
Unsaponifiable matter (%)[a]				
Unsaponifiable matter (%)[a,g]	1.11	0.315	0.3	0.37
Hydrocarbon (area%)[a,h]	1.2	0.7	1.2	1.35
alpha tocopherol (area %)[a,h]	0.3	2	-	0.9
gamma tocopherol (area %)[a,h]	0.7	6.3	3.1	3.55
delta tocopherol (area %)[a,h]	TR	3.7	4	3.25
Alcohol (area %)[a,h]	2.2	4.3	2.4	TR
Sterol (area %)[a,h]	95.1	75.5	88.8	90.85
Total polar components (area%)[a,h]	0.7	7.2	0.55	0.15
Unknown[a,h]		0.4		
Heavy metals				
Arsenic (ppm)			<0.10	<0.10
Cadmium (ppm)			<0.04	<0.04
Heavy metal as lead (ppm)			<5.0	<5.0
Mercury (ppm)			<0.025	<0.025
Lead (ppm)			<0.05	<0.05
Nickel (ppm)			<0.40	<0.40
Polymer content (area%)				
Triacylglycerol[a]	98	100.0	100.0	100.0
Polymer A[a]	0.2	-		
Polymer B[a]	0.85	TR		
Dimer			TR	TR
Unknown C[a]	TR	-		
Unknown D[a]	1.1	-		
Free fatty acid			-	-
Pesticides[f], phthalates, PCBs				
PCB[a,d]	ND	ND	ND	ND
ppDDE (ng/g)[a]	ND	ND	ND	ND
ppDDD[a]	ND	ND	ND	ND
ppDDT[a]	ND	ND	ND	ND
di-isobutyl phthalate[a]	ND	TR		
di-n-butyl phthalate[a]	ND	TR	TR[e]	TR[e]
di-(2-ethylexyl)-phthalate[a]	ND	TR	TR[e]	TR[e]
Acephate (ppb)			<80.0	<80.0
Alpha-BHC (ppb)			<25.0	<25.0

000040

TABLE II-4 Analysis of AA-rich oil: SUNTGA25 and SUNTGA40S				
	SUNTGA25	SUNTGA40S		
	Batch # 93122721	Batch # 94122121	Batch # 98071451	Batch # 99072651
Aldrin (ppb)			<25.0	<25.0
PCB 1254 (Arochlor 1254) (ppb)			<500.0	<500.0
Azinphos-methyl (ppb)			<80.0	<80.0
Beta-BHC (ppb)			<25.0	<25.0
Captafol (ppb)			<63.0	<63.0
Captan (ppb)			<100.0	<100.0
Chlorfenvinphos (ppb)			<80.0	<80.0
Chlorpyrifos-ethyl (ppb)			<50.0	<50.0
Chlorpyrifos-methyl (ppb)			<40.0	<40.0
Chlorothalonil (ppb)			<25.0	<25.0
Coumaphos (ppb)			<100.0	<100.0
Cypermethrin (ppb)			<188.0	<188.0
DCNA (ppb)			<37.0	<37.0
DCPA (ppb)			<37.0	<37.0
Demeton-S (ppb)			<50.0	<50.0
Delta-BHC (ppb)			<25.0	<25.0
Diazinon (ppb)			<40.0	<40.0
Dichlorofenthion (ppb)			<60.0	<60.0
Dicofol (ppb)			<63.0	<63.0
Dieldrin (ppb)			<25.0	<25.0
Dimethoate (ppb)			<40.0	<40.0
Disulfoton (ppb)			<50.0	<50.0
Endosufan I (ppb)			<25.0	<25.0
Endrin (ppb)			<37.0	<37.0
Endosufan II (ppb)			<37.0	<37.0
Endosufan sulfate (ppb)			<37.0	<37.0
EPN (ppb)			<80.0	<80.0
Ethion (ppb)			<40.0	<40.0
Fenitrothion (ppb)			<50.0	<50.0
Folpet (ppb)			<63.0	<63.0
Fonofos (ppb)			<50.0	<50.0
Gamma-BHC (ppb)			<25.0	<25.0
HCB (ppb)			<13.0	<13.0
Heptachlor epoxide (ppb)			<25.0	<25.0
Heptachlor (ppb)			<25.0	<25.0
Malathion (ppb)			<40.0	<40.0
Methamidophos (ppb)			<30.0	<30.0
Methidathion (ppb)			<60.0	<60.0
Methoxychlor (ppb)			<63.0	<63.0
Mevinphos (ppb)			<50.0	<50.0
Mirex (ppb)			<25.0	<25.0
Omethoate (ppb)			<70.0	<70.0
Oxadiazon (ppb)			<75.0	<75.0
Parathion-ethyl (ppb)			<60.0	<60.0
Parathion-methyl (ppb)			<50.0	<50.0
PCNB (ppb)			<20.0	<20.0
P,P'-DDD (ppb)			<37.0	<37.0

000041

TABLE II-4
Analysis of AA-rich oil: SUNTGA25 and SUNTGA40S

	SUNTGA25	SUNTGA40S		
	Batch # 93122721	Batch # 94122121	Batch # 98071451	Batch # 99072651
Permethrin (ppb)			<125.0	<125.0
Phosalone (ppb)			<80.0	<80.0
Phosmet (ppb)			<70.0	<70.0
Pirimiphos-methyl (ppb)			<50.0	<50.0
Propetamphos (ppb)			<60.0	<60.0
Propyzamide (ppb)			<50.0	<50.0
Prothiophos (ppb)			<60.0	<60.0
P,P'-DDE (ppb)			<25.0	<25.0
P,P'-DDT (ppb)			<50.0	<50.0
Ronnel (ppb)			<50.0	<50.0
Technical chlordane (ppb)			<500.0	<500.0
Tecnazene (ppb)			<25.0	<25.0
Tetradifon (ppb)			<37.0	<37.0
Thimet (ppb)			<40.0	<40.0
Toxaphene (ppb)			<1250.0	<1250.0
Trithion (ppb)			<60.0	<60.0
Vapona (ppb)			<30.0	<30.0

TR = Trace
ND = Not detected
In cases where no analysis is done, the cell remains blank.
[a] Average of duplicate analysis
[b] Peroxide value is expressed as milliequivalent of peroxide/1000g of sample
[c] Free fatty acid is expressed as percent oleic acid
[d] PCB expressed as Arochlor 1254
[e] The peak sizes for di-n-butyl phthalate and di-(2-ethylhexyl)-phthalate in the samples were so similar to the peak size of those components in the method/solvent blank, therefore those components were probably not true components of the sample oils but introduced during sample preparation.
[f] All pesticides were not detected at the detection limit given. The detection limit is listed in the table. Pesticide analysis was not done on batches 93122721 and 94122121.
[g] The lipid residue was weighed and that value divided by the weight of the oil saponified and multiplied by 100 to yield the percent unsaponifiable matter.
[h] The component parts of the unsaponifiable matter identified by TLC-FID expressed as area percents and uncorrected for detector response.

000042

B. Identity of DHA-Rich Oil

1. Common or Trade Name

High Docosahexaenoic acid (DHA) Tuna Oil; DHA-rich oil

2. Source

Tuna

3. Specifications

Product specifications for DHA-rich oil are presented in Table II-5

TABLE II-5
Product specifications for DHA-rich oil from tuna

Criteria	Specifications
Color/Clarity	Yellow clear liquid oil
Flavor	Bland, slight (or less) fishy, slight (or less/none) green, no painty flavor
Fatty acid composition[a] EPA DHA DHA/EPA ratio	 7.2% max (absolute); 8.0% max (relative) % of total fatty acids 20.0% min (absolute); 22.0% min (relative) % of total fatty acids ≥ 3.1
Free fatty acids[a]	0.1% max
Total tocopherols	0.7% maximum
Unsaponifiable material[a]	<1.5%
Arsenic[a]	<0.1 ppm
Lead[a]	<0.1 ppm
Mercury[a]	<0.5 ppm
Heavy metals as lead[a]	<1 ppm
Peroxide value[a]	<2.0 meq/Kg max
PCB[a]	Not detected
Dioxin[a]	Not detected
Pesticides[a]	Not detected

[a] per batch analysis

4. Composition/Impurities

Batches of DHA rich-oil have been analyzed for fatty acids, iodine value, peroxide, p-anisidine value, free fatty acid, tocopherols, trans isomers, unsaponifiable matter, heavy metals, polymer content, pesticides, phthalates and PCBs to demonstrate product quality and compliance with specifications. Results of these analyses are presented in Tables II-6 and II-7.

Batches of DHA rich-oil have also been evaluated for dioxin and furan contamination. The analytical data is found in Appendix A. With the exception of 1,2,3,4,6,7,8-HpCDD in one batch, all other analytes were not detected at their respective detection limits. The analysis of safety of consumption of the DHA-rich oil with respect to potential dioxin exposure is conducted in a manner consistent with the FDA assessment of the potential health risks associated with the dioxin exposure from bleached paper products (FDA 1990). The FDA determined that exposure for a full

lifetime (365 days/year for 70 years) to a dose of 2,3,7,8-TCDD of 0.064 pg/ kg/day corresponds to an upper limit on cancer risk of one in 1,000,000 or 1x 10^{-6}. This means that if one million people were exposed to 0.064 pg/kg/day of TCDD over their lifetimes (70 years), one additional cancer would result in this population.

This dose was derived from a two-year oral study in which rats exposed to 0.1 μg/kg/day 2,3,7,8-TCDD in the diet developed multiple-site carcinomas and/or sarcomas (FDA 1990). For a 3-kg infant, the corresponding allowable dose of TCDD (or TEQ) resulting in an estimated lifetime cancer risk of 1 x 10^{-6} is:

$$0.064 \text{ pg/kg/day} \times 3 \text{ kg} = 0.19 \text{ pg/day}$$

Employing the TEQ approach, ENVIRON calculated the estimated exposure to dioxins and furans from the dioxin and furan analytical results for two lots of DHA-rich tuna fish oil and the estimated intake of DHA-rich tuna oil of 106 mg per infant per day. This estimated intake is based on the maximum mean exposure by preterm infants consuming, SCC at term CA (estimated intakes presented in tables I-1, I-2, and I-3). For purposes of this analysis, all non-detects were set at one-half the detection limit. Then, the total TEQ value derived for each lot was multiplied by the estimated consumption of DHA-rich tuna oil of 106 mg per infant per day to arrive at an estimated exposure to dioxins and furans from the proposed uses in infant formula. This estimated exposure is then compared against the FDA's allowable lifetime dose of 0.19 pg per day. This comparison is extremely conservative since a short term exposure to tuna oil in infant formula is compared against an allowable lifetime exposure, however since infants are a very sensitive subpopulation, the conservatism is deemed to be appropriate. The chart below shows the results of these calculations for each of the two lots of oil.

LOT NUMBER	TOTAL TEQ (ppt)	ESTIMATED TCDD-EQUIVALENT INTAKE (pg/day)
990311	0.725	0.08
990921	0.729	0.08

The estimated TCDD-equivalent intakes for both lots of oil are below 0.19 pg per day, and thus the DHA-rich tuna oil does not present the potential for any adverse health effects from exposure to dioxins and furans under its intended conditions of use.

000044

TABLE II-6 Fatty acid analysis of DHA-rich oil (wt/wt%)						
Fatty acids Wt/Wt% (g/100g total fatty acids)	Batch # 971105		Batch # 990311		Batch # 990921	
	BF_3 FAMES[b]	NaOH FAMES[ab]	BF_3 FAMES[ab]	NaOH FAMES[ab]	BF_3 FAMES[ab]	NaOH FAMES[ab]
Saturates						
10:0	ND	ND				
11:0	ND	ND				
12:0	0.1	0.1				
13:0	TR	TR	0.1	0.1	0.1	TR
ISO14:0	TR	TR	TR	ND	ND	TR
14:0	3.8	3.4	3.55	3.5	3.65	3.35
ISO15:0	0.1	0.1	0.15	0.15	0.15	0.1
ANT15:0	TR	TR	0.1	0.1	0.1	0.1
15:0	0.9	0.85	0.8	0.8	0.85	0.8
ISO16:0	0.1	0.1	0.1	0.1	0.1	0.1
7MH-7M7H			0.15	0.15	0.15	0.2
ANT16:0	ND	TR				
16:0	20.8	19.65	19.45	19.25	19.6	19.25
7ME16:0	0.1	0.1				
ISO17:0	0.2	0.3				
ANT17:0	0.1	0.1	0.1	0.1	0.1	0.1
17:0	0.9	0.9	0.8	0.8	0.8	0.8
ISO18:0	0.2	0.2				
18:0	4.5	4.6	4.75	4.8	4.75	4.85
19:0	0.3	0.3				
20:0	0.3	0.3	0.3	0.3	0.3	0.3
21:0		0.1				
22:0	0.3	0.2	0.15	0.15	0.2	0.1
23:0		0.1				
24:0	0.2	0.2	0.2	0.1	0.3	0.15
Total saturates	32.9	31.55	30.5	30.35	30.9	30.15
Monoenes						
12:1	ND	ND				
14:1	0.1	0.1	0.2	0.2	0.3	0.2
15:1	TR	TR	0.1	TR	0.1	ND
16:1ω9	0.1	0.1				
16:1ω7	5.9	5.45	5.3	5.15	5.25	5.1
16:1ω5	0.4	0.4				
7M-16:1+ISO17:0			0.65	0.6	0.6	0.55
17:1	ND	0.9	0.8	0.8	0.8	0.8
18:1ω9	18.6	18.7	16.75	16.7	16.55	16.55
18:1ω7	2.4	2.4	2.55	2.55	2.55	2.55
18:1ω5	0.1	0.2	0.2	0.2	0.2	0.2
19:1		0.3				
20:1ω11	0.5	0.6	0.8	0.9	0.85	0.9
20:1ω9	2.1	2.2	1.65	1.7	1.65	1.7
20:1ω7	0.2	0.2	0.2	0.2	0.15	0.2
22:1ω11+13	1.1	1.05	1.25	1.3	1.25	1.3
22:1ω9	0.5	0.5	0.3	0.3	0.3	0.3
22:1ω7	0.2	0.1	0.1	0.1	0.1	0.1
24:1	0.7	ND				
24:1ω9			0.55	0.65	0.65	0.7
Total monoenes	32.9	33.15	31.35	31.35	31.15	31.1
Dienes						

000045

TABLE II-6 Fatty acid analysis of DHA-rich oil (wt/wt%)						
Fatty acids Wt/Wt% (g/100g total fatty acids)	**Batch # 971105**		**Batch # 990311**		**Batch # 990921**	
	BF_3 FAMES[b]	**NaOH FAMES[ab]**	**BF_3 FAMES[ab]**	**NaOH FAMES[ab]**	**BF_3 FAMES[ab]**	**NaOH FAMES[ab]**
16:2ω4			0.1	0.1	0.15	0.1
16:2ω4 + phytanic	0.9	1.0				
16:2ω6			0.7	0.9	0.7	0.9
18:2ω5,11			0.2	0.15	0.2	0.2
18:2ω7		ND				
18:2ω6	1.2	1.2	1.35	1.3	1.3	1.35
18:2ω4	0.1	0.2	0.2	0.2	0.2	0.2
20:2NMID	0.1	0.1	0.15	0.1	0.1	0.15
20:2ω6	0.3	0.3	0.3	0.25	0.3	0.25
Total dienes	2.6	2.8	2.95	3.0	2.9	3.2
Trienes						
16:3ω4	0.9		0.1	0.1	0.1	0.1
16:3ω3	TR	TR				
18:3ω6	ND	ND	0.2	0.2	0.2	0.2
18:3ω4	0.1		0.2	0.2	0.2	0.2
18:3ω3	0.4	0.4	0.8	0.75	0.75	0.7
18:3ω1	ND					
20:3ω6	0.2	0.1	0.1	0.1	0.1	0.1
20:3ω3	0.2	0.2	0.2	0.2	0.2	0.2
Total trienes	1.8	0.7	1.5	1.5	1.4	1.55
Tetraenes						
16:4ω1	ND	TR	0.1	0.1	0.1	0.1
16:4ω3	-0.6	TR	0.3	0.3	0.3	0.3
18:4ω3		0.65	1.2	1.15	1.2	1.2
18:4ω1			TR	TR	TR	TR
20:4ω6	1.9	1.85	1.5	1.55	1.5	1.6
20:4ω3	0.5	0.45	0.5	0.5	0.5	0.5
22:4ω6	0.5	0.5	0.2	0.2	0.2	0.2
22:4ω3	0.2	0.1	TR	TR		
Total tetraenes	3.7	3.55	3.85	3.75	3.7	3.8
Pentaenes						
20:5ω3	5.1	5.3	6.6	6.45	6.5	6.5
21:5ω3	0.3	0.2	0.2	0.2	0.2	0.2
22:5ω6	1.0	1.1	0.8	0.8	0.8	0.8
22:5ω3	1.3	1.3	1.2	1.2	1.2	1.2
Total pentaenes	7.7	7.9	8.7	8.65	8.65	8.7
Hexaenes[c]						
22:6ω3	18.3	19.9	20.75	21.25	20.85	21.25
Total hexaenes	18.3	19.9	20.75	21.25	20.85	21.25
Unknown between 18:3ω4 and 18:3ω3			0.2	0.1	0.2	0.1
Calculated Iodine value	162	166	174.25	175.75	173.6	176.1

ND = Not detected
TR = Trace
[a]average of duplicate analysis
[b]Fatty acid methyl esters (FAME) were prepared from aliquots of the oil sample in BF_3 – MeOH according to a modification (omitting the alkali step and heating for 60 minutes) of A.O.C.S. Official Method Ce1b-89. FAME were also prepared from aliquots of the oil sample with 0.5 N methanolic sodium hydroxide.
[c]The 22:6 distribution of high-DHA tuna oil is confined largely to the sn-2 and sn-3 positions (Myher et al. 1996)

000046

TABLE II-7 Analysis of DHA-rich oil				
	Batch # 971105	**Batch # 990311**	**Batch # 990921**	**Batch # 000122**
Components				
Iodine value[a]	181	185	184	
Peroxide value[a,b]	5.8	0.95	0.85	
p-Anisidine value[a]	7.9	5.7	5.35	
Free fatty acid[a,c]	0.025	0.025	0.03	
Alpha tocopherol (μg/g)[a]	65.5	128.9	131.57	
Gamma tocopherol (μg/g)[a]	144	149.7	152.19	
delta tocopherol (μg/g)[a]	5706.5	5176.9	4912.11	
Trans isomers	ND	ND	ND	
Molecular ion	387	386	386	
Cholesterol (mg/g)[a]	0.25	0.2	0.3	
Unsaponifiable matter				
Unsaponifiable matter (%)[a,g]	0.94	0.72	0.78	
Hydrocarbon (area%)[a,h]	1	2.45	0.95	
alpha tocopherol (area %)[a,h]	TR	0.35	TR	
gamma tocopherol (area %)[a,h]	1.75	0.95	1.2	
delta tocopherol (area %)[a,h]	79.25	84.7	83.5	
Alcohol (area %)[a,h]	2.85	-	-	
Sterol (area %)[a,h]	2.1	2.3	2.7	
Total polar components (area%)[a,h]	13	9.3	11.75	
Heavy metals				
Arsenic (ppm)		<0.10	<0.10	
Cadmium (ppm)		<0.04	<0.04	<0.04
Heavy metal as lead (ppm)		<5.0	<5.0	<5.0
Mercury (ppm)		<0.025	<0.025	<0.025
Lead (ppm)		<0.05	<0.05	<0.05
Nickel (ppm)		<0.40	<0.40	<0.40
Polymer content (area%)				
Triacylglycerol (TAG)[a]	95.65	96.7	97.65	
Trimeric TAG[a]	0.5			
Dimeric TAG[a]	2.45			
Dimer		1.25	1.55	
Monoglycerides[a]	1.4			
Free fatty acid		2.05	0.9	
Pesticides[f], phthalates, PCBs				
PCB[a,d]	ND	ND	ND	
ppDDE (ng/g)[a]	1.05	3.2	3.1	
ppDDD[a]	ND	ND	ND	
ppDDT[a]	ND	ND	ND	
di-isobutyl phthalate[a]	ND			
di-n-butyl phthalate[a,e]	TR			
di-(2-ethylexyl)-phthalate[a,e]	TR			
Acephate (ppb)		<80.0	<80.0	<80.0
Alpha-BHC (ppb)		<25.0	<25.0	<25.0
Aldrin (ppb)		<25.0	<25.0	<25.0
PCB 1254 (Arochlor 1254) (ppb)		<500.0	<500.0	<500.0
Azinphos-methyl (ppb)		<80.0	<80.0	<80.0
Beta-BHC (ppb)		<25.0	<25.0	<25.0
Captafol (ppb)		<63.0	<63.0	<63.0
Captan (ppb)		<100.0	<100.0	<100.0

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TABLE II-7
Analysis of DHA-rich oil

	Batch # 971105	Batch # 990311	Batch # 990921	Batch # 000122
Chlorfenvinphos (ppb)		<80.0	<80.0	<80.0
Chlorpyrifos-ethyl (ppb)		<50.0	<50.0	<50.0
Chlorpyrifos-methyl (ppb)		<40.0	<40.0	<40.0
Chlorothalonil (ppb)		<25.0	<25.0	<25.0
Coumaphos (ppb)		<100.0	<100.0	<100.0
Cypermethrin (ppb)		<188.0	<188.0	<188.0
DCNA (ppb)		<37.0	<37.0	<37.0
DCPA (ppb)		<37.0	<37.0	<37.0
Demeton-S (ppb)		<50.0	<50.0	<50.0
Delta-BHC (ppb)		<25.0	<25.0	<25.0
Diazinon (ppb)		<40.0	<40.0	<40.0
Dichlorofenthion (ppb)		<60.0	<60.0	<60.0
Dicofol (ppb)		<63.0	<63.0	<63.0
Dieldrin (ppb)		<25.0	<25.0	<25.0
Dimethoate (ppb)		<40.0	<40.0	<40.0
Disulfoton (ppb)		<50.0	<50.0	<50.0
Endosufan I (ppb)		<25.0	<25.0	<25.0
Endrin (ppb)		<37.0	<37.0	<37.0
Endosufan II (ppb)		<37.0	<37.0	<37.0
Endosufan sulfate (ppb)		<37.0	<37.0	<37.0
EPN (ppb)		<80.0	<80.0	<80.0
Ethion (ppb)		<40.0	<40.0	<40.0
Fenitrothion (ppb)		<50.0	<50.0	
Folpet (ppb)		<63.0	<63.0	<63.0
Fonofos (ppb)		<50.0	<50.0	<50.0
Gamma-BHC (ppb)		<25.0	<25.0	<25.0
HCB (ppb)		<13.0	<13.0	<13.0
Heptachlor epoxide (ppb)		<25.0	<25.0	<25.0
Heptachlor (ppb)		<25.0	<25.0	<25.0
Malathion (ppb)		<40.0	<40.0	
Methamidophos (ppb)		<30.0	<30.0	<30.0
Methidathion (ppb)		<60.0	<60.0	<60.0
Methoxychlor (ppb)		<63.0	<63.0	<63.0
Mevinphos (ppb)		<50.0	<50.0	<50.0
Mirex (ppb)		<25.0	<25.0	<25.0
Omethoate (ppb)		<70.0	<70.0	<70.0
Oxadiazon (ppb)		<75.0	<75.0	<75.0
Parathion-ethyl (ppb)		<60.0	<60.0	<60.0
Parathion-methyl (ppb)		<50.0	<50.0	<50.0
PCNB (ppb)		<20.0	<20.0	<20.0
P,P'-DDD (ppb)		<37.0	<37.0	<37.0
Permethrin (ppb)		<125.0	<125.0	<125.0
Phosalone (ppb)		<80.0	<80.0	<80.0
Phosmet (ppb)		<70.0	<70.0	<70.0
Pirimiphos-methyl (ppb)		<50.0	<50.0	<50.0
Propetamphos (ppb)		<60.0	<60.0	<60.0
Propyzamide (ppb)		<50.0	<50.0	<50.0
Prothiophos (ppb)		<60.0	<60.0	<60.0

TABLE II-7
Analysis of DHA-rich oil

	Batch # 971105	Batch # 990311	Batch # 990921	Batch # 000122
P,P'-DDE (ppb)		<25.0	<25.0	<25.0
P,P'-DDT (ppb)		<50.0	<50.0	<50.0
Ronnel (ppb)		<50.0	<50.0	<50.0
Technical chlordane (ppb)		<500.0	<500.0	<500.0
Tecnazene (ppb)		<25.0	<25.0	<25.0
Tetradifon (ppb)		<37.0	<37.0	<37.0
Thimet (ppb)		<40.0	<40.0	<40.0
Toxaphene (ppb)		<1250.0	<1250.0	<1250.0
Trithion (ppb)		<60.0	<60.0	<60.0
Vapona (ppb)		<30.0	<30.0	<30.0
Vinclozolin (ppb)		<50.0	<50.0	<50.0

TR = Trace
ND = Not detected
In cases where no analysis is done, the cell remains blank.
[a] Average of duplicate analysis. Method of detection limits are: Arochlor 1254, 0.0168 μg/ml; DDE, 0.0008 μg/ml; DDD, 0.0020 μg/ml; DDT, 0.0020 μg/ml; di-isobutyl phthalate, 0.10 μg/ml; di-butyl phthalate, 0.10 μg/ml; 2-ethyl-hexyl phthalate, 0.10 μg/ml.
[b] Peroxide value is expressed as milliequivalent of peroxide/1000g of sample
[c] Free fatty acid is expressed as percent oleic acid
[d] PCB expressed as Arochlor 1254
[e] The peak sizes for di-n-butyl phthalate and di-(2-ethylhexyl)-phthalate in the samples were so similar to the peak size of those components in the method/solvent blank, therefore those components were probably not true components of the sample oils but introduced during sample preparation.
[f] All pesticides were not detected at the detection limit given. The detection limit is listed in the table. Pesticide analysis not done for batch 971105.
[g] The lipid residue was weighed and that value divided by the weight of the oil saponified and multiplied by 100 to yield the percent unsaponifiable matter.
[h] The component parts of the unsaponifiable matter identified by TLC-FID expressed as area percents and uncorrected for detector response.

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C. Production Process

1. Manufacturing Process for AA-Rich Oil

a) Producing Organism

AA-rich oil is derived from *Mortierella alpina* Peyronel 1S-4. (AA-rich oil which contains approximately 40% AA is referred to as SUNTGA40S).

(AA-rich oil referred to as SUNTGA25, also derived from this organism, contains approximately 25% AA; SUNTGA25 is not the subject of this GRAS Notification).

b) Process Description

SUNTGA40S is derived from the fermentation of the fungal species *M. alpina*. The initial and second fermentation processes of seed culture are conducted for 2-3 days in a flask and a stirred tank, respectively, under aerobic conditions at 28°C. A yeast extract and glucose growth medium is used. The main culture is then fermented for 8-14 days under aerobic conditions at 26°C., also in a stirred tank. The biomass is filtered and dried using hot air. The biomass can be stored under refrigeration until extraction of the oil.

The oil purification process utilizes a hexane extraction. The extract is filtered, and the solvent is removed via evaporation. The extract is degummed and then deacidified using phosphoric acid and sodium hydroxide, respectively or column method. The oil slurry is washed with water, and the water is removed using vacuum evaporation. The oil undergoes a two step deodorization process. Mixed tocopherols are added to the oil to help maintain the oxidative quality, and the oil is filled into storage containers.

Critical control points in the manufacturing and purification processes of SUNTGA40S are presented in Tables II-8 and II-9, respectively.

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TABLE II-8 Critical Control Points in the Manufacturing Process		
Manufacturing step	**Control item**	**Specification**
Stock culture	microorganism	N.D. other than *M. alpina*
	appearance of mycelia on plate culture	white fluffy colony observed
	fatty acid composition	AA produced
Seed cultures	culture temperature	28 ± 2°C throughout the culture
	contamination check	characteristic appearance and odor of *M. alpina* culture
	fatty acid composition	AA produced
Main culture	sterilization of culture medium	at not less than 121°C for not less than 20 minutes
	pH of the sterilized medium	pH 5.5-6.5
	culture temperature	26 ± 2°C throughout the culture
	contamination check	characteristic appearance and odor of *M. alpina* culture
	fatty acid composition at the end of the culture	AA content: not less than 40%
Drying	moisture of dry cell	not more than 5%
	fatty acid composition after drying	AA content: not less than 40%
Storage	temperature of the storage room	not more than 10°C
Abbreviations: *M. alpina*, *Mortierella alpina*; N.D., not detected; AA, arachidonic acid		

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TABLE II-9 Critical Control Points in the Purification Process		
Manufacturing step	**Control item**	**Specification**
Column treatment	Break of filter	Pressure of 0.5 micron filter: 0.2kgf/cm^2 (1.521x10^{4}Pa)
Hexane removal	Degree of vacuum & Temperature	Vacuum: <720mmHg (9.597x10^{4}Pa) Temperature: >35°C
Wash with water	Residual of NaOH	pH of wash out water: = pH of use water (+0.5, -0.5)
First deodorization	Degree of vacuum & Temperature	Vacuum: <740mmHg (9.863x10^{4}Pa) Temperature: >160°C
	Leak a medium oil from a pinhole of jacket[a]	1. Organoleptic test 2. Pressure test before/after using the apparatus: no break at 2.9kgf/cm^2 (2.205x10^{5}Pa)
Second deodorization	Degree of vacuum & Temperature	Vacuum: <740mmHg (9.863x10^{4}Pa) Temperature: >150°C
	Leak a medium oil from a pinhole of jacket[a]	1. Organoleptic test 2. Pressure test before/after using the apparatus: no break at 2.9kgf/cm^2 (2.205x10^{5}Pa)
Mix-toco addition & Filling	environment	>Class 100,000
[a] Break test is done at the same time (first and second deodorization).		

There were several changes made to the manufacturing process for SUNTGA25 to produce an improved product, SUNTGA40S. A comparison of the key steps in the manufacturing process used for oil production is presented in Table II-10

TABLE II-10
Comparison of manufacturing process used to produce SUNTGA25 and SUNTGA40S

Manufacturing Process Conditions	SUNTGA25	SUNTGA40S
Strain	*Mortierella alpina* 1S-4	*Mortierella alpina* 1S-4
Medium	Glucose (fed batch), yeast extract, soybean oil	Glucose (fed batch), soy flour, soybean oil, minerals
Sterilization conditions	Not less than 121°C. for not less than 20 minutes.	Not less than 121°C. for not less than 20 minutes
Fermentation temperature	28°C. ± 2°C. through the cultivation for the oil	26°C± 2°C. through the cultivation for the oil
Fermentation period	7 days	8-14 days
Agitation and Aeration	Agitated in the fermentation tank and sparged by clean air during the fermentation period.	Agitated in the fermentation tank and sparged by clean air during the fermentation period.

The changes in the production process used to produce SUNTGA40S do not introduce new impurities or alter the composition of the oil in a qualitative manner compared to SUNTGA25. The final product comparison indicates that the major change is an increase in the AA content (from approximately 25% in SUNTGA25 to approximately 40% in SUNTGA40S) and a reduction in the unsaponifiable matter and reduction in the sterol impurity, 24,25-Methylenecholest-5en-3β-ol. A comparison of the fatty acid composition for both SUNTGA oils is given in Table II-11.

TABLE II-11
Comparison of final products

SUNTGA25 (original medium used)		SUNTGA40S (improved medium used)	
triglyceride	>95%	triglyceride	>95%
unsaponifiable matter	1.8%	unsaponifiable matter	<1.0%
24,25-Methylenecholest-5en-3β-ol	0.284%	24,25-Methylenecholest-5en-3β-ol	0.025%
Fatty acid composition (%)		Fatty acid composition (%)	
14:0	0.4	14:0	0.7
16:0	14.3	16:0	16.4
18:0	6.3	18:0	8.7
18:1(n-9)	14.4	18:1(n-9)	6.6
18:2	20.7	18:2	10.7
18:3(n-6)	3.0	18:3(n-6)	2.8
18:3(n-3)	1.6	18:3(n-3)	0.5
20:0	0.7	20:0	0.4
20:3	2.9	20:3	2.8
20:4	23.6	20:4	42.3
22:0	2.0	22:0	1.4
24:0	4.0	24:0	4.8
others	6.1	others	1.9

2. Manufacturing Process for DHA-Rich Oil

a) Source of Oil

Tuna

b) Process Description

DHA-rich Tuna Oil is supplied to Abbott Laboratories, Ross Products Division, by Mochida International Co., Limited. The oil is manufactured by Nippon Suisan Kaisha, Ltd.

Tuna is currently sourced from the waters off Peru. The heads and bones from the fish are harvested and cooked using a hot water extraction process. A filterpress is used to separate the oil/water fraction from the solid material. A crude tuna oil extract is collected via centrifugation. The tuna oil (crude) is transported to a Nissui facility in Japan.

The crude tuna oil is degummed and then neutralized. The slurry is then bleached and run through an activated clay column. These processes remove phospholipid and protein fractions, neutralize free fatty acids, and improve the color and odor of the tuna oil. A molecular distillation process is used to further purify the oil, removing any PCBs and other environmental pollutants. The oil is then deodorized. Mixed tocopherols or ascorbyl palmitate and monoglycerides are added to the oil to help maintain the oxidative quality of the oil, and the oil is then filled into containers which conform to all applicable food regulations.

All processes, reagents and processing aids used in the manufacture of the DHA-rich tuna oil are approved as food grade.

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III. USE

A. Historical Exposure to AA and DHA through Human Milk

Human milk is the optimal source of nutrition for infants for up to 3 months of age (Innis, 1991). Milk is a complex fluid containing carbohydrates, salts, proteins, cells and cellular debris and lipids. Lipids in milk have been extensively investigated because the total lipid and fatty acid concentrations can vary considerably. The total lipid content of human milk is affected by the following factors: duration of nursing or feeding, age postpartum, stage of lactation, diurnal rhythm, gestation age at birth (preterm vs. term), diet, infections, metabolic disorders, parity, and adiposity (Jensen, 1996).

It is not clear if long-chain polyunsaturated fatty acids (LCPs) are present in human milk to maintain the physical properties of milk fat globules or if they are specifically secreted in milk to fulfill the essential fatty acid requirement of the infant (Innis 1991). However, the LCPs in the diet of breast fed infants clearly contribute to the infant's plasma and/or red blood cell glycerophospholipid LCP (Carlson et al., 1986; Delucchi et al., 1987; Olegard and Svennerholm, 1971; Ponder et al., 1991; Putnam et al., 1982; Sanders et al., 1978; Sanders et al., 1979). Human milk provides AA and DHA, which may substantially decrease the infant's need for endogenous LCP synthesis to meet the requirements for new structural lipid synthesis (Innis 1991).

The LCP content of human milk is affected by gestation age, stage of lactation and fat in the maternal diet. The essential fatty acids (LA and ALA) and their LCP derivatives including AA and DHA are proportionally higher in colostrum and transition milk compared to mature milk. LCP levels including AA and DHA are also higher in colostrum and milk of mothers of preterm infants than mothers of full term infants (Bitman et al., 1983).

Changes in maternal diet cause variation in DHA levels but not in AA levels in human milk (Finley et al., 1985, Harzer et al. 1984, Innis et al., 1988, Sanders et al., 1978, Specker et al., 1987). The diet-related increase in DHA levels in human milk is not accompanied by a decrease in AA levels in human milk, thereby suggesting the possibility of a specific mechanism to regulate the secretion of AA in human milk independent of n-3 levels in the diet (Innis 1991). The variation in DHA levels in human milk due to their dependence on maternal diet (Chappell et al., 1985; Finley et al., 1985; Harris et al., 1984; Harzer et al., 1984; Innis et al., 1988; Sanders et al., 1978), precludes the determination of dietary requirements for DHA and AA for the developing infants based solely on human milk fatty acid concentration. However, it is assumed that the n-3 and n-6 fatty acid content of human milk of all well-nourished mothers meets or exceeds the n-3 and n-6 fatty acid requirement of the infant for normal physiological and biochemical development and may provide a guide for determination of appropriate levels of AA and DHA for formula supplementation (Innis 1991).

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The DHA and AA levels in human milk from 27 published studies are reported in Table III-1. The human milk samples analyzed and summarized in this table were obtained from mothers in different regions of the world with diverse cultural background, different dietary habits, at different stage of lactation, and from mothers who delivered preterm or term infants. The mean DHA level in human milk was highly variable and ranged from 0.07 to 2.78% (g/100 g total fatty acids), primarily due to the differences in maternal dietary patterns. The highest levels were reported in Chinese mothers consuming seafood and the lowest levels were reported in milk from well nourished Sudanese mother consuming diets low in fish. The AA level in human milk was less variable than the DHA content and ranged from 0.31 to 1.22%. The mean AA and DHA content in mature human milk of women in the US ranged from 0.40 to 0.69% and 0.1 to 0.29%, respectively.

TABLE III-1
Levels of AA, DHA and EPA in human milk

Reference	Country	Characteristics	Wt % DHA (g/100 g total fatty acids)	Wt % AA (g/100 g total fatty acids)	Wt % EPA (g/100 g total fatty acids)
Chen et al. 1995	Canada	3-4 weeks postpartum	0.14 ± 0.10 (SD) (range Trace-0.53)	0.35 ± 0.11 (SD) (range 0.05–0.69)	0.05 ± 0.05 (SD) (range Trace–0.25)
Innis et al. 1988	Canada	4-28th week of lactation, Inuit women from Arctic community	1.4 ± 0.4 (SE) (range 0.4-3.8)	0.6 ± 0.0 (SE) (range 0.2-2.4)	1.1 ± 0.3 (SE) (range 0.2-2.4)
Innis et al. 1988	Canada	2-4 months postpartum, women from Vancouver consuming mixed North American diet	0.4 ± 0.1 (SE)	0.7 ± 0.0 (SE)	0.2 ± 0.2 (SE)
Koletzko et al. 1988	Germany	mature hindmilk	0.22 (0.15-0.60)*	0.36 (0.30-0.54)*	0.04 (ND-0.16)*
Genzel-Boroviczeny et al. 1997	Germany	term, day 5 postpartum	0.46 (0.08)*	0.72 (0.07)*	0.04 (0.08)*
Genzel-Boroviczeny et al. 1997	Germany	preterm, day 5 postpartum	0.43 (0.08)*	0.74 (0.12)*	0.00 (0.04)*
Genzel-Boroviczeny et al. 1997	Germany	term,day 10 postpartum	0.39 (0.06)*	0.59 (0.09)*	0.00 (0.03)*
Genzel-Boroviczeny et al. 1997	Germany	preterm, day 10 postpartum	0.35 (0.06)*	0.64 (0.08)*	0.00 (0.05)*
Genzel-Boroviczeny et al. 1997	Germany	term day, 20 postpartum	0.27 (0.07)*	0.50 (0.06)*	0.04 (0.05)*
Genzel-Boroviczeny et al. 1997	Germany	preterm, day 20 postpartum	0.24 (0.05)*	0.51 (0.04)*	0.05 (0.05)*
Genzel-Boroviczeny et al. 1997	Germany	term, day 30 postpartum	0.23 (0.06)*	0.45 (0.07)*	0.05 (0.07)*
Genzel-Boroviczeny et al. 1997	Germany	preterm day, 30 postpartum	0.24 (0.05)*	0.48 (0.03)*	0.00 (0.04)*
Harzer et al. 1983	Germany	day 1 postpartum, term infants	0.22	0.75	0.64
Harzer et al. 1983	Germany	day 3 postpartum, term infants	0.21	0.55	0.43
Harzer et al. 1983	Germany	day 5 postpartum, term infants	0.29	0.54	0.18
Harzer et al. 1983	Germany	day 8 postpartum, term infants	0.26	0.50	0.22
Harzer et al. 1983	Germany	day 15 postpartum, term infants	0.17	0.43	0.16
Harzer et al. 1983	Germany	day 22 postpartum, term infants	0.15	0.36	0.14
Harzer et al. 1983	Germany	day 29 postpartum, term infants	0.16	0.39	0.13
Harzer et al. 1983	Germany	day 36 postpartum, term infants	0.16	0.39	0.05

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TABLE III-1
Levels of AA, DHA and EPA in human milk

Reference	Country	Characteristics	Wt % DHA (g/100 g total fatty acids)	Wt % AA (g/100 g total fatty acids)	Wt % EPA (g/100 g total fatty acids)
Chardigny et al. 1995	France	day 0 ≥ 90 postpartum	0.32 ± 0.08 (SEM) (range 0–0.82)	0.50 ± 0.06 (SEM) (range 0.25-0.87)	0.02 ± 0.01 (SEM) (range 0–0.08)
Guesnet et al. 1995[a]	France	days 2-5 postpartum	0.55 ± 0.29 (SD)	0.67 ± 0.30 (SD)	-
Guesnet et al. 1995[a]	France	day 15 postpartum	0.43 ± 0.18 (SD)	0.53 ± 0.13 (SD)	-
Guesnet et al. 1995[a]	France	day 30 postpartum	0.32 ± 0.13 (SD)	0.41 ± 0.12 (SD)	-
Guesnet et al. 1995[a]	France	day 60 postpartum	0.37 ± 0.19 (SD)	0.41 ± 0.14 (SD)	-
Guesnet et al. 1995[a]	France	day 90 postpartum	0.38 ± 0.17 (SD)	0.50 ± 0.21 (SD)	-
Jansson et al. 1981	Sweden	Colostral milk	1.0 ± 0.2 (SD)	0.8 ± 0.2 (SD)	-
Jansson et al. 1981	Sweden	Transitional milk	0.5 ± 0.2 (SD)	0.5 ± 0.2 (SD)	-
Jansson et al. 1981	Sweden	Mature milk	0.3 ± 0.1 (SD)	0.4 ± 0.1 (SD)	-
Putnam et al. 1982	US	8 week of lactation	0.1 ± 0.01 (SEM)	0.6 ± 0.03 (SEM)	0.1 ± 0.03 (SEM)
Clark et al. 1982	US	2 weeks postpartum	-	0.55 ± 0.09 (SD)	-
Clark et al. 1982	US	6 weeks postpartum	-	0.45 ± 0.12 (SD)	-
Clark et al. 1982	US	12 weeks postpartum	-	0.44 ± 0.06 (SD)	-
Clark et al. 1982	US	16 weeks postpartum	-	0.40 ± 0.07 (SD)	-
Bitman et al. 1983	US	very premature infants, day 42 of lactation (mature milk)	0.24 ± 0.09 (SE)	0.55 ± 0.18 (SE)	0.04 ± 0.05 (SE)
Bitman et al. 1983	US	premature infants, day 42 of lactation (mature milk)	0.21 ± 0.09 (SE)	0.58 ± 0.19 (SE)	0
Bitman et al. 1983	US	term infants, day 42 of lactation (mature milk)	0.23 ± 0.14 (SE)	0.60 ± 0.29 (SE)	0
Harris et al. 1984	US	2-7 months postpartum	0.1 ± 0.1	0.4 ± 0.1	Trace
Specker et al. 1987	US	women on macrobiotic diet for 36-132 months, median duration of lactation 5-months	0.27 ± 0.08 (SEM)	0.68 ± 0.03 (SEM)	-
Specker et al. 1987	US	Omnivorous diet, median duration of lactation 2-months	0.29 ± 0.05 (SEM)	0.69 ± 0.03 (SEM)	-
Sanders et al. 1978	England	vegans	0.23 ± 0.11 (SE) (range 0.02-0.55)	0.72 ± 0.21 (SE) (range 0.21-1.21)	0.04 ± 0.01 (SE) (range 0.02-0.08)
Sanders et al. 1978	England	Omnivore controls	0.59 ± 0.23 (SE) (range 0.24-1.22)	0.54 ± 0.13 (SE) (range 0.34-0.92)	0.20 ± 0.08 (SE) range (0.04-0.36)
Sanders et al. 1979	England	3-months of lactation	0.59 ± 0.23 (SE)	0.54 ± 0.01 (SE)	0.20 ± 0.08 (SE)

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TABLE III-1
Levels of AA, DHA and EPA in human milk

Reference	Country	Characteristics	Wt % DHA (g/100 g total fatty acids)	Wt % AA (g/100 g total fatty acids)	Wt % EPA (g/100 g total fatty acids)
Huisman et al. 1996	Dutch	day 14 postpartum	0.23 ± 0.06 (SD)	0.41 ± 0.07 (SD)	0.05 ± 0.02 (SD)
Huisman et al. 1996	Dutch	day 42 postpartum	0.19 ± 0.11 (SD)	0.34 ± 0.06 (SD)	0.06 ± 0.04 (SD)
Huisman et al. 1996	Dutch	day 89 postpartum	0.19 ± 0.12 (SD)	0.34 ± 0.05 (SD)	0.07 ± 0.06 (SD)
Muskiet et al. 1987	Netherlands	Tanzania, >10 days after birth	0.27 ± 0.11 (SD)	0.60 ± 0.13 (SD)	0.19 ± 0.38 (SD)
Muskiet et al. 1987	Netherlands	Curacao, >10 days after birth	0.43 ± 0.14 (SD)	0.71 ± 0.17 (SD)	0.05 ± 0.03 (SD)
Muskiet et al. 1987	Netherlands	Surinam, >10 days after birth	0.41 ± 0.18 (SD)	0.56 ± 0.15 (SD)	0.06 ± 0.05 (SD)
Beijers and Schaafsma 1996	Netherlands	very preterm, mature milk	0.24 ± 0.13 (SD)	0.31 ± 0.11 (SD)	0.07 ± 0.03 (SD)
Beijers and Schaafsma 1996	Netherlands	preterm, mature milk	0.34 ± 0.15 (SD)	0.37 ± 0.12 (SD)	0.07 ± 0.03 (SD)
Makrides et al. 1995b	Australia	week 6 postpartum	0.26 ± 0.13 (SD)	0.45 ± 0.08 (SD)	0.07 ± 0.04 (SD)
Makrides et al. 1995b	Australia	week 16 postpartum	0.21 ± 0.13 (SD)	0.40 ± 0.07 (SD)	0.07 ± 0.04 (SD)
Makrides et al. 1995b	Australia	week 30 postpartum	0.19 ± 0.10 (SD)	0.39 ± 0.07 (SD)	0.06 ± 0.03 (SD)
Gibson and Kneebone 1981	Australia	Colostral milk (day 3-5)	0.64 ± 0.27 (SD)	0.71 ± 0.18 (SD)	0.43 ± 0.20 (SD)
Gibson and Kneebone 1981	Australia	Mature milk (day 40-45)	0.32 ± 0.17 (SD)	0.40 ± 0.10 (SD)	0.16 ± 0.07 (SD)
de la Presa-Owens et al. 1996	Spain	20-30 days postpartum	0.34 (range 0.15–1.03)*	0.50 (range 0.22–0.79)*	0.14 (range 0.06–0.42)*
Idota, 1991[a]	Japan	3-5 days postpartum	1.33	0.59	0.20
Idota, 1991[a]	Japan	6-10 days postpartum	1.24	0.54	0.22
Idota, 1991[a]	Japan	11-15 days postpartum	1.09	0.47	0.23
Idota, 1991[a]	Japan	16-30 days postpartum	1.02	0.42	0.23
Idota, 1991[a]	Japan	31-60 days postpartum	1.00	0.40	0.24
Idota, 1991[a]	Japan	61-120 days postpartum	1.00	0.39	0.24
Idota, 1991[a]	Japan	120-240 days postpartum	0.95	0.37	0.23
Idota, 1991[a]	Japan	240-482 days postpartum	1.04	0.36	0.22
Ogunleye et al. 1991	Japan	well-nourished mothers, 70-100 days postpartum	0.53 ± 0.10 (SD) (mol %)	0.36 ± 0.01 (SD) (mol % of milk fat)	0.20 ± 0.01 (SD) (mol %)
Ogunleye et al. 1991	Nigeria	well-nourished mothers, 60-90 days postpartum	0.34 ± 0.01 (SD) (mol %)	0.56 ± 0.02 (SD) (mol %)	0.09 ± 0.01 (SD) (mol %)
Koletzko et al. 1991	Nigeria	mature milk, rural area, 3-5	0.93 (0.70-2.16)*	0.82 (0.38-1.48)* (ratio	0.48 (0.17-1.57)*

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TABLE III-1
Levels of AA, DHA and EPA in human milk

Reference	Country	Characteristics	Wt % DHA (g/100 g total fatty acids)	Wt % AA (g/100 g total fatty acids)	Wt % EPA (g/100 g total fatty acids)
		months postpartum		0.72 (0.44-3.9))	
Budowski et al. 1994	Israel	6-10 weeks postpartum, foremilk	0.38 ± 0.16 (SD)	0.60 ± 0.12 (SD)	-
Budowski et al. 1994	Israel	6-10 weeks postpartum, aftermilk	0.37 ± 0.17 (SD)	0.58 ± 0.12 (SD)	-
Laryea et al. 1995	Sudan	2-24 months of lactation, well nourished mothers	0.07 ± 0.05 (SD) (range 0–0.28)	0.51 ± 0.10 (SD) (range 0.28-0.86)	0.01 ± 0.02 (SD) (range 0–0.13)
Chulei et al. 1995	China	marine, 2 weeks – 6-months postpartum	2.78 ± 1.20 (SD)	1.17 ± 0.33 (SD)	1.01 ± 0.50 (SD)
Chulei et al. 1995	China	urban 1, 2 weeks – 6-months postpartum	0.88 ± 0.34 (SD)	0.89 ± 0.14 (SD)	0.50 ± 0.29 (SD)
Chulei et al. 1995	China	urban 2, 2 weeks – 6-months postpartum	0.82 ± 0.35 (SD)	1.01 ± 0.50 (SD)	0.66 ± 0.43 (SD)
Chulei et al. 1995	China	rural, 2 weeks – 6-months postpartum	0.68 ± 0.29 (SD)	0.80 ± 0.14 (SD)	0.35 ± 0.18 (SD)
Chulei et al. 1995	China	pastoral, 2 weeks – 6-months postpartum	0.44 ± 0.29 (SD)	1.22 ± 0.32 (SD)	0.66 ± 0.27 (SD)
Kneebone and Gibson 1985	Malaysia	Chinese, 1-6 months postpartum	0.71 ± 0.14	0.64 ± 0.17	-
Kneebone et al. 1985	Malaysia	Malay, 1-6 months postpartum	0.90 ± 0.29	0.47 ± 0.20	-
Kneebone et al. 1985	Malaysia	Indian, 1-6 months postpartum	0.90 ± 0.36	0.57 ± 0.17	-

* data reported as median (interquartile range)
- Not reported
[a] Data obtained from Jensen, 1999
SD = standard deviation
SE = standard error
SEM = standard error of the mean

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B. Intended Uses

1. Introduction

Similac® Special Care® and Similac NeoSure® are infant formulas designed for preterm infants and for preterm infants after hospital discharge, respectively. Similac® Special Care® is an exempt infant formula used in the hospital management of premature infants. Similac NeoSure® is an exempt infant formula used in the post-discharge management of premature infants. Similac® With Iron is a non-exempt infant formula used for the routine feeding of term infants. Ross Products Division conducted two prospective, randomized, double-blinded, multicenter, clinical trials in which AA and DHA-rich oil were added to one or more of these three formulas and the effects on growth, safety, tolerance, and efficacy were evaluated as compared to infants fed unsupplemented formulas through approximately the first year of life.

In Ross Study AG38/AH95, the study of the preterm infants through 14-m corrected age (CA), AA and DHA were added to SSC at average levels of 0.43 and 0.27% (g/100 g total fatty acids), respectively, while AA and DHA were added to Similac NeoSure® at levels of 0.43 and 0.16% (g/100 g total fatty acids), respectively. Infants in the AF92 study, the study of term infants through the 12-months of life, consumed formulas supplemented with 0.46 % AA and 0.13% DHA (g/100 g total fatty acids).

As part of each study protocol, Ross Products collected data detailing the amount of formula consumed by infants. During the in-hospital phase of the preterm study, formula intake was monitored daily. Post-discharge, parents were requested to maintain 3-day dietary intake records prior to each scheduled visit at term-, 1-, 2-, 3-, 4-, 5-, 6-, 9-, and 12-months CA. Three-day dietary intake records were also maintained by parents of infants in the term study during the days prior to the 1-, 2-, 4-, 6-, 9-, and 12-month visits. The information in these hospital and diet records therefore provide our best estimates of consumption of these formulas by the target populations, namely preterm and term infants. Knowledge of the formula intake along with the fatty acid content of the oils and the oil content of the formulas can be used to estimate intake of the individual LCPs. Similarly, data on the concentrations of the enriched oils in the formulas allows us to estimate intakes of the AA and DHA-rich oils.

The estimated intakes of AA, DHA and the AA and DHA-rich oils in the supplemented SSC, Similac NeoSure®, and SWI formulas used in the clinical trials are presented below. These data will be used to establish the EDI of the AA and DHA-rich oils for the purposes of this GRAS determination. It should be noted, however, that the EDI derived for the AA-rich oil is based on the use of SUNTGA25 a fungal oil that provides approximately 25% AA by weight of fatty acids. The AA-rich oil that is the subject of this GRAS document is SUNTGA40S. It is more concentrated than SUNTGA25 and provides

approximately 40% AA by weight of fatty acids. Given the higher concentration of AA in SUNTGA40S, compared to SUNTGA25, less oil will need to be added to the infant formula in order to provide the same amount of AA. The estimated intake of SUNTGA40S will be reduced by 37.5% compared to the intake values derived from these clinical trials using SUNTGA25.

2. Preterm Infant Formula (SSC)

Table III-2 provides estimates of the fatty acid and oil intakes by infants consuming SSC supplemented with 0.43% AA and 0.27% DHA in the preterm study. These estimates are based on the formula intakes of individuals categorized as exclusive formula feeders, i.e. <100 mL human milk/kg birth weight for the duration of the initial hospital stay and AA + DHA supplemented study formula >80% of the times fed daily at term CA. Comparison of the in-hospital intakes, averaged daily intakes, and 3-day intake reported at the term CA visit, reveals that the highest intakes, as calculated per kilogram bodyweight, were estimated at the term CA visit. At the term visit, estimates of intake indicate that infants consumed a mean of 134.1 and 105.9 mg/kg bodyweight of the AA- and DHA-enriched oils, respectively. At the term visit, mean intakes of AA, DHA and EPA fatty acids were 32.4, 20.3, and 5.8, mg/kg bodyweight, respectively. The DHA:EPA ratio was 3.5, and the target AA:DHA ratio was 1.6.

The AA-rich oil used in the AG38 study of preterm infants was SUNTGA25, a fungal oil that is approximately 25% AA by weight of fatty acids. The AA-rich oil that is the subject of this GRAS document is SUNTGA40S, a more pure form of the fungal oil that contains approximately 40% AA by weight of fatty acids. Given the higher concentration of AA in SUNTGA40S as compared to SUNTGA25, less oil will need to be added to the infant formula in order to provide the same amount of AA, namely 0.43 g per 100 gram of fatty acids in SSC as consumed in this study. The estimated intake of AA-rich oil from SSC, 134.1mg/kg/d, will be reduced by 37.5% when SUNTGA40S is used, thereby reducing the estimated mean intake of AA-rich oil to 84 mg/kg/d.

TABLE III-2
Estimated Intake of AA-rich oil, DHA-rich oil, and AA, DHA and EPA Fatty Acid Intake by Preterm Infants Consuming Similac® Special Care® Formula Supplemented with 0.43% AA and 0.27% DHA *
Exclusive Formula Feeders**

		AA-rich oil	DHA-rich oil	AA	DHA	EPA
		mg/kg/d		mg/kg/d		
Time	N	Mean ± SD		Mean ± SD		
In-hospital	34	86.8 ± 12.5	68.5 ± 9.9	21.0 ± 3.0	13.1 ± 1.9	3.7 ± 0.5
Term CA	28	134.1 ± 35.4	105.9 ± 27.9	32.4 ± 8.5	20.3 ± 5.3	5.8 ± 1.5

* AA provided by AA-rich oil; DHA provided by DHA-rich oil: intakes based upon oil content of formulas, mean fatty acid content of the oils, and formula intake data collected from in-hospital records and 3-day diet records collected prior to each the term CA visit in Ross Clinical Trial AG38.
** Exclusive Formula Feeders are defined as those infants receiving less than 100 mL human milk/kg of birth weight for the duration of the initial hospital stay and receiving study formula more than 80% of the times fed daily to term CA.

3. Post-Discharge Formula for Preterm Infants (Similac NeoSure®)

Table III-3 provides estimates of the fatty acid and oil intakes by infants consuming Similac NeoSure® supplemented with 0.43% AA and 0.16% DHA in the preterm study. These estimates are based on the formula intakes of individuals categorized as exclusive formula feeders, i.e. <100 mL human milk/kg birth weight for the duration of the initial hospital stay and AA + DHA supplemented study formula >80% of the times fed daily at term CA. Comparison of the mean intakes across the 5 time points reveals that the highest intakes, as calculated per kilogram bodyweight, were estimated at the 2-month CA visit. At the 2-month CA visit, estimates of intake indicate that infants consumed a mean of 114.3 and 49.0 g per kilogram bodyweight of the AA- and DHA-enriched oils, respectively. At the 2-month CA visit, mean intakes of AA, DHA and EPA fatty acids were 27.5, 10.1, and 2.7, mg per kilogram bodyweight, respectively. The DHA:EPA ratio was 3.7, and the target AA:DHA ratio was 2.7.

In Clinical Trial AG38, the source of the AA-rich oil was SUNTGA25. The estimated intake of AA-rich oil from Similac NeoSure®, 114.3 mg/kg/d, will be reduced by 37.5% when SUNTGA40S is used, thereby reducing the estimated intake of AA-rich oil to 71.4 mg/kg/d.

TABLE III-3
Estimated Intake of AA-rich oil, DHA-rich oil, and AA, DHA and EPA Fatty Acid Intake by Preterm Infants Consuming Similac NeoSure® Formula Supplemented with 0.43% AA and 0.16% DHA *
Exclusive Formula Feeders**

		AA-rich oil	DHA-rich oil	AA	DHA	EPA
		mg/kg/d		mg/kg/d		
Month CA	N	Mean ± SD		Mean ± SD		
2	22	114.3 ± 45.9	49.0 ± 19.7	27.5 ± 11.0	10.1 ± 4.0	2.7 ± 1.1
4	23	113.7 ± 47.0	48.8 ± 20.1	27.3 ± 11.3	10.0 ± 4.1	2.7 ± 1.1
6	20	97.1 ± 36.3	41.6 ± 15.6	23.3 ± 8.7	8.5 ± 3.2	2.3 ± 0.9
9	18	82.6 ± 47.1	35.4 ± 20.2	19.8 ± 11.3	7.3 ± 4.1	2.0 ± 1.1
12	14	69.9 ± 40.7	30.0 ± 17.4	16.8 ± 9.8	6.2 ± 3.6	1.7 ± 1.0

* AA provided by AA-rich oil; DHA provided by DHA-rich oil: intakes based upon oil content of formulas, mean fatty acid content of the oils, and formula intake data collected from 3-day diet records collected prior to study visits in Ross Clinical Trial AG38.

** Exclusive Formula Feeders are defined as those infants receiving less than 100 mL human milk/kg of birth weight for the duration of the initial hospital stay and receiving study formula more than 80% of the times fed daily to term CA.

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4. Term Infant formula (SWI)

Table III-4 provides estimates of the fatty acid and oil intakes by exclusively formula fed term infants consuming SWI supplemented with 0.46% AA and 0.13% DHA. Comparison of the mean intakes across the 6 time points reveals that the highest intakes, as calculated per kilogram bodyweight, were estimated at the 2-month visit. At the 2-month visit, estimates of intake indicate that infants consumed a mean of 107.8 and 38.9 g per kilogram bodyweight of the AA- and DHA-enriched oils, respectively. At the 2-mo visit, mean intakes of AA, DHA and EPA fatty acids were 26.3, 7.31, and 1.94, mg per kilogram bodyweight, respectively. The DHA:EPA ratio was 3.8, and the target AA:DHA ratio was 2.7.

As in Clinical Trial AG38, the source of the AA-rich oil in the clinical trial of term infants, Clinical Trial AF92, was also SUNTGA25. The estimated intake of AA-rich oil from SWI, 107.8 mg/kg/d, will be reduced by 37.5% when SUNTGA40S is used, thereby reducing the estimated intake of AA-rich oil to 67.4 mg/kg/d.

TABLE III-4
Estimated Intake of
AA-rich oil, DHA-rich oil, and
AA, DHA and EPA Fatty Acid Intake by Exclusively Formula Fed Term Infants Consuming Similac® with Iron Formula Supplemented with 0.46% AA and 0.13% DHA *

		AA-rich oil	DHA-rich oil	AA	DHA	EPA
Visit (month)	N	mg/kg/d Mean ± SD		mg/kg/d Mean ± SD		
1	69	105.3 ± 13.4	38.0 ± 4.8	25.7 ± 3.3	7.15 ± 0.91	1.89 ± 0.24
2	63	107.8 ± 16.3	38.9 ± 5.9	26.3 ± 4.0	7.31 ± 1.10	1.94 ± 0.29
4	60	94.5 ± 16.6	34.1 ± 6.0	23.1 ± 4.1	6.41 ± 1.13	1.70 ± 0.30
6	59	75.7 ± 16.5	27.3 ± 5.9	18.5 ± 4.0	5.14 ± 1.12	1.36 ± 0.30
9	59	60.2 ± 14.0	21.7 ± 5.1	14.7 ± 3.4	4.08 ± 0.95	1.08 ± 0.25
12	54	48.2 ± 17.9	17.4 ± 6.5	11.8 ± 4.4	3.27 ± 1.21	0.87 ± 0.32

* AA provided by AA-rich oil; DHA provided by DHA-rich oil: intakes based upon oil content of formulas, mean fatty acid content of oils, and formula intakes reported in 3-day diet records collected in Ross Clinical Trial AF92

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5. Published Recommendations for Fatty Acid Addition to Infant Formulas

Three expert panels, the British Nutrition Foundation, FAO/WHO, and the International Society for the study of Fatty Acids and Lipids (ISSFAL), have published recommendations for the levels of AA and DHA in infant formulas (Table III-5). These recommendations range from 20 to 60 mg AA per kilogram bodyweight per day, and from 20 to 40 mg DHA per kilogram bodyweight per day in infant formulas for preterm formulas. For term formulas, the recommended intakes for AA are 20 to 40 mg per kilogram bodyweight per day, while recommended intakes of DHA are 20 mg per kilogram bodyweight per day. Additionally, ISSFAL suggests that the DHA:EPA ratio of all infant formulas exceed 3.5. Comparison of these recommendations to the estimated daily intakes and maximum intakes of AA and DHA from the formulas used in the clinical trials reveals that the proposed formulas provided LCPs at levels of intake at or slightly below the recommendations of these international expert panels. The ratio of DHA:EPA was also within the recommendations.

Given the absence of any adverse outcomes from either Clinical Trial AG38, the study of preterm infants, and Clinical Trial AF92, the study of term infants, we can conclude that estimated intakes of enriched oils and AA and DHA fatty acids from these formulas as provided in the studies are safe levels of intake for the intended uses.

TABLE III-5 Recommendations of Expert Panels for the Inclusion of AA and DHA in Infant Formulas			
	AA	DHA	DHA:EPA
	(mg/kg body weight/d)		
Preterm Formula			
BNF, 1992	20	20	--
FAO/WHO, 1994	60	40	--
ISSFAL, 1999*[a]	33	23	> 3.5
Term Formula			
BNF, 1992	20	20	--
FAO/WHO, 1994	40	20	--
ISSFAL, 1999*[a]	28	19	> 3.5

* Data were converted from % fatty acids to mg/kg/d using these assumptions: preterm infants consume 120 kcal/kg/d; term infants consume 100 kcal/kg/d; fatty acids account for 50% of the energy in preterm and term formulas; and 1 gram of fat has 9 kcals.
[a] Data obtained from Simopoulos, 1999

C. Finished Product: Composition with Respect to Levels of AA, DHA, EPA, LA and ALA in the Infant Formulas

The fatty acid composition of the control (i.e., unsupplemented) and AA + DHA supplemented SSC, Similac NeoSure®, and SWI formulas used in the AG38 and AF92 studies are presented in Tables III-6, III-7, and III-8.

Comparison of the fatty acid composition of each control formula to the corresponding AA + DHA supplemented formula reveals that the addition of the AA and DHA-rich oils affected only the long chain polyunsaturated fatty acid composition of the formulas. The levels of linoleic (LA) and α-linolenic (ALA) were comparable between each control and supplemented formula, as was the ratio of LA:ALA. This is because the addition rate of the oils is very low and the oils is are concentrated of AA and DHA.

In addition, the intakes of linoleic acid (LA) and α-linolenic acid (ALA) fatty acids by infants consuming supplemented or control formulas from clinical trials AG38 (preterm infants; SSC and Similac NeoSure® phases) and AF92 (term infants; SWI) were calculated to determine the impact of AA-rich and DHA-rich oil supplementation in formulas providing adequate intakes of the essential fatty acids. As shown in Tables III-9, III-10, and III-11, there was no meaningful difference in intake levels of these fatty acids from supplemented formulas compared to the controls.

TABLE III-6 Fatty Acid Composition (wt% or g/100 g total fatty acids) of Similac® Special Care® Formula[a]		
Fatty Acid (FA)	**Control**	**AA- and DHA-rich oil supplemented formula**
Saturated FA		
6:0	0.38	0.34
8:0	30.4	29.8
10:0	21.3	21.2
12:0	9.4	8.1
14:0	3.6	3.2
15:0		0.04[b]
16:0	5.3	5.5
17:0	0.06	0.06
18:0	2.6	2.7
20:0	0.12	0.13
22:0	0.11	0.15
24:0	0.27[c]	0.10
Monounsaturated FA		
16:1n-7	0.22	0.45
18:1n-9	8.2	8.4
20:1n-9	0.08	0.10
Omega-6 FA		
18:2n-6 (linoleic acid)	16.0	16.8
20:2n-6		
20:3n-6		0.06
20:4n-6 (AA)[d]	ND	0.43
22:5n-6		
Omega-3 FA		
18:3n-3 (α-linolenic acid)	2.4	2.6
18:4n-3		
20:5n-3 (EPA)	ND	0.08
22:6n-3 (DHA)[d]	ND	0.27
18:2n-6:18:3n-3	6.7	6.5

[a] data represent average values of the three formula batches used in the Ross Products Division Clinical Trial AG38
[b] single batch
[c] average of two batches
[d] Range of AA from 0.42 – 0.45%
Range of DHA from 0.24 – 0.32%
ND = Not detected

TABLE III-7 Fatty Acid Composition (wt% or g/100 g total fatty acids) of the Similac NeoSure® Formula[a]		
Fatty Acid	**Control[b]**	**AA- and DHA-rich oil supplemented formula**
Saturated FA		
6:0	0.24	0.27
8:0	16.1	17.0
10:0	11.2	10.6
12:0	9.5	8.5
14:0	3.7	3.4
15:0	ND	ND
16:0	6.3	6.4
17:0	0.05	ND
16:3	ND	ND
18:0	2.4	2.4
20:0	0.24	0.25
22:0	0.17	0.22
24:0	0.09	0.15
Monounsaturated FA		
16:1n-7	0.07	0.11
18:1n-9	28.3	27.9
20:1n-9	NA	NA
24:1n-9		
Omega-6		
18:2n-6 (linoleic acid)	19.1	19.5
18:3n-6		
20:2n-6	ND	ND
20:3n-6		
20:4n-6 (AA)[d]	ND	0.43
22:4n-6		
22:5n-6	NA	NA
Omega-3		
18:3n-3 (α-linolenic acid)	2.4	2.4
18:4n-3	NA	NA
20:5n-3 (EPA)	ND	ND
22:5n-3		
22:6n-3 (DHA)[d]	ND	0.16
18:2n-6:18:3n-3	8.0	8.1

[a] data represent average values of the formula batches used in the Ross Products Division Clinical trial AG38
[b] average of 5 batches
[c] average of 6 batches
[d] Range of AA from 0.41 – 0.44%
Range of DHA from 0.15 – 0.18%
NA = not available
ND = not detected

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TABLE III-8
Fatty Acid Composition (wt% or g/100 g total fatty acids) of Similac® with Iron[a]

Fatty Acid	Control	AA + DHA Fungal/fish
Saturated FA		
6:0	0.27	0.24
8:0	2.73	2.49
10:0	2.00	1.78
12:0	10.48	12.87
14:0	5.76	5.17
16:0	8.37	8.16
18:0	3.83	3.81
20:0	0.33	0.35
22:0	0.25	0.29
24:0	0.12	0.19
Monounsaturated FA		
16:1	0.09	0.12
18:1	40.53	40.00
20:1	0.22	0.26
24:1n-9	0.09	0.09
Omega-6		
18:2n-6 (linoleic acid)	22.23	21.00
18:3n-6		
20:2n-6		
20:3n-6	ND	0.05
20:4n-6 (AA)[b]	-	0.46
22:4n-6		
22:5n-6		
Omega-3		
18:3n-3 (α-linolenic acid)	2.58	2.37
20:5n-3 (EPA)	-	0.04
22:5n-3		
22:6n-3 (DHA)[b]	-	0.13
Ratios		
18:2n-6/18:3n-3	8.63	8.90
20:4n-6/22:6n-3	-	3.60

[a] data represent average values of the three formula batches used in the Ross Products Division Clinical trial AF92
[b] Range of AA from 0.42 – 0.48%
Range of DHA from 0.11 – 0.15%
ND = Not detected

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TABLE III-9
Estimated Intake of Linoleic and α-Linolenic Fatty Acids by Preterm Infants Consuming Control Formula or Similac® Special Care® Formula Supplemented with 0.43% AA and 0.27% DHA*
(g/100 g total fatty acids)
Exclusive Formula Feeders**

	AA + DHA (fungal/fish) Formula			Control Formula		
		Linoleic	α-Linolenic		Linoleic	α-Linolenic
Time	N	g/kg/d Mean ± SD		N	g/kg/d Mean ± SD	
In-hospital	34	0.81 ± 0.12	0.12 ± 0.02	37	0.74 ± 0.14	0.11 ± 0.02
Term CA	28	1.26 ± 0.33	0.19 ± 0.05	33	1.06 ± 0.24	0.16 ± 0.01

* AA provided by AA-rich oil; DHA provided by DHA-rich oil; DHA 0.16% from term CA through 12-mo CA; intakes based upon oil content of formulas, mean fatty acid content of the oils, and formula intake data collected from in-hospital records and 3-day diet records collected prior to the term CA visit in Ross Clinical Trial AG38.

** Exclusive Formula Feeders are defined as those infants receiving less than 100 mL human milk/kg of birth weight for the duration of the initial hospital stay and receiving formula more than 80% of the times fed daily at term CA.

TABLE III-10
Estimated Intake of Linoleic and α-Linolenic Fatty Acids by Preterm Infants Consuming Control Formula or Similac NeoSure® Formula Supplemented with 0.43% AA and 0.16% DHA*
(g/100 g total fatty acids)
Exclusive Formula Feeders**

	AA + DHA (fungal/fish) Formula			Control Formula		
		Linoleic	α-Linolenic		Linoleic	α-Linolenic
Month CA	N	g/kg/d Mean ± SD		N	g/kg/d Mean ± SD	
2	22	1.25 ± 0.50	0.16 ± 0.06	32	1.07 ± 0.27	0.13 ± 0.03
4	23	1.25 ± 0.52	0.16 ± 0.06	31	0.95 ± 0.26	0.12 ± 0.03
6	20	1.07 ± 0.40	0.13 ± 0.05	29	0.87 ± 0.36	0.11 ± 0.04
9	18	0.91 ± 0.52	0.11 ± 0.06	28	0.83 ± 0.31	0.10 ± 0.04
12	14	0.77 ± 0.45	0.10 ± 0.06	23	0.73 ± 0.24	0.09 ± 0.03

* AA provided by AA-rich oil; DHA provided by DHA-rich oil; DHA 0.27% until infants reached term CA, and 0.16% from term CA through 12-mo CA; intakes based upon oil content of formulas, mean fatty acid content of the oils, and formula intake data collected from 3-day diet records collected prior to each post term CA visit in Ross Clinical Trial AG38.

** Exclusive Formula Feeders are defined as those infants receiving less than 100 mL human milk/kg of birth weight for the duration of the initial hospital stay and receiving formula more than 80% of the times fed daily at term CA.

TABLE III-11
Estimated Intake of
Linoleic and α-Linolenic
Fatty Acids by Exclusively Formula Fed Term Infants Consuming Control Formula or Similac® with Iron Formula Supplemented with 0.46% AA and 0.13% DHA *
(g/100 g total fatty acids)

	AA + DHA (fungal/fish) Formula			Control Formula		
		Linoleic	α-Linolenic		Linoleic	α-Linolenic
Visit (month)	N	g/kg/d Mean ± SD		N	g/kg/d Mean ± SD	
1	69	1.17 ± 0.15	0.13± 0.02	63	1.24 ± 0.19	0.14± 0.02
2	63	1.20 ± 0.18	0.14 ± 0.02	54	1.29 ± 0.23	0.15 ± 0.03
4	60	1.05 ± 0.18	0.12 ± 0.02	53	1.14 ± 0.20	0.13 ± 0.02
6	59	0.84 ± 0.18	0.10 ± 0.02	50	0.92 ± 0.18	0.11 ± 0.02
9	59	0.67 ± 0.16	0.08 ± 0.02	48	0.74 ± 0.18	0.09 ± 0.02
12	54	0.53 ± 0.20	0.06 ± 0.02	42	0.63 ± 0.19	0.07 ± 0.02

* AA provided by AA-rich oil; DHA provided by DHA-rich oil; intakes based upon oil content of formulas, mean fatty acid content of oils, and formula intakes reported in 3-day diet records collected prior to each visit in Ross Clinical Trial AF92

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IV. Intended Effect

A. Rationale for Adding AA and DHA to Preterm and Term Infant Formulas

1. Introduction

Linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) cannot be synthesized by human tissues and are classified as dietary essential nutrients. The dietary essentiality of both n-6 and n-3 fatty acids is related in part to their role as precursors for the synthesis of the long chain polyunsaturated fatty acids (LCP), arachidonic acid (AA)(20:4n-6) and docosahexanoic acid (DHA)(22:6n-3), respectively. The n-6 and n-3 fatty acid composition of developing tissues is dependent on the quantity and type of diet lipid, the activity of the desaturase enzymes responsible for synthesis of LCP from the essential fatty acids, and the partitioning of the various n-6 and n-3 fatty acids among oxidation, desaturation and acylation.

Dietary linoleate and linolenate are metabolized, respectively, to 5,8,11,14-20:4 and 5,8,11,14,17-20:5 via the use of position-specific Δ6 and Δ5 desaturases and a malonyl-CoA-dependent chain elongation step as follows (Sprecher 2000):

- the n-6 pathway starting from dietary linoleate, 9,12-18:2→6,9,12-18:3→8,11,14-20:3→5,8,11,14-20:4;
- the n-3 pathway starting from dietary linolenate, 9,12,15-18:3→6,9,12,15-18:4→8,11,14,17-20:4→5,8,11,14,17-20:5.

For many years, it was assumed, but not proven, that the synthesis of 22-carbon acids with their first double bond at position 4 required an acyl-CoA-dependent Δ4 desaturase. It has now been established that the synthesis of these compounds requires a degradative step and proceeds as follows (Sprecher 2000):

- the n-6 pathway, 5,8,11,14-20:4→7,10,13,16-22:4→9,12,15,18-24:4→6,9,12,15,18-24:5→4,7,10,13,16-22:5;
- the n-3 pathway, 5,8,11,14,17-20:5→7,10,13,16,19-22:5→9,12,15,18,21-24:5→6,9,12,15,18,21-24:6→4,7,10,13,16,19-22:6

The revised pathways for the biosynthesis of PUFA require the participation of both microsomes and peroxisomes as they relate to the synthesis of 22-carbon acids with their first double bond at position 4 (Sprecher 2000).

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Linoleic and α-linolenic acids are precursors for the synthesis of AA and DHA which are important structural fatty acids in neural tissues and perform an array of membrane associated functions (Al et al., 2000). During the last trimester of pregnancy, fetal requirements for AA and DHA are high. Large amounts of AA and DHA are incorporated into the central nervous system as well as the retina. Before birth, the fetus depends primarily on the placental transfer of AA and DHA and thus on the essential fatty acid status and supply of the mother (Al., 2000; Innis, 1991). After birth, AA and DHA are supplied to the infant from diet, i.e. human milk or infant formula or are formed from their dietary fatty acid precursors. Although

linoleic and α-linolenic acids are precursors for AA and DHA respectively, it is thought that for infants born prematurely, AA and DHA may actually be conditionally essential. Infants born at term have incorporated LCP stores in the liver and brain, but infants born prematurely contain lower amounts and concentrations of these LCPs (Martinez, 1992).

Therefore, the question that arises is, is it helpful to supplement infant formula with AA and DHA? There are several lines of evidence, which are considered when evaluating this question. It is known that fetal requirements of AA and DHA are high in part due to the incorporation of these fatty acids into the retina and CNS. Intrauterine and postnatal estimates of AA and DHA accretion give a determination of the amount of AA and DHA which need to be supplied to the infants either directly or through conversion of their precursors. Data on *in vivo* synthesis of AA and DHA in preterm and term infants can also be analyzed to examine whether essential fatty acid metabolism is sufficient to provide an infant with enough AA and DHA to meet the needs of the developing tissues. Additionally, biochemical and functional measurements can be used to determine fatty acid requirements. One can compare plasma and RBC phospholipid AA and DHA levels in infants fed human milk to infants fed formula with or without supplemental AA and DHA as a measure of the biologic availability and incorporation from dietary intake of these fatty acids. It is assumed that the n-3 and n-6 fatty acid content of human milk of all well-nourished mothers meets or exceeds the n-3 and n-6 fatty acid requirement of the infant for normal physiological and biochemical development. It is therefore reasonable to examine the levels of AA and DHA commonly found in human milk as a guide to the appropriate levels of AA and DHA supplementation for infant formula. The results of formula supplementation with AA and DHA can be compared from a functional perspective by evaluating the consequences of AA and DHA supplementation on vision and mental development.

The following discussion presents these arguments to provide the rationale for adding AA and DHA to preterm and term infant formulas.

2. Intrauterine and Postnatal Estimates of AA and DHA Accretion

Intrauterine and extrauterine accretion estimates for the long-chain n-3 and n-6 fatty acids in the brain are published by several investigators (Clandinin et al., 1980a, 1980b, 1981; Makrides et al., 1994; Farquharson et al., 1992). Clandinin et al. (1981) reported an estimate of 22.2 mg/week and 42.7 mg/week for intrauterine accretion of n-3 and n-6 fatty acids respectively, in the brain. Martinez (1992, 1998) later reported accretion estimates for individual fatty acids in the forebrain. DHA is the dominant n-3 fatty acid and AA and its derivatives (22:4n-6; 22:5n-6) are the major n-6 fatty acids in the brain. Accretion estimates from 34 preterm and postnatal normally fed infants up to 2 years of age were provided by Martinez (1992) as equations (e.g. μmol DHA/forebrain; $= -4519.446 + 226.459x - 2.245x^2 + 0.017x^3 - 5.244\ (10^{-5})x^4$; x = gestation age in weeks). Based on this equation and assuming the percentage of DHA in the forebrain is similar to that in other brain regions and the forebrain represents 90% of the total brain weight, the third trimester intrauterine and postnatal accretion rates for DHA are 46 mg/week and 34mg/week, respectively. Using the equations provided by Martinez (1992) for accretion of AA, estimates for third trimester accretion are 60 mg/week and for postnatal accretion are 33 mg/week.

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Cunnane et al. (2000) estimated postnatal DHA accretion for infants over 6-months after birth using previously published information (Clandinin et al., 1980a, 1980b, 1981; Martinez 1998; Makrides et al., 1994; Farquharson et al., 1992), assumptions about storage pools of DHA, estimates for accretion from synthesis of DHA from α-linolenic acid *in vivo*, and estimates for accretion of DHA in lean tissue based on published studies with animals. They estimated that DHA accretion in brain represented 48% of the total DHA accretion, and total accretion of DHA during the first 6-months after birth is 72 mg/week. Estimates for accretion of AA were not presented.

The assumptions required to estimate the intrauterine and postnatal accretion rates for AA and DHA in non-neural tissues, however, limit the reliability of using accretion estimates alone for establishing supplementation levels of AA and DHA in infant formulas. Clandinin and colleagues (1980b) estimated accretion of n-3 and n-6 fatty acids in the whole brain, but not specifically DHA. Martinez (1992) estimated accretion of individual fatty acids but in the forebrain only.

3. *In vivo* Synthesis of DHA and AA in Preterm and Term Infants

The biosynthesis of AA and DHA from n-6 and n-3 fatty acids, respectively, consists of a series of chain desaturation and elongation reactions at the carboxyl terminal (Uauy et al. 1989). In infants who are breast fed, essential fatty acid metabolism is based in part on a direct supply of LCPs which by-pass the regulatory desaturation steps (Uauy and Hoffman 2000). Earlier studies on n-3 and n-6 fatty acids requirements of newborn infants suggested that the activity of the enzymes, particularly desaturase, were low during the newborn period (Innis, 2000). In addition, the effects of AA and DHA are not replicated by providing equivalent amount of linoleic and α-linolenic acid because there appears to be limited conversion of α-linolenic acid to DHA in infants. The synthesis of DHA from α-linolenic acid may be reduced due to the inhibition of the desaturase enzyme by excess of dietary linoleic acid (Uauy and Hoffman, 2000). Thus, it is unclear if the rates of AA and DHA synthesis in young infants are sufficient to meet the needs of developing tissues (Innis 2000).

4. Fatty Acids Levels in Plasma and RBC Phospholipid Fractions

Several studies have reported higher circulating plasma and/or RBC phospholipid AA and DHA levels in term infants fed formula enriched with AA and DHA compared to the infants receiving unsupplemented formula (Agostoni et al. 1994, 1995; Auestad et al. 1997; Decsi et al. 1998). The circulating DHA levels were similar in infants fed 1.2% or 5% fatty acids as α-linolenic acid (Putnam et al., 1982), suggesting that circulating DHA levels are not affected by increased α-linolenic acid intake.

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Plasma and/or red blood cell phospholipid AA levels were similar in infants fed human milk or infant formula (Sanders et al., 1978; Olegard and Svennerholm, 1971). In contrast to these findings, Putnam et al. (1982) and DeLucchi et al. (1987) reported lower RBC phospholipid AA levels and higher linoleic acid levels in formula fed infants. The AA level in human milk of mothers on vegan diet was similar to the mothers on omnivorous diet despite higher amount of linoleic acid in human milk of vegan mothers. The RBC AA level was also similar in infants of mothers on vegan and infants of mothers on omnivorous diet

(Sanders et al., 1978). This suggests that the AA levels in human milk is independent of the mothers linoleic acid intake and the RBC AA level of infants is independent of linoleic acid level in human milk. The relationship between AA and DHA levels in milk and in the infants circulating lipids favors the hypothesis that the dietary intake of AA and DHA is a major determinant of the level of these fatty acids in the plasma and red blood cell glycerophospholipids of young infants (Innis, 1991).

Similarly, circulating plasma and/or RBC phospholipid AA and DHA levels were higher in preterm infants fed AA and DHA supplemented formula compared to the infants fed unsupplemented formula, and the levels were comparable to the infants fed human milk (Vanderhoof et al. 1999; Foreman-Van Drogelen et al. 1996; Koletzko et al. 1995; Clandinin et al. 1997; Carlson et al. 1998; Birch et al. 1998). The circulating plasma phospholipid and/or RBC phospholipid AA and DHA levels are lower in very premature infants born at less than 33 weeks gestation compared to term infants, when fed either human milk or formula (Innis et al., 1989; Innis et al., 1990). It has been observed that the DHA levels decreased in premature infants during intravenous nutrition and transition to full enteral feeding. Subsequent feeding with human milk prevented the decline in DHA levels, but repletion to the levels expected *in utero* at corrected age did not occur (Innis et al., 1990). Older premature infants (33-34 weeks gestation) fed conventional infant formula containing α-linolenic acid had lower plasma phospholipid DHA than infants fed human milk (Koletzko et al., 1989). A decline in RBC phospholipid AA and DHA from birth to 30 days was observed in infants (26-35 weeks gestation) fed formula with 1.9% α-linolenic acid rather than human milk (Pita et al., 1988). This inverse relationship between higher levels of precursors and lower levels of AA and DHA in very premature and premature infants fed formula rather than human milk is similar to the effects of these diets in term infants (Innis, 1991).

Evening primrose oil (EPO) has been used in some preterm (Woltil et al. 1999; Koletzko et al. 1995) and term studies (Makrides et al. 1995a; Agostoni et al. 1994; Desci et al. 1998; Koletzko et al. 1996; Desci and Koletzko, 1995) as a precursor of AA. Evening primrose oil (EPO) contains γ-linolenic acid, which is a metabolic precursor of AA that bypasses the rate-limiting Δ6-desaturase-enzyme step (Makrides et al., 1995a). In the studies by Woltil et al. (1999) and Makrides et al. (1995a), the RBC AA levels were lower in the fish oil and EPO supplemented group compared to the group given formula without LCPs or the human milk group, suggesting that EPO cannot fully compensate for decreased AA levels in infants RBC. In studies where egg lipids (Koletzko et al. 1995; Agostoni et al. 1994; Desci et al. 1998; Koletzko et al. 1996; Desci and Koletzko, 1995) were used as a source of AA and DHA in addition to EPO, the plasma and/or RBC AA levels were higher than the LCP unsupplemented formula group, but lower or comparable to the human milk group. Evening primrose oil in the presence of EPA and the absence of AA does not increase the RBC AA levels, and the effect of EPO on AA levels in plasma and/or RBC in presence of AA cannot be determined due to lack of control groups without AA supplementation. The effects of EPO as a precursor of AA on plasma and/or RBC AA are inconclusive.

5. Human Milk Levels of AA and DHA

In Table III-1, an extensive review of the levels of AA and DHA in human milk was presented. Due to the dependence of human milk DHA levels on the maternal diet, it is informative to consider the differences among the western and non-western diets. Jensen (1999) performed a systematic review of the levels of AA and DHA in human milk from women consuming western and non-western diets. As can be seen in Table IV-1, for women consuming Western diets, the AA levels in human milk range from 0.05 - 0.87 wt % and the DHA levels range from trace to 1.03 wt %. EPA levels are low with a mean of 0.09 wt %. For women consuming non-western diets, DHA levels tend to be higher. The AA/DHA ratio, calculated by dividing reported mean values of AA and DHA in human milk, is presented in Table IV-2 and ranges from 0.35 to 7.29. The ratio of means of AA/DHA for women from the U.S. and Europe ranges from 0.43 to 6.00. This is the normal range of ratios of means of AA/DHA from human milk to which human infants are exposed. It is reasonable to examine the levels of AA and DHA found in human milk as well as the range of ratios of means of AA/DHA as a guide to the appropriate levels of AA and DHA in infant formula from the supplementation of that formula with any GRAS sources of these fatty acids.

TABLE IV-1 Fatty Acid Composition (wt%) of Milks from Women Consuming Western and Non-Western Diets				
Fatty acids	**Western**		**Non-Western**	
	Mean[a]	Range[a]	Mean[a]	Range[a]
AA (20:4n-6)	0.42	0.05-0.87	0.92	0.28-1.12
DHA 22:6n-3	0.45	Tr-1.03	0.88	0.00-2.78
EPA 20:5n-3	0.09	0.00-0.71	0.52	0.00-1.07

Note: Table adapted from Jensen (1999)
[a] reported in Jensen (1999), mature milk used

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TABLE IV-2 Ratio of Means of AA and DHA in Human Milk					
Reference	**Country**	**Characteristics**	**Mean Wt % DHA**	**Mean Wt % AA**	**Ratio of means of (AA/DHA)**
Chen et al. 1995	Canada	3-4 weeks postpartum	0.14	0.35	2.50
Innis et al. 1988	Canada	4-28th week of lactation, inuit women from Arctic community	1.4	0.6	0.43
Innis et al. 1988	Canada	2-4 months postpartum, women from Vancouver consuming mixed North American diet	0.4	0.7	1.75
Koletzko et al. 1988	Germany	mature hindmilk	0.22	0.36	1.64
Genzel-Boroviczeny et al. 1997	Germany	term, day 5 postpartum	0.46	0.72	1.57
Genzel-Boroviczeny et al. 1997	Germany	preterm, day 5 postpartum	0.43	0.74	1.72
Genzel-Boroviczeny et al. 1997	Germany	term, day 10 postpartum	0.39	0.59	1.51
Genzel-Boroviczeny et al. 1997	Germany	preterm, day 10 postpartum	0.35	0.64	1.83
Genzel-Boroviczeny et al. 1997	Germany	term, day 20 postpartum	0.27	0.50	1.85
Genzel-Boroviczeny et al. 1997	Germany	preterm, day 20 postpartum	0.24	0.51	2.13
Genzel-Boroviczeny et al. 1997	Germany	term, day 30 postpartum	0.23	0.45	1.96
Genzel-Boroviczeny et al. 1997	Germany	preterm, day 30 postpartum	0.24	0.48	2.00
Harzer et al. 1983	Germany	day 1 postpartum, term infants	0.22	0.75	3.41
Harzer et al. 1983	Germany	day 3 postpartum, term infants	0.21	0.55	2.62
Harzer et al. 1983	Germany	day 5 postpartum, term infants	0.29	0.54	1.86
Harzer et al. 1983	Germany	day 8 postpartum, term infants	0.26	0.50	1.92
Harzer et al. 1983	Germany	day 15 postpartum, term infants	0.17	0.43	2.53
Harzer et al. 1983	Germany	day 22 postpartum, term infants	0.15	0.36	2.40
Harzer et al. 1983	Germany	day 29 postpartum, term infants	0.16	0.39	2.44
Harzer et al. 1983	Germany	day 36 postpartum, term infants	0.16	0.39	2.44
Chardigny et al. 1995	France	day 0 - 90 postpartum	0.32	0.50	1.56
Guesnet et al. 1993	France	days 2-5 postpartum	0.55	0.67	1.22
Guesnet et al. 1993	France	day 15 postpartum	0.43	0.53	1.23
Guesnet et al. 1993	France	day 30 postpartum	0.32	0.41	1.28
Guesnet et al. 1993	France	day 60 postpartum	0.37	0.41	1.11
Guesnet et al. 1993	France	day 90 postpartum	0.38	0.50	1.32
Jansson et al. 1981	Sweden	Colostral milk	1.0	0.8	0.80

TABLE IV-2 Ratio of Means of AA and DHA in Human Milk					
Reference	**Country**	**Characteristics**	**Mean Wt % DHA**	**Mean Wt % AA**	**Ratio of means of (AA/DHA)**
Jansson et al. 1981	Sweden	Transitional milk	0.5	0.5	1.00
Jansson et al. 1981	Sweden	Mature milk	0.3	0.4	1.33
Putnam et al. 1982	US	8 week of lactation	0.1	0.6	6.00
Clark et al. 1982	US	2 weeks postpartum	-	0.55	
Clark et al. 1982	US	6 weeks postpartum	-	0.45	
Clark et al. 1982	US	12 weeks postpartum	-	0.44	
Clark et al. 1982	US	16 weeks postpartum	-	0.40	
Bitman et al. 1983	US	very premature infants, day 42 of lactation (mature milk)	0.24	0.55	2.29
Bitman et al. 1983	US	premature infants, day 42 of lactation (mature milk)	0.21	0.58	2.76
Bitman et al. 1983	US	term infants, day 42 of lactation (mature milk)	0.23	0.60	2.61
Harris et al. 1984	US	2-7 months postpartum	0.1	0.4	4.00
Specker et al. 1987	US	women on macrobiotic diet for 36-132 months, median duration of lactation 5 months	0.27	0.68	2.52
Specker et al. 1987	US	Omnivorous diet, median duration of lactation 2 months	0.29	0.69	2.38
Sanders et al. 1978	England	vegans	0.23	0.72	3.13
Sanders et al. 1978	England	Omnivore controls	0.59	0.54	0.92
Sanders et al. 1979	England	3 months of lactation	0.59	0.54	0.92
Huisman et al. 1996	Dutch	day 14 postpartum	0.23	0.41	1.78
Huisman et al. 1996	Dutch	day 42 postpartum	0.19	0.34	1.79
Huisman et al. 1996	Dutch	day 89 postpartum	0.19	0.34	1.79
Muskiet et al. 1987	Netherlands	Tanzania, >10 day after birth	0.27	0.60	2.22
Muskiet et al. 1987	Netherlands	Curacao, >10 day after birth	0.43	0.71	1.65
Muskiet et al. 1987	Netherlands	Surinam, >10 day after birth	0.41	0.56	1.37
Beijers and Schaafsma 1996	Netherlands	very preterm, mature milk	0.24	0.31	1.29
Beijers and Schaafsma 1996	Netherlands	preterm, mature milk	0.34	0.37	1.09
Makrides et al. 1995b	Australia	week 6 postpartum	0.26	0.45	1.73
Makrides et al. 1995b	Australia	week 16 postpartum	0.21	0.40	1.90
Makrides et al. 1995b	Australia	week 30 postpartum	0.19	0.39	2.05
Gibson and Kneebone 1981	Australia	Colostral milk (day 3-5)	0.64	0.71	1.11
Gibson and Kneebone 1981	Australia	Mature milk (day 40-45)	0.32	0.40	1.25
de la Pressa-Owens et al. 1996	Spain	20-30 days postpartum	0.34	0.50	1.47
Idota, 1991[a]	Japan	3-5 days postpartum	1.33	0.59	0.44
Idota, 1991[a]	Japan	6-10 days postpartum	1.24	0.54	0.44

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TABLE IV-2 Ratio of Means of AA and DHA in Human Milk					
Reference	**Country**	**Characteristics**	**Mean Wt % DHA**	**Mean Wt % AA**	**Ratio of means of (AA/DHA)**
Idota, 1991[a]	Japan	11-15 days postpartum	1.09	0.47	0.43
Idota, 1991[a]	Japan	16-30 days postpartum	1.02	0.42	0.41
Idota, 1991[a]	Japan	31-60 days postpartum	1.00	0.40	0.40
Idota, 1991[a]	Japan	61-120 days postpartum	1.00	0.39	0.39
Idota, 1991[a]	Japan	120-240 days postpartum	0.95	0.37	0.39
Idota, 1991[a]	Japan	240-482 days postpartum	1.04	0.36	0.35
Ogunleye et al. 1991	Japan	well-nourished mothers, 70-100 days postpartum	0.53	0.36	0.68
Ogunleye et al. 1991	Nigeria	well-nourished mothers, 60-90 days postpartum	0.34	0.56	1.65
Koletzko et al. 1991	Nigeria	mature milk, rural area, 3-5 months postpartum	0.93	0.82	0.88
Budowski et al. 1994	Israel	6-10 weeks postpartum, foremilk	0.38	0.60	1.58
Budowski et al. 1994	Israel		0.37	0.58	1.57
Laryea et al. 1995	Sudan	2-24 months of lactation, well nourished mothers	0.07	0.51	7.29
Chulei et al. 1995	China	marine, 2 weeks – 6 months postpartum	2.78	1.17	0.42
Chulei et al. 1995	China	urban 1, 2 weeks – 6 months postpartum	0.88	0.89	1.01
Chulei et al. 1995	China	urban 2, 2 weeks – 6 months postpartum	0.82	1.01	1.23
Chulei et al. 1995	China	rural, 2 weeks – 6 months postpartum	0.68	0.80	1.18
Chulei et al. 1995	China	pastoral, 2 weeks – 6 months postpartum	0.44	1.22	2.77
Kneebone et al. 1985	Malaysia	Chinese, 1-6 months postpartum	0.71	0.64	0.90
Kneebone et al. 1985	Malaysia	Malay, 1-6 months postpartum	0.90	0.47	0.52
Kneebone et al. 1985	Malaysia	Indian, 1-6 months postpartum	0.90	0.57	0.63
[a] Data obtained from Jensen, 1999					

6. Functional assessment

Some clinical studies in preterm infants have shown that dietary supplementation of infant formula with LCP results in improvement in some mental and visual functions. One study showed improvement in electroretinogram (ERG) threshold (Uauy et al. 1990; Birch et al. 1992a, Uauy et al. 1994) and visual acuity as measured by visual evoked potential (VEP) (Hoffman et al. 1993; Birch et al. 1992b; Uauy et al. 1994) in preterm infants fed formula enriched with DHA from marine oil compared to infants fed corn oil. Faldella et al. (1996) and Carlson et al (1999) reported maturation of visual evoked potential and higher visual acuity using the acuity card procedure, respectively, in infants fed formula containing AA and DHA compared to the infants receiving formula with no DHA. Carlson et al. (1999) observed higher visual acuity in the group that was

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supplemented with AA and DHA from birth than the group who was supplemented from term and the control group. In contrast, studies by Hansen et al. (1997) and Koletzko et al. (1995b) showed no effect of DHA or DHA and AA supplementation on visual acuity in preterm infants fed formula supplemented with DHA and AA. However, in the studies by Hansen et al. (1997) and Koletzko et al. (1995b), the duration of supplementation was 28 and 21 days, respectively compared to 52 weeks of PCA supplementation in the Faldella et al. (1996) and Carlson et al. (1999) studies. Perhaps long-term supplementation with AA and DHA is required for beneficial effects on vision. Enhanced VEP in preterm infants who consumed formulas supplemented with AA and DHA-rich oils was found at 6-months but not at 4-months in a study conducted by Ross Products (AH95).

Only a few studies in term infants have reported beneficial effects of AA and DHA supplementation on visual acuity and mental development. In a study by Birch et al. (1998), sweep VEP acuity was higher in the AA and DHA supplemented formula group compared to the control formula group, and the acuity in the supplemented group was comparable to the human milk group. The mental development index (MDI) scores, cognitive, language and motor scores in the Bayley scales of Infant Development (Birch et al. 2000), development quotient (DQ) scores in the Bornet-Lezine at 4-months or Agostoni at 24 months (Agostoni et al. 1995) and problem solving ability (Willatts et al. 1998a, 1998b) was better in AA and DHA supplemented group compared to the control formula group. These studies, however, had a low number of subjects. In contrast, most studies have shown no effect of AA and DHA enriched infant formula on visual acuity (Auestad et al. 1997), sweep VEP (Auestad et al. 1997; Makrides et al. 2000), psychomotor development index (PDI) (Birch et al. 2000; Lucas et al. 1999; Makrides et al. 2000), MDI (Lucas et al. 1999; Makrides et al. 2000), behavioral rating scale (Birch et al. 2000), developmental screening inventory tests (Lucas et al. 1999), IQ and vocabulary at 3.25 years (Scott et al. 1997), and on mental and motor scores at 12-months of age. Scott et al. (1997, 1998) observed lower vocabulary scores at 14-months of age, but not at 39-months, in infants supplemented with DHA without AA.

7. Conclusion

There is a growing concern that current methods for the nutritional management of infants, particularly very premature infants, with standard formulas, may not support deposition of optimal amounts of n-6 and n-3 fatty acids in the CNS during the period of rapid brain growth. Similarly, biochemical measurements have shown essential fatty acid deficiency develops rapidly during the first days of life if critically ill infants are administered intravenous solutions with little or no lipid. Plasma and RBC levels of AA and DHA are also lower in premature and term infants fed some commercial formulas, or bovine rather than human milk. Human milk fatty acids contain about 7-18% LA, 0.4-1.5% ALA and small amounts (<1-2%) of n-6 and n-3 LCP. Infant formula and intravenous fats, on the other hand, provide similar or higher levels of LA and ALA but little or no LCPs. The accretion of large amounts of LCP in the growing CNS, presence of LCPs in human milk, lower circulating LCP levels in plasma and RBC of infants fed formula rather than human milk, and improvement in some mental and visual functions in some clinical studies of infants fed AA and DHA supplemented formula, have been

offered as reasons to consider that LCPs may be essential in infant nutrition (Innis, 1991).

B. Rationale For Specific Use Levels of AA-rich oil and DHA-rich oil in Preterm Infant Formulas (SSC, Similac NeoSure®) and Term Infant Formula (SWI)

1. Use Levels in Preterm Formulas

Similac® Special Care® (SSC) and Similac NeoSure® are infant formulas containing AA and DHA designed for preterm infants in the hospital and for preterm infants who have been discharged from the hospital, respectively. The results of clinical studies with preterm infants available during the planning stages of the AG38/AH95 study in 1996 provided the basis for the levels of AA and DHA that were added to SSC (0.43 and 0.25%, respectively) and Similac NeoSure® (0.43 and 0.15%, respectively).

The studies by Carlson and colleagues (1993; 1996) and Uauy and colleagues (Birch et al., 1992a; Uauy et al., 1990) provided the basis for supplementing SSC with 0.25% total fatty acids as DHA. These published studies reported improved visual development in preterm infants fed formulas with 0.20 to 0.35% as DHA from fish oil. These formulas however, did not contain AA. In a study by Clandinin et al. (1997), the plasma phospholipid AA and DHA levels in preterm infants fed low AA and DHA (0.32 and 0.24%, respectively) and medium AA and DHA (0.49 and 0.35%, respectively) formulas were comparable to the levels observed in human milk fed infants. However, AA and DHA levels in the RBC phospholipid fractions in the group receiving medium levels were comparable to the human milk group. The group that received high AA and DHA (1.1 and 0.76%, respectively) formula had higher AA and DHA levels in the plasma and RBC phospholipids compared to the human milk group. The levels used in SSC and Similac NeoSure® are comparable to the levels used for the low and medium group in the Clandinin et al. (1997) study.

The results of studies on term infants formed the basis for supplementing Similac NeoSure® with 0.15% total fatty acids as DHA. A dose-response study with term infants fed human milk or formulas containing 0 to 0.24% DHA from fish oil (Innis et al. 1996a) together with the results of another DHA supplementation study with term infants fed formula containing 0 to 0.23% DHA found that formula with 0.15% DHA (Auestad et al. 1997) resulted in plasma and red blood cell levels of DHA most similar to those of the age-matched breastfed infants studied concurrently.

The results of the latter study with term infants also formed the basis for supplementing both SSC and Similac NeoSure® with 0.43% total fatty acids as AA. The study by Auestad et al. (1997) found that plasma and red blood cell levels of AA closely

matched those of breastfed infants studied concurrently when formulas contained 0.43% as AA.

Further, the levels of AA and DHA used in SSC and Similac NeoSure® are within the ranges of the levels of AA (0.05 – 0.87%) and DHA (Tr-1.03%) found in human milk of mothers from western countries (Jensen 1999). The levels are comparable or lower than the recommended levels by several expert panels BNF (1992) (AA 0.4%, DHA 0.4%), FAO/WHO (1994) (AA 0.9%, DHA 0.6%)[1], and Simopoulos et al., (1999) (AA 0.5%, DHA 0.35%)) for preterm infant formulas. The ratio of AA/DHA in SSC and Similac NeoSure® is 1.6 and 2.7, respectively and is comparable to the ratio in human milk of mothers from western and non-western countries.

2. Use Levels in Term Formula

The results of studies on term infants formed the basis for supplementing the term infant formula with 0.15% total fatty acids as DHA. A dose-response study with term infants fed human milk or formulas containing 0 to 0.24% DHA from fish oil (Innis et al. 1996a) together with the results of another DHA supplementation study with term infants fed formula containing 0 to 0.23% DHA found that formula with 0.15% DHA (Auestad et al. 1997) resulted in plasma and red cell levels of DHA most similar to those of the age-matched breastfed infants studied concurrently.

The results of the latter study with term infants also formed the basis for supplementing term infant formula with 0.43% total fatty acids as AA. The study by Auestad et al. (1997) found that plasma and red cells levels of AA closely matched those of breastfed infants studied concurrently when formulas contained 0.43% as AA.

The levels of AA (0.43%) and DHA (0.15%) used in term infant formula are within the ranges of the levels of AA (0.05-0.87%) and DHA (Tr-1.03%) found in human milk of mothers from western countries (Jensen 1999). The levels are comparable or lower than the recommended levels by several expert panels: BNF 1992 (AA 0.4%, DHA 0.4%), FAO/WHO (1994) (AA 0.72%, DHA 0.36%)[1], and Simopoulos et al., (1999) (AA 0.5%, DHA 0.35%)) for term infant formulas. The target ratio of AA/DHA (2.7) in the term infant formula falls well within the normal range of ratios from human milk of mothers from western and non-western countries (0.43 to 6.0).

[1] Assumptions for calculating AA and DHA % values are 1) preterm and term infants consume 120 kcal/kg/d and 100 kcal/kg/d, respectively, 2) fatty acids comprise 50% of energy of the formula, and 3) 1 g fat has 9 kcal.

V. Analytical Methodology

The analytical methods used to provide the characterization for several batches of both DHA-rich oil and SUNTGA25 and 40S are provided in Appendix B.

Fatty acid methyl esters (FAME) were prepared from aliquots of the SUNTGA oil samples. Gas liquid chromatography of the methyl esters was performed. Additional fatty acid identification was performed from the alkali-esterification product. Absolute fatty acid composition (g/100 g oil) was completed using methyl esters for analysis and GC-MS for identification. The oil sample of SUNTGA40S was analyzed in duplicate for the Wijs iodine value, peroxide value, free fatty acid content and p-anisidine value. The amount of tocopherol present in SUNTGA40S was calculated by comparison to known amounts of reference standard materials injected into the same HPLC system. Identification of sterols was performed on SUNTGA40S and was facilitated by comparison with standard reference materials and comparison of the molecular ion produced by the ITDS with the molecular weights of standard reference materials. Quantitation of the identified sterols and unknowns was made relative to the internal standard. Duplicate aliquots of SUNTGA40S were saponified according to A.O.C.S. Official Method Ca 6b-53 and the unsaponifiable matter was recovered. Analysis for organochlorine material and phthalate esters in SUNTGA40S was also completed. Pesticide analysis was completed. Dioxin analysis was completed for DHA-rich oil samples only.

VI. Safety Assessment

A. AA-Rich and DHA-Rich Oils

1. Safety of the Source of AA-Rich Oil (*Mortierella alpina*)

SUNTGA25 and 40S are AA containing oils derived from *Mortierella alpina* Peyronel 1S-4. *Mortierella alpina* is GRAS as a source of ARASCO, an AA containing oil, for use in infant formulas (Letter of May 17, 2001 from FDA in response to GRN 000041). SUNTGA40S is essentially identical to ARASCO; the major differences observed between the two oils are in the unsaponifiable matter and sterol content (information taken from GRN 00041). SUNTGA40S has a much lower amount of unsaponifiable matter (0.49, 0.39 and 0.46% analyzed in three batches) compared to Martek's ARASCO (DSM Gist Optimar) oil (1.73, 1.36, 1.69 and 1.18% analyzed in four batches). Sterol content for SUNTGA40S (0.237, 0.125 and 0.100% analyzed in three batches) is also much lower than Martek's ARASCO (1.5% as reported by Martek). The manufacturing process for SUNTGA40S removes these impurities more completely than the manufacturing process for ARASCO.

The genus *Mortierella* is presently classified as a member of the family Morteirellaceae within the order of the Mucorales, class Zygomycetes. In general, members of the Mucorales have been associated with Mucormycosis. The disease is the result of an opportunistic infection. Classic risk groups are patients with uncontrolled diabetes and immunocompromised patients. In general, all strains capable of growing at 37° C. should be regarded as potentially pathogenic, whereas strains that are unable to grow at body temperatures should be regarded as safe with respect to this disease (Streekstra 1997).

Mortierellaceae, including *M. alpina*, is a common soil fungus to which humans are frequently exposed (Domsch et al. 1980). The pathogenic potential of the genus seems to be quite low (Streekstra 1997). The species of Mortierella can be divided fairly sharply into two ecological groups according to their preference for particular pH ranges. *M. alpina*, probably the most common species of the genus, together with *M. gamsii*, *M exigua* and *M. minutissima*, belongs to the inhabitants of neutral and/or alkaline habitats. *M. alpina* possesses a relatively low growth rate. The production of mycoferritin, ethanol and acetic acid has been reported, as well as AA (Domsch et al. 1980).

Species of the genus *Mortierella* have not been used extensively for food production, but a number of examples exist. The ATCC list of Industrial Fungi (Jong et al. 1994, cited in Streekstra 1997) mentions *Mortierella* species as producers of several food-related products (apart from lipids), such as enzymes (α-galactosidase and lipase) and β-carotene. Most of these examples concern the species *M. isabellina*, *M. ramanniana* and *M. vinacaea*, which are currently no longer considered to be valid

members of the genus *Mortierella*. On the other hand, related fungi within the *Mucorales* have a long track-record as a source of commercial food enzymes.

The only reliable reports of pathogenicity for the genus Mortierellaceae concern the species *M. wolfii*. It has been recovered from cases of abortion and pneumonia in cattle (Di Menna et al. 1972; Seviour et al. 1987; Carter et al. 1973; Corbel and Eades 1991; Cordes et al. 1972). In this regard, it is relevant that all isolates described in the literature have been isolated at temperatures of 35° C and higher. *M. alpina* Peyronel 1S-4, used for the production of SUNTG25 and 40S, is fermented initially at 28° C with fermentation of the main culture at 26° C. Therefore, evidence from optimal growth temperature of this strain supports the view that it is not pathogenic.

There has been no reference to any specific toxin production by members of the genus Mortierella in the literature, with the exception of the pathogenic species *M. wolfii* which excretes a water-soluble heat-labile trypsin-labile nephrotoxin (Davey and Kalmakoff 1973; Davey et al. 1973; Davey and Kalmakoff 1974; Davey et al. 1974). Several lines of evidence indicate that *M. alpina* does not produce any toxins of concern. A mycotoxin screen of Batch 97121951 of SUNTGA40S demonstrates that there is no detectable mycotoxin contamination from oil produced by *M. alpina* 1S-4. Results are shown in Table VI-1.

TABLE VI-1
Mycotoxin Analysis*

Mycotoxin	Result	Limit of Detection	Analytical Method
Aflatoxin B1	Not detected	5 ppb	HPLC
Aflatoxin B2	Not detected	5 ppb	HPLC
AflatoxinG1	Not detected	5 ppb	HPLC
AflatoxinG2	Not detected	5 ppb	HPLC
Sterigmatocystin	Not detected	0.05 ppm	HPLC
Ochratoxin	Not detected	0.05 ppm	HPLC
Patulin	Not detected	0.05 ppm	HPLC
Nivalenol	Not detected	0.05 ppm	GLC
Zearalenone	Not detected	0.05 ppm	HPLC
Fumonisin B1	Not detected	0.05 ppm	HPLC
Fumonisin B2	Not detected	0.05 ppm	HPLC

* Completed for three different batches (97121951, 98071451, 99072651)
HPLC = High performance liquid chromatography
GLC = Gas liquid chromatography

Additionally, results of toxicity tests which have been conducted on SUNTGA25, unsaponifiable matter of SUNTGA25 and the fungus culture have indicated that *M. alpina* does not produce any toxins of concern. These toxicology studies are described in more detail in the following section.

A paper by Shimizu et al. (1992) reported the finding of a novel sterol, 24, 25-Methylenecholest-5-en-3β-ol from *M. alpina* Peyronel 1S-4. An analysis of three lots of

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SUNTGA40S and one lot of SUNTGA25 was completed to quantify sterol impurity content. These results are presented in Table VI-2.

TABLE VI-2
Sterol Impurities from Batches of Oil Produced by *M. alpina* 1S-4

Lot Number	Total Sterol (g/100 g oil)	Desmosterol (g/100 g oil)	24,25 methylene- cholest-5-en-3β-ol (g/100 g oil)
SUNTGA40S			
98071451	0.13	0.074	0.015
99072651	0.27	0.18	0.022
99111651	0.22	0.16	0.037
SUNTGA25			
93122721	0.84	0.20	0.284

The biological significance of exposure to the novel sterol produced by *M. alpina* Peyronel 1S-4 was evaluated by examining its potential for estrogenic activity. The SUNTGA40S product was evaluated for estrogenic activity in three *in vitro* studies. Results of these studies demonstrate that SUNTGA40S does not bind *in vitro* to the estrogen receptor. The total unsaponifiable components and cyclopropanesterol, by-products in SUNTGA40S, were able to weakly displace [^{3}H]estradiol from the estrogen receptor. Therefore, estrogenic and anti-estrogenic activity of SUNTGA40S and total unsaponifiable components was studied *in vitro*. Neither test article functioned as an agonist or antagonist of human estrogen receptor in yeast.

In one study (Suntory 1998d), the binding of SUNTGA40S and its components to the estrogen receptor was evaluated using the receptor fraction from homogenized calf uterus. Standard solutions of test substances (SUNTGA40S (lot 97022151), total unsaponifiable matter (lot 970715), cyclopropane sterol) and reference substance (17-β-Estradiol) were prepared by serial dilution and the binding of the test substances to the receptor was determined by calculation of the inhibition rate from binding of tracer (^{3}H-Estradiol) to the receptor. Five concentrations were tested: 10^{-3}, 10^{-2}, 10^{-1}, 1, and 10 μg/ml. The inhibition rate of SUNTGA40S from binding of tracer to the receptor was low and was not dose-dependent, suggesting no binding of SUNTGA40S to the receptor. The inhibition rate of total unsaponifiable matter was dose-dependent, being 32.40% at 10 μg/ml, 20.52% at 1 μg/ml and 0.90% at 0.1 μg/ml. The inhibition rate of cyclopropane sterol was also dose-dependent, being 46.22% at 10 μg/ml, 22.30% at 1 μg/ml and 15.58% at 0.1 μg/ml. The results demonstrate the binding of the total unsaponifiable matter and cyclopropane to the receptor.

In another study (Suntory 1998e), a comparison of the binding of several test substances (SUNTGA40S, total unsaponifiable matter, Oil M, Oil R, Soybean oil, Fish oil, Rapeseed oil, Cholesterol, Cholic acid, β-Sitosterol, and ergosterol) to the estrogen receptor was evaluated using the receptor fraction from homogenized calf uterus.

Standard solutions of test substances and reference substance (17-β-estradiol) were prepared by serial dilution and the binding of the test substances to the receptor was determined by calculation of the inhibition rate from binding of tracer (^{3}H-Estradiol) to the receptor. Five concentrations were tested: 10^{-3}, 10^{-2}, 10^{-1}, 1, and 10 μg/ml. Inhibition rates of the test substances from binding of the tracer with the receptor indicated that the highest inhibition was produced by soybean oil (>20%) followed by rapeseed oil, β-sitosterol and ergosterol (>10%). The inhibition rates of the other test substances were <10%; inhibition produced by SUNTGA40S was 8% and inhibition by the total unsaponifiable matter was 6%.

The previous two studies evaluated the effects of SUNTGA40S and its components on the estrogen receptor using a competitive binding assay. Results indicated that total unsaponifiable components and cyclopropanesterol, by-products in SUNTGA40S, were able to weakly displace [^{3}H]estradiol from the estrogen receptor. Therefore, yeast expressing the human estrogen receptor (hER) and a reporter containing estrogen response element linked to β-galactosidase were used to assess estrogenic and anti-estrogenic activity of SUNTGA40S and total unsaponifiable components in comparison with representative oils and sterols in animals and plants (ARASCO, Soybean oil, Fish oil, Rapeseed oil, Cholesterol, Cholic acid, β-Sitosterol and Ergosterol) (Suntory 1998f). 17β-Estradiol caused β-galactosidase activity to increase in the yeast in a dose-dependent fashion. SUNTGA40S and total unsaponifiable components, at 0.1 - 100 μg/ml, caused no significant increase in β-galactosidase activity. None of the other test materials produced a marked effect on β-galactosidase activity. Tamoxifen reduced 17β-estradiol dependent β-galactosidase activity by 40%. Therefore, it can be concluded that since there was no significant increase in β-galactosidase activity induced by SUNTGA40S or the total unsaponifiable component fraction, the test article did not function as an agonist of hER. To determine if SUNTGA40S and the total unsaponifiable component fraction could function as an antagonist of hER, these test articles were incubated with yeast with 10 nM 17β-estradiol. SUNTGA40S and the total unsaponifiable component fraction had no effect on 17β-estradiol-induced β-galactosidase activity.

In conclusion, there is no evidence from the literature that *M. alpina* is pathogenic or toxigenic. SUNTGA40, containing as an impurity a small quantity of the sterol 24, 25-methylenecholest-5-en-3β-ol from *M. alpina* Peyronel 1S-4, is not likely to produce a clinically relevant adverse response through its action at the estrogen receptor.

2. Safety of the Source of DHA-Rich Oil (Tuna)

There is a substantial body of literature addressing the physiological and biochemical effects, and the safety of, diets rich in fish and/or fish oils. Much of this information was reviewed by FDA as part of the GRAS petition on menhaden oil. Concerns over possible adverse effects of fish oil consumption that were discussed by FDA included effects on bleeding time, glycemic control, and low-density lipoprotein (LDL) cholesterol. In summary, the FDA limited consumption of EPA plus DHA from fish oils is limited to 3 grams/person/day at this level there is no significant risk for increased bleeding time beyond the normal range. In addition, there is no clinically significant effect on glycemic control in diabetics. Consumption of menhaden oil by

healthy persons with normal cholesterol levels, as well as in persons with diabetes melitus, hypertension, abnormal blood lipid levels and cardiovascular disease was evaluated with respect to any adverse effect on LDL cholesterol or apolipoprotein B, a principal component of LDL. As a result of the evaluation FDA found that although there is a trend toward increased LDL cholesterol values with increased fish oil consumption in all population subgroups, the effect was associated with high levels of consumption (more than 5 g or more per day of EPA plus DHA). There would not be a safety concern from the perspective of adverse effects on LDL cholesterol of EPA plus DHA at a level of 3 grams/person/day or below.

A comparison of the composition of menhaden oil with DHA-rich tuna oil reveals that they are qualitatively similar with respect to the polyunsaturated fatty acid composition. EPA levels, however, are much lower in DHA-rich tuna oil than in menhaden oil. Table VI-3 presents a summary of the mean weight percentages for polyunsaturated fatty acids in menhaden oil and DHA-rich oil from tuna.

TABLE VI-3
Comparison of Polyunsaturated Fatty Acid (PUFA) Composition of Menhaden Oil and DHA-rich oil from Tuna

Menhaden Oil		**DHA-rich oil from Tuna**	
PUFA	**Mean Weight Percent**	**PUFA**	**Mean Percent**
C18:4	2.3	C18:4	1.1
C20:4 (AA)	2.0	C20:4 (AA)	2.1
C20:5 (EPA)	13.1	C20:5 (EPA)	6.2
C22:5	2.5	C22:5	2.1
C22:6 (DHA)	6.7	C22:6 (DHA)	20.6

A determination of the EPA levels in infant formulas to which DHA-rich oil are added and a calculation of the intake of EPA on a body weight basis from ingestion indicate that there is no concern for adverse effects on bleeding time. The GRAS affirmation of menhaden oil (21 CFR §184) specifies that at levels of intake of EPA plus DHA less than 3000 mg/person/day (50 mg/kg/day for a 60 kg person), there is no significant risk for increased bleeding time beyond the normal range. In a prior human infant study of a high EPA fish oil (menhaden oil) as 1% of fat in an infant formula, bleeding times were reported to be well within normal limits although the study was not designed with the power to detect adverse changes in this parameter (Uauy 1994). A calculation of the DHA plus EPA intake by infants from formulas supplemented with DHA-rich oil, as proposed in this GRAS document, demonstrates that intake is much lower than that reported by Uauy (1994). Calculations indicate that ingestion is a maximum of 26 mg/kg/day for preterm infants from SSC, a maximum of 13 mg/kg/day from Similac NeoSure® and a maximum of 10 mg/kg/day from SWI. An expert panel evaluating the composition of LCP for infant formula concluded "there is no concern about modest intakes of EPA, such as those found in human milk" (Koletzko 2001). The levels of EPA infant formulas supplemented with DHA-rich tuna oil are similar to those in human milk.

Compositional analysis of DHA-rich oil, presented in Section II. B, supports the safety of tuna oil as a source of DHA for infant formula. Appropriate specifications have

been set for fatty acid composition as well as any potential contaminants or impurities and factors that reflect oil quality, including: free fatty acids, heavy metals, pesticides, PCB, dioxins, unsaponifiable material, and peroxide value.

Currently, DHA-rich oil from tuna is used in infant formulas, yogurt, bread and fish sausage in Japan and in infant formulas, bread and fish sausage in Korea.

B. Adult Rodent Toxicology Studies of AA-Rich Oil and DHA-Rich Oil

M. alpina is a source for AA containing oil that has been tested extensively in preclinical assays. SUNTORY has tested its products, SUNTGA25 and 40s in acute, subacute, and subchronic rodent assays, in addition to mutagenicity assays. DHA-rich oil has been tested in acute and subchronic rat studies.

1. Acute Toxicity of SUNTGA25 (Suntory 1991a) and SUNTGA40S (Suntory 1998a)

SUNTGA25 and SUNTGA40S were tested in acute toxicity assays using three week old male ICR (SPF) strain mice (5 mice/group). Test compound was dissolved in olive oil (total volume of 10 ml/kg) and gavaged at a dose of 2 g/kg. Controls received olive oil. None of the animals showed any signs of abnormality as determined by clinical symptoms, weight changes and necropsy results, during the seven-day study period. The LD_{50} is determined to be > 2 g/kg for both oils.

2. Acute Toxicity of Unsaponifiable Matter of SUNTGA25 (Suntory 1994a,b)

Unsaponifiable matter from two lots of SUNTGA25 (Lots. 91111311 and 93122721) was tested in two acute toxicity assays in three week old male ICR (SPF) strain mice (5 mice/group). Test compound was dissolved in olive oil (total volume of 10 ml/kg) and gavaged at a dose of 2 g/kg. Controls received olive oil. None of the animals showed any signs of abnormality as determined by clinical symptoms, weight changes and necropsy results, during the seven-day study period. The LD_{50} of both lots from the two assays is determined to be > 2 g/kg.

3. Acute Toxicity of DHA-Rich Oil (Japan Food Research Laboratories 1991)

DHA-rich containing tuna oil was tested in an acute toxicity assay using male and female ddy-N mice (5 mice/sex/group). Test sample was diluted with olive oil to 5.8, 6.9, 8.3 and 10% (w/v) solution. Each mouse was administered test material by gavage at doses of 2000, 1660, 1380, and 1160 mg/kg. No mortality occurred. Soft stool was seen one hour after administration of test article, however, this resolved by 20 hours after dosing. No abnormal findings were seen at necropsy. The LD_{50} was determined to be > 2 g/kg.

4. **Subacute Toxicity Test of SUNTGA Producing Fungus Culture in Mice (Suntory 1994c)**

A two-week subacute test was conducted to determine the safety of orally fed SUNTGA producing fungus culture in mice (four week old male ICR strain (SPF) 10 mice/group). SUNTGA producing fungus culture was added to a γ-ray sterilized powder diet CE-2 at 0, 1.6, 4, or 10% providing 0, 0.8, 2, or 5% of SUNTGA, respectively. All test diets were made nutritionally similar by adding the fungus preparation (assuming 50% lipid and 50% undigestible fiber) at the expense of cellulose and olive oil. Controls received 5 g cellulose and 5 g olive oil per 100 g feed. No test article related abnormalities were observed in clinical signs, body weight, feed and water intake or gross pathology. The average feed intake was 13.9 g/kg/day for the highest dose level (10% addition).

5. **Mutagenicity/Chromosome Aberration**

Ames/Salmonella Mutagenicity of AA-Rich Oil (SUNTGA25; Suntory 1991b and SUNTGA40S; Suntory 1998b)

An Ames/Salmonella assay was conducted on SUNTGA25 using *S. typhimurium* tester strains TA97, TA98, TA100, TA102 and five different concentrations of test compound with the maximum concentration set at 5 mg/plate. A negative control and positive control were included (with S-9 for TA 97, 98, 100: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; TA 102: tert-burylhydroperoxide; without S-9 for TA97: 2-aminofluroene; TA98, 100: benzo[a]pyrene). No revertants appeared at 5 mg/plate. No mutagenicity was detected in the presence of S9 metabolic activation. (A slight increase in the appearance of TA102 revertants was noted in the presence of S9. AA is known to be easily oxidized and oxidized AA is very mutagenic. TA102 is an oxygen radical sensitive strain.) In conclusion, SUNTGA25 is not mutagenic in the Ames/Salmonella assay.

An Ames/Salmonella assay was conducted on SUNTGA40S using *S. typhimurium* tester strains TA97, TA98, TA100, TA102 and five different concentrations of test compound with the maximum concentration set at 5 mg/plate. A negative control and positive controls were included (2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; tert-burylhydroperoxide; 2-aminoanthracene; 2-aminofluroene; benzo[a]pyrene). No increase in the number of revertant colonies was seen with or without metabolic activation. In conclusion, SUNTGA40SAis not mutagenic in the Ames/Salmonella assay.

Chromosome Aberration of SUNTGA40S (Suntory 1998c)

SUNTGA40S was tested for potential to produce chromosome aberration in a fibroblast cell line from Chinese hamster. No cytotoxicity was observed with a maximum concentration of 1 mg/ml; therefore this level was used as the maximum concentration for the test in the presence and absence of S9 mix. The test was performed on 3 dose levels. For the direct method, cells were seeded in a plastic petri plate. Test agent was allowed to remain in the cultures until chromosome specimens

were prepared at 24 and 48 hours after the start of treatment. Mitomycin C was adopted as the positive control for the direct method. For the metabolic activation with S9 mix method, cells were seeded in a petri plate. After 3 days in culture, 2.5 ml of medium was removed. Different concentrations of the sample were simultaneously treated with S9 mix for 6 hours. Chromosome specimens were prepared after additional culture for 18 hours. Benzo[a]pyrene was used as the positive control for the S9 method. Cells were arrested in metaphase; a hundred well-spread metaphases per plate (two hundred metaphases per each concentration) were analyzed.

The results indicated that the incidence of cells with either structural or numerical aberrations was less than 5%. SUNTGA40S did not produce chromosome aberrations under the conditions of this assay.

6. Subacute Toxicity of SUNTGA25 (Suntory 1992a)

A two week range-finding toxicity study in rats was conducted on SUNTGA25 at 2.5%, 5% and 10% in the diet (study summarized in Table VI-4). At the low, mid and high dose levels, rats consumed up to 2960, 5470 and 12,750 mg AA containing oil/kg/day, respectively. The only effect noted was a non-significant increase in absolute and relative liver weight. Livers from both control and high dose animals demonstrated hepatic vacuolization and fatty change. All vacuoles found in control and high-dose test animal livers were Sudan III stain positive.

TABLE VI-4 Suntory (1992a) Two-Week Dietary Study in Rats		
AA Source and/or DHA Source	**AA (*Mortierella alpina*) SUNTGA25 (25% AA)**	
Dose	**Dose of Oil *(mg/kg b.w.)** **Low dose:** AA Oil = 2.5% of diet = 2835 mg/kg **Mid dose:** AA Oil = 5% of diet = 5280 mg/kg **High dose:** AA Oil = 10% of diet = 11,845 mg/kg * mean for male and female combined	**Dose of AA or DHA *(mg/kg b.w.)** **Low dose:** AA = 709 mg/kg **Mid dose:** AA = 1320 mg/kg **High dose:** AA = 2961 mg/kg *mean for male and female combined
Standardization of Test Groups for Dietary Lipid	Dose groups were not controlled for fat content.	
Control Groups	Basal diet (4.4% fat) only used as control	
Adverse Effects		
Clinical Chemistry	No consistent, dose-related changes.	
Hematology	No consistent dose-related changes.	
Organ Weight	Absolute and relative liver weight is increased in all dose groups, however, not statistically significant.	
Histopathology	Hepatic vacuolization and fatty change of the liver (only control and high dose examined). All vacuoles found in the control and compound dosed groups were Sudan III stain positive.	
Other	Animals fasted before sacrifice.	
Threshold for Effects of Oil Ingestion	Not applicable - range finding study	

7. Subchronic Toxicity of SUNTGA25 (Suntory 1992b)

A ninety day toxicity study of SUNTGA25 was conducted in rats (10 per sex/group) using a basal diet fortified by weight with 0, 0.8, 2.0 or 5.0% SUNTGA25 (study summarized in Table VI-5). Test group diets were not standardized for lipid content. The dose levels used resulted in total fat composition of the diet of 5.2% in the low dose, 6.4% in the mid dose and 9.4% in the high dose. The control group was fed basal diet with 4.4% fat. There was no high fat intake control group.

No mortality occurred in the study and there were no clinical observations indicative of test article toxicity. There were no differences in body weight gain of treated groups compared to controls. There were decreases in feed intake for male rats in the 2% dose group during weeks 4, 6, 7, 10, 11 and 13 and in the 5% dose group during weeks 1 to 3, 7 to 10, 12, and 13. There were decreases in feed intake

for female rats in the 0.8% dose group during week 4 and 10, in the 2.0% dose group during week 4 and in the 5.0% dose group during weeks 2 to 4, 6, and 9 to 11.

Sporadic changes in urinalysis and hematology parameters were not dose-related and not considered to be a treatment effect. Clinical chemistry indicated that there was increased blood urea nitrogen in males at the high dose and females in the mid and high doses. Other changes in clinical chemistry parameters were sporadic and not treatment related. Test compound related decreases in kidney weight and increases in liver weight were seen at the mid and high doses.

No adverse treatment related changes were seen in histopathology of the kidney or any other organ examined with the exception of liver. Histopathologic changes as determined by H&E staining were seen in the liver. As reported in the original pathology summary, six cases of fatty changes (small droplets) were noted in the liver of high dose male rats. One case was noted in low dose females, nine cases in mid dose females and four cases in high dose females. Sudan III staining showed fatty changes in all male and female rats, including control.

A re-evaluation of the histopathology data from this study, focused on the liver changes reported, was conducted by Dr. Dawn Goodman (full report found in Appendix C). Two sets of photomicrographs of liver sections were reviewed, in addition to the study report. No individual animal data or summary incidence tables were available for the necropsy or histopathology data. One set of photomicrographs was of liver sections stained by H&E. Individual animal numbers and diagnoses of the lesions depicted were provided with these photomicrographs. Caution must be used in interpreting this information as there was no indication how the lesions were selected for photomicrography and whether or not all of the lesions observed in the study are illustrated or were only representative examples. Review of these photomicrographs, however, indicates an apparent increase in the incidence of vacuolar degeneration (fatty change) in the high dose males and in mid dose and high dose females. This increase in incidence was slight for the high dose groups compared to controls. The increase was most striking in the mid dose females that does not correlate well with the increase in liver weight. The severity of the vacuolar degeneration observed in the photomicrographs was recorded as minimal to slight in all dose groups and would be considered to be a reversible change. The second set of photomicrographs consisted of H&E stained sections and comparable Sudan III stained sections from 3 animals/sex/group. Selection criteria for the animals or the lesions illustrated were not available. Based on these photomicrographs, however, there appeared to be a very slight increase in the severity of the Sudan III positive staining (indicating the presence of fats) in the high dose females. In all dose groups, the degree of positive staining was minimal to slight.

In conclusion, based on the photomicrographs provided, fatty change may have contributed <u>slightly</u> to the liver weight increase, however, the data is contradictory and inconclusive at best. Absence of a high fat control group limits interpretation of these study results.

TABLE VI-5 SUNTORY (1992b) Ninety-Day Dietary Rat Toxicity Test of an AA Containing Oil (SUNTGA25)		
AA Source and/or DHA Source	**AA (*Mortierella alpina*) SUNTGA25 (25% AA)**	
Dose	**Dose of Oil (mg/kg b.w.)** **Low dose:** AA Oil = 0.8% of diet = 600 mg/kg **Mid dose:** AA Oil = 2.0% of diet = 1200 mg/kg **High dose:** AA Oil = 5.0% of diet = 3000 mg/kg	**Dose of AA or DHA (mg/kg b.w.)** **Low dose:** AA: 150 mg/kg **Mid dose:** AA = 300 mg/kg **High dose:** AA = 750 mg/kg
Standardization of Test Groups for Dietary Lipid	Dose groups were not controlled for fat content.	
Control Groups	Control group had basal diet with 4.4% fat.	
Adverse Effects		
Clinical Chemistry	Increased BUN in males at the high dose and females at mid and high doses. No other consistent treatment related changes	
Hematology	No consistent treatment related changes	
Organ Weight	Increased absolute liver weight at high dose; increased relative liver weight at mid- and high dose. Decreased relative kidney weight at mid and high doses in males and high dose in females.	
Histopathology	No adverse treatment-related changes in histopathology seen in any organ examined except for the liver. Six cases of fatty changes (small droplets) in the liver of high dose male rats. One case in low dose females, nine cases in mid dose females and four cases in high dose females of fatty changes (small droplets), as seen by H&E staining. Sudan III stain showed fatty changes in all male and female rats, including control. Re-analysis of photomicrographs of liver sections indicated that the lesions depicted were of the sort and degree of severity that might be observed in control rats of this age. There was an apparent increase in the incidence of vacuolar degeneration (fatty change) in the high dose males and in mid and high dose females. This increase in incidence was marginal in the high dose groups compared to controls. The increase was most evident in the mid dose females which dose not correlate well with the increases in liver weights. The severity of vacuolar degeneration observed in the photomicrographs was recorded as minimal to slight in all dose groups and would be considered a reversible change. Based on the Sudan III stained photomicrographs, there appeared to be a very slight increase in the severity of the Sudan III positive staining (indicating the presence of fats) in the high dose females only. In all dose groups, the degree of positive staining was generally minimal to slight. Based on the photomicrographs, fatty change may have contributed slightly to the increased liver weight, but the data are contradictory and inconclusive at best.	
Other	Animals were fasted before sacrifice.	
Threshold for Effects of Oil Ingestion	600 mg/kg (based on liver weight increases)	

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8. Subchronic Toxicity of SUNTGA40S and DHA-Rich Oil (Suntory 1997)

a) Materials and Methods

Animals

Five-week old male and female rats Crj:CD(SD strain) rats were purchased (Charles River Japan, Inc.), quarantined (2-3 animals per cage during the quarantine period), and acclimatized for 7 days. During the pretest period, the rats were weighed and examined for clinical signs, and 6 animals (3 male and 3 female) were necropsied to confirm their health status. At initiation of the study, the animals were 6 weeks old, with body weights ranging from 186.0 to 219.9 g for males and from 128.7 to 158.2 g for females. The animals were individually housed in stainless steel wire bottom cages in animal rooms under controlled environmental conditions (temperature: 23-26°C; humidity: 55 – 68%; air exchange: 13-15 times per hour; 12-hour light/dark cycle). Cage racks were exchanged every 4 weeks, cages every 2 weeks, and sanitary trays 3 times per week. The animal room was cleaned and disinfected on a daily basis.
The animals were provided food and water *ad libitum*. The feed was provided as a powder (MF, Oriental Yeast Co., Ltd., sterilized by γ-ray). Drinking water was well water supplemented with sodium hypochlorite (approximately 2 ppm).

Test Articles

Test articles, AA-rich oil (SUNTGA40S) and DHA-rich oil (derived from tuna), and the control article, soybean oil, were provided by Suntory Limited (Tokyo, Japan). Table VI-6 provides the fatty acid composition for each of the articles.

The stability of the test substances was determined prior to the initiation of the toxicity study, after one month of refrigeration, and after completion of dosing in the toxicity study. AA-rich oil and soybean oil were stored in polyethylene containers filled with nitrogen gas under darkened, refrigerated conditions (approximately 4°C), whereas DHA-rich oil was stored in a metal container filled with nitrogen gas also under darkened, refrigerated conditions. Stability assessments were based on changes over time in fatty acid composition and by peroxide content. All three materials were found to be stable under their specified storage conditions.

The test substance admixtures were produced by Chiba Factory, Oriental Yeast Co. Ltd. The admixtures were produced more than once a month, sterilized by γ-radiation, received in sealed containers, and stored under dark, refrigerated (approximately 4°C) conditions. The test article admixtures were found to be stable and uniform for 6 weeks under dark, refrigerated conditions, followed by 2-week storage under dark and room temperature conditions, and 10-day storage under light and room temperature conditions. In addition, the primary

constituents of each article (AA for the AA-rich oil, DHA for the DHA-rich oil, and linoleic acid for the control article) were analyzed; results of the analysis of the test and control oils are found in Table VI-6.

Experimental Design

As shown in Table VI-7, the toxicity study utilized six groups of rats; five of which contained 15 male and 15 female rats, and 1 group (the basal diet control group with no oils added to the feed) with 10 male and 10 female rats. [Dose levels were chosen on the basis of a previous 2-week toxicity study of AA-rich oil, in which animals were treated with the test material at doses of 2.5, 5, or 10% oil by weight in the diet. In that study, animals in the 10% treatment group showed accumulation of fat droplets at periportal areas in the liver.] Thus, 2% was chosen as the highest dose for total amount of oils to be added to the diet. Accordingly, concentrations of AA-rich oil were set at 0.5% (plus 1.5% soybean oil) (AA low dose test group), 1% (plus 1% soybean oil) (AA middle dose test group), and 2 % (no additional oil) (AA high dose test group). A fourth test group was given diet containing 1% AA-rich oil and 1% DHA-rich oil (AA + DHA test group). Two control groups were used. Animals in the first control group, Control 1, were given basal diet; animals in the second control group, Control 2, were given diet containing 2% soybean oil.

Animals were allocated into groups by the stratified sequenced randomization method based on body weights one day before the start of treatment. Treatment was performed by feeding diet containing the test articles ad libitum for 13 weeks. The food admixture in the hoppers was changed every week.

Clinical Examinations

During the treatment period, all animals were observed for clinical signs and mortality twice a day (AM and PM). Body weights were measured once a week and before necropsy. Food consumption was measured twice a week to calculate mean consumption per day per animal. In addition, actual intake of test article was calculated from food consumption and expressed relative to individual body weight.

Opthalmological examination was carried out at week 13 of the treatment period. Five animals from each group were examined for effects on the cornea, iris, conjunctiva, lens, and vitreous body using a slit lamp (KL-5, Kowa), followed by examination of ocular fundus using an opthalmofundscope (RC-2, Kowa). Inspection of lens, vitreous body, and ocular fundus was performed after treatment with a mydriatic (Midrin P, Santen Pharmaceutical Co. Ltd.).

Biochemical Examinations

Hematological examination was carried out at the end of the treatment period. At the time of necropsy, blood samples of all animals were collected from the posterior vena cava under anesthesia with intraperitoneal injection of sodium

phenobarbital (30 mg/kg). Leukocytes, erythrocytes, hemoglobin, hematocrit, and platelets were measured in an aliquot of blood treated with EDTA-2K by using a multi blood counter (Sysmex CC-780, Toa Medical Electronics Co., Ltd.). Mean corpuscle volume (MCV), mean corpuscle hemoglobin (MCV), and mean corpuscle hemoglobin concentration (MCHC) were calculated from erythrocytes, hemoglobin, and hematocrit values. Differential leukocyte count and reticulocyte count were calculated under the microscope. In addition, plasma samples, obtained from blood samples treated with 3.8% citric acid and centrifuged at 3,000 rpm for 15 minutes, were measured for prothrombin time and activated partial thromboplastin time, using an automated blood coagulometer (Sysmex CA-5000, Toa Medical Electronics Co., Ltd.).

Sera, obtained from blood samples collected concurrently with those used for hematological examination and centrifuged for 10 min at 3,000 RPM, were examined for total protein (T. protein), albumin, albumin/globulin (A/G) ratio, total bilirubin (T. bilirubin), aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), alkaline phosphatase (ALP), total cholesterol (T. cholesterol), free cholesterol (F. cholesterol), triglyceride, non-esterified fatty acids (NEFA), phospholipids, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorous (IP), and calcium with an autoanalyzer (Hitachi 736-10). Electrophoresis of serum protein was carried out with a Cosmo FDE-III. Sodium, potassium, and chloride were anaylzed with via electrolyte analyzer (PVA-αIII, Analytical Instruments Co., Ltd.).

Necropsy and Histological Examinations

After blood collection, animals were sacrificed by exsanguination from the lateral iliac artery and observed macroscopically. After necropsy, the following tissues were collected and weighed: brain, pituitary, submaxillary glands (with sublingual glands), thyroids (with parathyroids), heart, lungs (with bronchi), thymus, liver, spleen, kidneys, adrenals, seminal vesicles, prostate, testes, ovaries, and uterus. The ratio of organ weight to body weight was calculated on the basis of body weight on the day of necropsy. The following tissues were removed and fixed in 10% neutral buffered formalin: spinal cord, eyeballs, Harderian glands, optic nerves, submaxillary lymph nodes, parotid glands, trachea, tongue, pancreas, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, mesenteric lymph node, urinary bladder, epididymus, vagina, femur (with marrow), sternum (with marrow), mammary gland, skin (lower abdominal region), aorta (thoracic region), sciatic nerve, and biceps femoris. Eyeballs, optic nerve, and Harderian glands were fixed in 2.5% glutaraldehyde solution prior to fixation with formalin. Testes were prefixed in Bouin's solution. The organs described above, for animals in treatment groups control 1, control 2, comparative control, and high dose group, were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Statistical Analysis

The statistical analysis was performed on body weights, food consumption, data from hematological and biochemical examinations, plasma hormone levels, organ weights, and the ratio of organ weight to body weight. Multi-comparison procedures were used for analysis of data. Bartlett's test was used to compare the variances among groups of data. If groups were accepted to be homogenous, Dunnet's multiple comparison test was used for comparisons of groups of data. If the groups were not normally distributed, Steel's multiple comparison test was used. The comparison was performed among treatment groups and for the first and second control groups. The level of $p < 0.05$ was considered to be significant.

b) Results

Clinical signs

There were no appreciable changes in clinical signs related to test articles in any group. Defects of maxillary incisors or abnormal bites were mainly observed in several male animals, due to maxilla or mandible being caught in food vessels. A few of these animals showed low food consumption, followed by transient or intermittent prone position, hypoactivity, bradypnea, lacrimation, salivation, or soiled lower abdomen. One male showing abnormal bite in control group 2 died at week 9. One male in the AA low dose group became moribund at week 13 and was sacrificed for necropsy. In addition, a few males and females in all groups showed loss of hair, trauma, or swelling of the skin.

Body weight

Body weight gains were similar among all study groups, and no significant differences in body weight were observed between the treated groups and control groups 1 or 2. Body weights of male rats are presented in Table VI-8 and Figure VI-1; body weights of female rats are presented in Table VI-9 and Figure VI-2.

Food Consumption

A slight decrease in food consumption, which was statistically significant at several time points, was observed in males from all treatment groups and control group 2, compared to control group 1. This decrease was not noted for females from all treated groups compared with control groups 1 or 2. There was also no significant difference in food consumption for females from control group 1 compared to control group 2.

Test article intake

The mean daily intake of AA-rich oil and DHA-rich oil, on a body weight basis, is presented in Table VI-7. The actual intakes of AA-rich oil were: 182 – 531 mg/kg/day for males and 258 - 462 mg/kg/day for females in the AA low dose test group; 384 - 1087 mg/kg/day for males and 509 - 886 mg/kg/day for females in the AA middle dose test group; 820 - 2125 mg/kg/day for males and

1045 - 1732 mg/kg/day for females in the AA high dose test group and; 420 - 1074 mg/kg/day for males and 508 - 910 mg/kg/day for females in the AA + DHA test group. Actual intakes of DHA-rich oil were 420 - 1074 mg/kg/day for males and 508 - 910 mg/kg/day for females in the AA + DHA test group.

Ophthalmologic examination

No changes were observed in any animals upon opthamological examination at week 13.

Biochemical Observations

The results of hematological examinations are shown in Table VI-10. Males in the AA low, middle, and high dose groups showed significantly increased activated partial thromboplastin time (APTT) in comparison to control group 1. Males in the AA middle dose group only showed increased APTT in comparison to control group 2. All values were within historical control range (14.0 – 29.6 seconds). No significant changes were seen in APTT in the females from the AA low, middle and high dose groups. Prothrombin time (PT) was statistically significantly elevated in males, but not females, from the AA middle dose group, compared to control group 2. This value was within historical control range (9.2 – 26.4 seconds). No significant changes in PT were seen in males or females from the AA low or high dose groups compared to either control group.

Males in the DHA + AA test group showed significantly increased APTT and PT in comparison to control groups 1 and 2. The value for PT was within historical control range (cited above), however, the APTT value was slightly higher than the historical control range (30.1 ±4.7 compared to the maximum historical value of 29.6 seconds). No significant changes were seen in PT or APTT in the females compared to either control group. The elevated values in males were not associated with other hematologic/biochemical variations and there was no evidence of anemia or pathologic bleeding.

The results for APTT and PT are confounded in the AA + DHA test group by the contribution of basal diet to the levels of DHA and EPA ingested by the test animals. Basal diet provided animals with 122 mg/kg body weight/day of DHA + EPA. When this is added to DHA + EPA provided by the test article, animals in the AA + DHA test group consumed up to 303 mg DHA + EPA/kg body weight/day. (Although the level of DHA + EPA ingestion that would result in clinically significant adverse effects on PT and APTT in rats is not known, the level of ingestion is 6-fold higher than the recommended maximum level of ingestion of DHA + EPA in humans (50 mg/kg body weight/day). Beyond this level of ingestion in humans, there is an increased risk of an adverse effect on the hematologic parameter of bleeeding time. The clinical significance of increased bleeding time is not known, however, since incrased bleeding itme does not correlate with clinically significant bleeding (21 CFR §184.1472))

Another factor to consider when evaluating the biologic significance of the finding of increased PT and APTT is that vitamin K activity and vitamin K dependent blood coagulation factors can be reduced by fish oil. When vitamin K is limiting, prolongation of blood coagulation tests may be seen (Andriamampandry 1998; Nieuwenhuys 1998). The current AIN dietary recommendations for rats include 750 µg vitamin K/kg diet (Reeves 1993). In this study, only 400 µg vitamin K/kg diet was used. A mild vitamin K deficiency may have exacerbated effects of the fish oil in the AA + DHA test group.

In addition, because this finding was only seen in males, who consumed a lower dose of DHA + EPA on a mg/kg bodyweight basis, and not females, it is unclear that the increased PT and APTT can be determined to be related solely to test article administration. The finding of increase PT and APTT was not associated with an adverse health consequence to the animals. (The finding in this rat study of increased APTT and PT was not reproduced in a neonatal piglet study of AA-rich and DHA-rich oils).

The results of clinical chemistry analyses are shown in Table VI-11. Sporadic clinical chemistry values were significantly changed in test groups, however, these were within historical control range and not considered to be test article related or biologically significant.

Organ Weights

There was a statistically significant effect on absolute (compared to control 2) and relative (compared to controls 1 and 2) spleen weights in female rats from the middle dose group. There was no dose response for this effect; no increase in absolute or relative spleen weights were seen in the high dose group. No adverse effects on spleen weight were seen in male rats from any treated group compared to controls. In addition, there was no evidence in spleen of any adverse histopathologic outcome. This suggests a non-specific finding.

There was a statistically significant increase in absolute liver weight, for males only, in the AA + DHA test group and AA middle dose test group compared to control group 2. No difference in absolute liver weight was seen in males in these groups compared to control group 1. No significant differenence in absolute liver weights were seen in the AA low or high dose groups compared to control groups 1 or 2. No significant differences in relative liver weights were seen in males or females from any test group compared to either control.

Although a statistically significant effect on absolute liver weight was seen in the AA middle dose group and the AA + DHA dose group compared to control group 2, there was no effect on absolute liver weight seen in the high dose AA test group. Therefore, there was no dose resonse effect for this endpoint. In addition, there was no evidence in liver of any adverse histopathologic outcome. This suggests a non-specific finding. Absolute and relative organ weights for males and females are given in Tables VI-12 and VI-13, respectively.

Necropsy and Histological Observations

Upon necropsy, no macroscopic changes related to treatment were observed in any of the animals. No treatment-related histopathological changes were observed in any organs from control or treated animals.

c) **Discussion**

The ingestion of the test articles was well tolerated, as evidenced by the normal growth and appearance of the rats. Observed increases in PT and APTT values were within historical control range for all exclusive AA-rich oil test groups. Furthermore, there was no evidence of concurring observations, such as hepatic lesions or hemorrhagic lesions, suggesting that these findings of slight changes in coagulation parameters would not result in an adverse health consequence to the animals.

The finding of significantly increased APTT and PT levels may have been confounded in the AA + DHA test group by the contribution of basal diet to the levels of DHA and EPA ingested by the test animals. It is also possible that a mild vitamin K deficiency produced by the basal diet may have exacerbated effects of the fish oil in the AA + DHA test group. In addition, because this finding was only seen in males, who received a lower dose of DHA + EPA on a mg/kg bodyweight basis, and not females, it is unclear that the increased PT and APTT can be determined to be related solely to test article administration. No dose or test article related adverse effects were noted on organ weights, gross pathology or histopathology.

Although a statistically significant effect on absolute liver weight was seen in the AA middle dose group and the AA + DHA dose group compared to control group 2, there was no dose resonse effect for this endpoint. No adverse effect on relative liver weight was seen in any dose group. In addition, there was no evidence in liver of any adverse histopathologic outcome. There was a statistically significant effect on absolute (compared to control 2) and relative (compared to controls 1 and 2) spleen weights in female rats from the middle dose group. There was no dose response for this effect. In addition, there was no evidence in spleen of any adverse histopathologic outcome. This suggests that the liver and spleen organ weight changes were non-specific findings.

Based on the results of this study, administration of AA-rich oil as 2% of the diet and administration of 1% DHA-rich oil in combination with 1% AA-rich oil in the diet, did not produce test article related effects associated with adverse health consequences in adult rats.

TABLE VI-6 Fatty Acid Composition of Test Articles and Control Oil Used in Subchronic Study			
Fatty Acid	**AA-Rich Oil (Weight %)**	**DHA-Rich Oil (Weight %)**	**Soybean Oil (Weight %)**
14:0	0.3	3.3	0.1
15:0	0.1	0.7	ND
16:0	10.3	16.8	10.4
16:1 (n-7)	0.1	5.1	0.1
17:0	0.2	0.8	0.1
18:0	6.6	4.3	3.9
18:1 (n-9)	7.4	16.4	23.1
18:1 (n-7)	0.4	2.6	1.5
18:2 (n-6)	13.6	1.3	52.0
18:3 (n-6)	2.8	ND	ND
18:3 (n-3)	0.6	0.6	5.6
18:4 (n-3)	ND	1.1	ND
20:0	0.8	0.3	0.4
20:1 (n-9)	0.5	2.1	ND
20:2 (n-6)	1.1	0.3	ND
20:3 (n-6)	2.9	ND	ND
20:4 (n-6)	39.7	1.9	ND
20:5 (n-3)	0.3	7.8	ND
22:0	2.7	ND	0.4
22:1 (n-11)	ND	1.5	ND
22:1 (n-9)	0.2	ND	ND
22:4 (n-6)	0.3	0.3	ND
22:5 (n-6)	ND	1.1	ND
22:5 (n-3)	ND	1.7	ND
22:6 (n-3)	ND	23.1	ND
24:0	6.6	ND	0.1
24:1 (n-9)	0.4	ND	ND
Others	2.1	6.9	2.3
Total	100.0	100.0	100.0

ND = Not Detected

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TABLE VI-7 Experimental Design of Subchronic Study				
Treatment Group	**Dosage Preparation**	**Sex**	**Number of Animals**	**Mean Daily Intake of AA-rich oil [DHA-rich oil] (mg/kg body weight/day)**
Control 1	Basal diet only	Male	10	0
		Female	10	0
Control 2	Basal diet + 2% soybean oil	Male	15	0
		Female	15	0
AA + DHA Test Group	Basal diet + 1% AA-rich oil + 1% DHA-rich oil	Male	15	591 [591]
		Female	15	696 [696]
AA Low Dose Test Group	Basal diet + 0.5% AA-rich oil + 1.5% Soybean oil	Male	15	283
		Female	15	341
AA Middle Dose Test Group	Basal diet + 1% AA-rich oil + 1% Soybean oil	Male	15	578
		Female	15	674
AA High Dose Test Group	Basal diet + 1.5% AA-rich oil + 0.5% Soybean oil	Male	15	1158
		Female	15	1353

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TABLE VI-8 BODY WEIGHTS OF MALE RATS (grams)																		
Day	Group and Dose																	
	Control 1			Control 2			AA Low Dose Test Group			AA Middle Dose Test Group			AA High Dose Test Group			AA + DHA Test Group		
	n=10			n=15			n=15			n=15			n=15			n=15		
	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD
0	201.9	±	9.2	202.6	±	9.1	202.1	±	9.5	202.2	±	6.9	201.8	±	8.2	201.4	±	5.8
6	257.6	±	14.7	255.9	±	12.5	252.2	±	15.1	256.0	±	12.7	252.9	±	14.0	256.6	±	10.8
13	306.4	±	19.6	302.1	±	14.4	301.8	±	19.1	304.6	±	18.4	297.5	±	22.1	306.6	±	15.7
20	347.9	±	21.6	341.9	±	20.2	343.7	±	27.0	350.1	±	25.2	337.9	±	30.0	348.2	±	22.9
27	385.5	±	26.1	373.8	±	23.4	376.5	±	32.0	385.2	±	30.7	371.9	±	38.6	389.7	±	26.9
34	412.7	±	29.7	397.4	±	28.1	401.6	±	32.9	414.2	±	34.4	402.7	±	41.9	414.6	±	31.0
41	442.6	±	32.4	420.7	±	33.4	422.6	±	33.7	440.8	±	35.5	426.6	±	44.3	444.2	±	30.6
48	460.8	±	35.3	438.8	±	34.9	438.3	±	37.9	461.7	±	37.1	447.5	±	48.9	466.9	±	35.3
55	481.6	±	33.9	455.9	±	39.2	462.6	±	39.0	485.4	±	39.1	468.7	±	46.2	489.2	±	36.0
62	499.5	±	29.7	473.9	±	40.1*	479.7	±	44.4	501.7	±	44.7	487.2	±	51.3	507.7	±	35.3
69	518.7	±	32.3	485	±	40.5*	492.5	±	45.0	516.1	±	48.3	504.8	±	56.7	518.1	±	41.2
76	528.3	±	34.1	500.3	±	42.1*	504.4	±	45.3	529.6	±	49.1	516.8	±	56.2	532.8	±	40.8
83	539.6	±	35.0	508.5	±	40.3*	521.7	±	48.0	545.3	±	50.4	531.9	±	59.3	546.5	±	44.4
90	544.1	±	33.7	520.3	±	41.2*	522.8	±	46.0	552.3	±	50.7	540.3	±	58.8	554.6	±	44.1

* n=14
SD= Standard Deviation

000110

TABLE VI-9 BODY WEIGHTS OF FEMALE RATS (grams)																		
Day	Group and Dose																	
	Control 1			Control 2			AA Low Dose Test Group			AA Middle Dose Test Group			AA High Dose Test Group			AA + DHA Test Group		
	n=10			n=15			n=15			n=15			n=15			n=15		
	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD
0	146.5	±	6.5	145.6	±	6.4	143.4	±	7.3	146.8	±	4.9	144.4	±	7.0	147.0	±	5.2
6	169.0	±	9.2	164.6	±	7.7	163.7	±	9.0	165.8	±	8.7	161.6	±	7.9	167.0	±	7.8
13	190.6	±	13.2	187.3	±	8.5	186.1	±	12.8	187.2	±	12.0	182.1	±	10.4	189.3	±	11.0
20	205.8	±	15.1	204.8	±	8.9	204.6	±	13.6	202.6	±	12.3	197.0	±	11.4	209.1	±	14.7
27	219.7	±	18.0	218.5	±	10.1	217.5	±	14.6	217.4	±	13.6	211.8	±	13.3	222.0	±	16.1
34	235.3	±	17.9	233.5	±	12.3	228.2	±	12.1	231.4	±	16.5	222.7	±	12.8	236.5	±	16.0
41	247.5	±	19.1	245.0	±	14.7	239.7	±	14.3	241.5	±	15.7	233.9	±	13.5	249.2	±	17.9
48	252.3	±	19.6	254.0	±	14.3	248.7	±	15.7	251.6	±	19.3	243.5	±	14.7	258.3	±	21.2
55	261.6	±	21.8	259.6	±	15.5	255.4	±	17.4	258.9	±	19.1	249.1	±	16.1	267.0	±	24.0
62	267.9	±	22.7	268.5	±	18.0	264.7	±	19.5	264.6	±	24.2	255.6	±	15.9	273.8	±	22.7
69	271.9	±	22.9	274.4	±	18.5	270.7	±	19.3	272.9	±	24.1	263.6	±	17.6	279.4	±	24.0
76	279.8	±	22.7	279.6	±	20.2	275.4	±	20.1	277.4	±	24.7	267.3	±	19.1	286.8	±	26.4
83	282.8	±	23.3	281.5	±	21.5	278.1	±	19.6	279.5	±	26.4	270.8	±	19.3	290.2	±	27.9
90	286.4	±	24.8	286.2	±	23.3	283.9	±	22.4	282.1	±	28.2	272.6	±	19.2	294.2	±	31.2

SD= Standard Deviation

000111

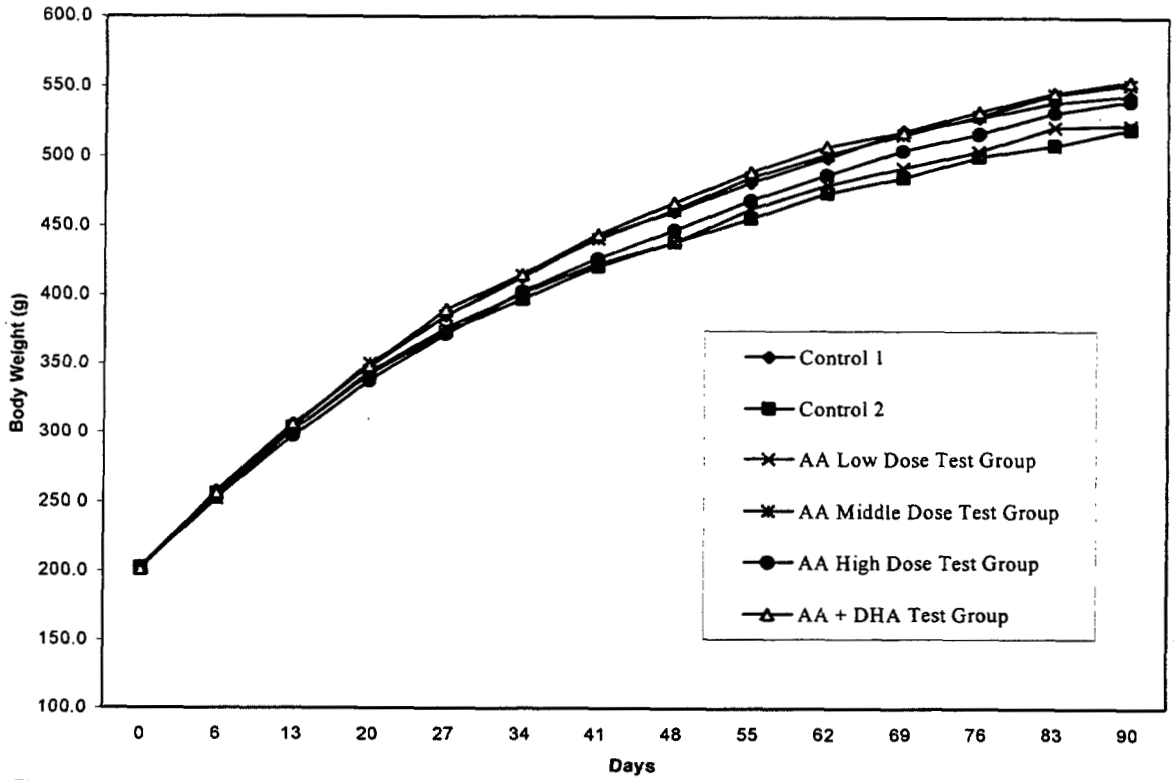

Figure VI-1. Group Mean Body Weights of Male Rats

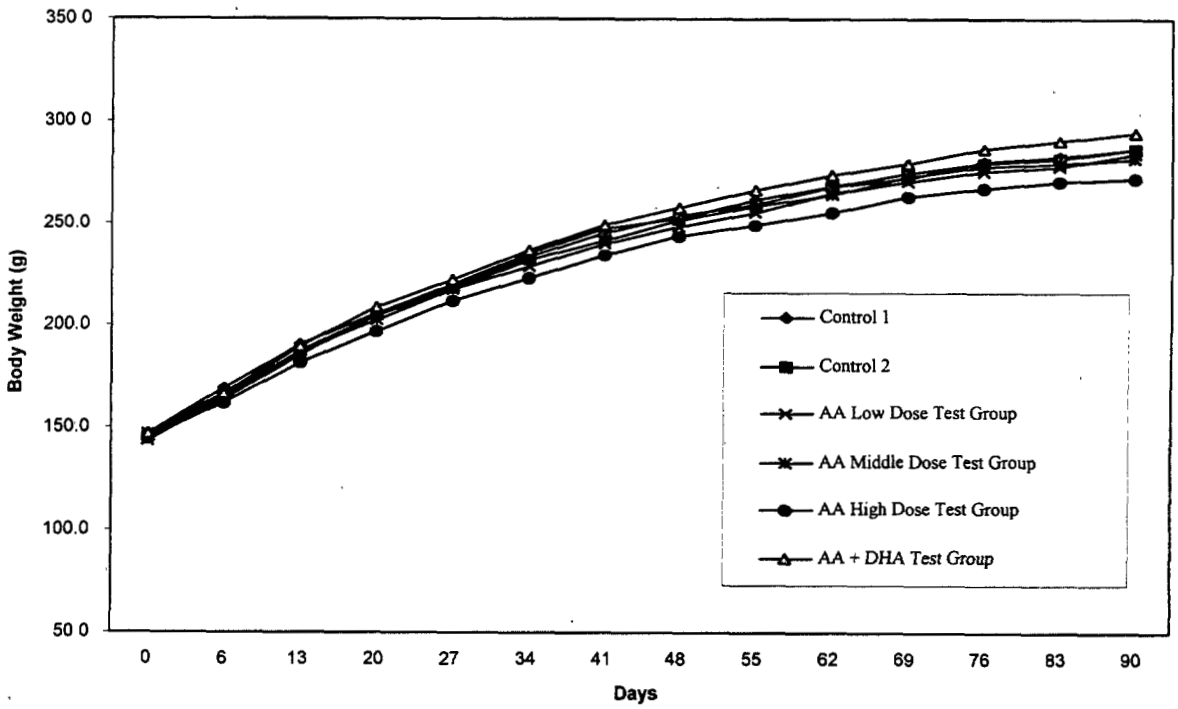

Figure VI-2. Group Mean Body Weights of Female Rats

000112

TABLE VI-10
Hematological Findings in Rats Fed Diet Containing SUNTGA40S for 13 Weeks

Group	Sex	Number of Animals	Leucocyte ($10^2/\mu l$)	Erythrocyte ($10^4/\mu l$)	Hemoglobin (g/dl)	Hematocrit (%)	Platelet ($10^4/\mu l$)	MCV (fl)
Control 1	Male	10	76 ± 17	877 ± 20	15.2 ± 0.3	47.0 ± 1.2	101.8 ± 7.7	54 ± 2
	Female	10	52 ± 12	824 ± 35	14.7 ± 0.3	46.1 ± 1.3	101.0 ± 6.7	56 ± 2
Control 2	Male	14	73 ± 28	886 ± 42	15.4 ± 0.5	47.7 ± 1.9	97.2 ± 12.7	54 ± 1
	Female	15	48 ± 8	825 ± 42	14.8 ± 0.6	46.2 ± 2.1	101.0 ± 7.3	56 ± 2
AA + DHA Test Group	Male	15	80 ± 15	895 ± 45	15.1 ± 0.5	47.1 ± 1.8	102.8 ± 7.8	53 ± 2
	Female	15	54 ± 25	819 ± 50	14.7 ± 0.6	45.8 ± 1.9	101.0 ± 14.4	56 ± 2
AA Low Dose Test Group	Male	14	74 ± 17	863 ± 47	14.9 ± 0.7	46.5 ± 2.0	101.1 ± 10.5	54 ± 1
	Female	15	51 ± 13	820 ± 31	14.7 ± 0.6	46.2 ± 1.8	97.5 ± 11.2	56 ± 2
AA Middle Dose Test Group	Male	15	90 ± 28	889 ± 52	15.1 ± 0.8	47.2 ± 2.5	99.6 ± 9.4	53 ± 2
	Female	15	56 ± 19	812 ± 50	14.5 ± 0.5	44.9 ± 1.9	100.6 ± 12.6	56 ± 2
AA High Dose Test Group	Male	15	86 ± 19	888 ± 35	15.1 ± 0.4	47.2 ± 1.3	96.6 ± 10.7	53 ± 2
	Female	15	55 ± 24	819 ± 46	14.8 ± 0.7	45.6 ± 2.2	100.9 ± 12.6	56 ± 2

TABLE VI-10 (continued)

Group	Sex	Number of Animals	MCH (pg)	MCHC (%)	Reticulocyte (%)	Prothrombin time (sec)	APTT (sec)
Control 1	Male	10	17.3 ± 0.4	32.3 ± 0.5	33 ± 6	12.5 ± 0.8	23.3 ± 3.5
	Female	10	17.8 ± 0.7	31.8 ± 0.4	32 ± 7	12.2 ± 1.1	21.3 ± 3.6
Control 2	Male	14	17.3 ± 0.7	32.2 ± 0.7	27 ± 5	12.7 ± 1.9	24.3 ± 3.0
	Female	15	17.9 ± 0.5	31.9 ± 0.4	34 ± 6	12.0 ± 1.0	20.3 ± 2.5
AA + DHA Test Group	Male	15	16.9 ± 0.5	32.0 ± 0.5	30 ± 5	18.5 ± 5.4 [b, d]	30.1 ± 4.7 [b, d]
	Female	15	18.0 ± 0.6	32.2 ± 0.5	34 ± 5	11.7 ± 0.7	20.0 ± 3.3
AA Low Dose Test Group	Male	14	17.3 ± 0.5	32.1 ± 0.4	28 ± 6	14.7 ± 3.4	27.8 ± 2.9 [a]
	Female	15	18.0 ± 0.6	31.9 ± 0.6	34 ± 8	11.8 ± 1.1	20.0 ± 2.3
AA Middle Dose Test Group	Male	15	17.0 ± 0.6	32.0 ± 0.4	34 ± 11	15.0 ± 2.9 [c]	28.7 ± 5.2 [b, c]
	Female	15	17.9 ± 0.6	32.3 ± 0.6 [a]	33 ± 5	12.1 ± 1.2	20.6 ± 2.1
AA High Dose Test Group	Male	15	17.1 ± 0.6	32.1 ± 0.5	31 ± 8	15.2 ± 4.1	28.1 ± 4.5 [a]
	Female	15	18.1 ± 0.6	32.5 ± 0.4 [b, c]	33 ± 8	12.5 ± 1.2	19.5 ± 1.5

000113

TABLE VI-10 (continued)								
Group	**Sex**	**Number of Animals**	**Differential leucocyte count**					
			Eosinophil (%)	**Neutro-Stab. (%)**	**Neutro-Seg. (%)**	**Lymphocyte (%)**	**Basophil (%)**	**Monocyte (%)**
Control 1	Male	10	1.0 ± 0.9	0.0 ± 0.0	20.6 ± 9.3	77.0 ± 10.0	0.1 ± 0.3	1.3 ± 0.9
	Female	10	0.9 ± 1.0	0.1 ± 0.3	19.8 ± 6.4	77.3 ± 6.4	0.1 ± 0.3	1.8 ± 1.6
Control 2	Male	14	0.9 ± 1.0	0.0 ± 0.0	19.6 ± 8.1	77.6 ± 8.6	0.1 ± 0.3	1.7 ± 1.2
	Female	15	1.5 ± 1.5	0.0 ± 0.0	20.1 ± 9.4	76.4 ± 9.5	0.0 ± 0.0	2.0 ± 1.1
AA + DHA Test Group	Male	15	1.1 ± 1.3	0.0 ± 0.0	24.0 ± 10.1	72.7 ± 11.0	0.0 ± 0.0	2.1 ± 1.6
	Female	15	1.5 ± 1.2	0.0 ± 0.0	21.4 ± 11.0	74.5 ± 11.2	0.0 ± 0.0	2.6 ± 1.6
AA Low Dose Test Group	Male	14	1.4 ± 1.2	0.0 ± 0.0	20.5 ± 11.4	75.9 ± 12.3	0.1 ± 0.3	2.1 ± 1.4
	Female	15	1.3 ± 1.4	0.0 ± 0.0	17.9 ± 9.5	78.6 ± 9.3	0.0 ± 0.0	2.3 ± 1.4
AA Middle Dose Test Group	Male	15	1.0 ± 0.8	0.0 ± 0.0	24.1 ± 11.3	73.4 ± 11.2	0.0 ± 0.0	1.5 ± 1.1
	Female	15	1.1 ± 1.5	0.0 ± 0.0	17.5 ± 8.1	79.5 ± 8.6	0.0 ± 0.0	1.9 ± 1.7
AA High Dose Test Group	Male	15	0.9 ± 1.3	0.1 ± 0.3	29.1 ± 12.5	67.1 ± 12.4	0.1 ± 0.3	2.7 ± 1.9
	Female	15	1.1 ± 1.2	0.0 ± 0.0	21.8 ± 10.0	75.0 ± 10.1	0.0 ± 0.0	2.1 ± 1.2

Note. Values are mean ± S.D.
a: $P<0.05$, b: $P<0.01$ (significantly different from control 1)
c: $P<0.05$, d: $P<0.01$ (significantly different from control 2)

000114

TABLE VI-11
Clinical Chemistry Values in Rats Fed Diet Containing SUNTGA40S for 13 Weeks

Group	Sex	Number of Animals	T. protein (g/dl)	Albumin (g/dl)	A/G ratio	T. bilirubin (mg/dl)	SGOT (IU/l)	SGPT (IU/l)	ALP (IU/l)
Control 1	Male	10	5.7 ± 0.2	3.6 ± 0.1	1.71 ± 0.08	0.0 ± 0.0	104 ± 16	22 ± 4	136 ± 25
	Female	10	6.3 ± 0.4	4.5 ± 0.3	2.58 ± 0.30	0.0 ± 0.0	101 ± 19	20 ± 10	72 ± 29
Control 2	Male	14	5.6 ± 0.2	3.6 ± 0.2	1.84 ± 0.19	0.0 ± 0.0	98 ± 24	20 ± 2	140 ± 23
	Female	15	6.2 ± 0.5	4.5 ± 0.4	2.70 ± 0.37	0.0 ± 0.1	92 ± 11	19 ± 6	79 ± 22
AA + DHA Test Group	Male	15	5.7 ± 0.2	3.5 ± 0.2	1.62 ± 0.17 [d]	0.0 ± 0.0	87 ± 17	20 ± 4	128 ± 30
	Female	15	5.9 ± 0.3	4.2 ± 0.4	2.47 ± 0.46	0.0 ± 0.0	93 ± 23	19 ± 7	73 ± 16
AA Low Dose Test Group	Male	14	5.5 ± 0.3	3.5 ± 0.3	1.72 ± 0.19	0.0 ± 0.0	96 ± 11	20 ± 3	114 ± 26
	Female	15	5.8 ± 0.4 [a]	4.1 ± 0.4	2.48 ± 0.35	0.0 ± 0.1	98 ± 24	19 ± 8	70 ± 19
AA Middle Dose Test Group	Male	15	5.8 ± 0.2	3.5 ± 0.1	1.55 ± 0.12 [d]	0.0 ± 0.0	98 ± 23	22 ± 7	136 ± 32
	Female	15	6.0 ± 0.5	4.2 ± 0.5	2.37 ± 0.37	0.0 ± 0.1	109 ± 51	29 ± 32	74 ± 21
AA High Dose Test Group	Male	15	5.6 ± 0.2	3.4 ± 0.1 [a,c]	1.57 ± 0.15 [d]	0.0 ± 0.0	87 ± 13	21 ± 4	120 ± 25
	Female	15	5.8 ± 0.3 [a]	4.0 ± 0.4 [a,d]	2.20 ± 0.33 [d]	0.0 ± 0.1	101 ± 26	20 ± 11	79 ± 16

TABLE VI-11 (continued)

Group	Sex	Number of Animals	T. cholesterol (mg/dl)	F. cholesterol (mg/dl)	Triglycerides (mg/dl)	NEFA (mEq/l)	Phospho-lipids (mg/dl)	Glucose (mg/dl)
Control 1	Male	10	68 ± 14	15 ± 4	89 ± 33	0.68 ± 0.12	127 ± 22	114 ± 12
	Female	10	73 ± 12	17 ± 4	30 ± 10	0.87 ± 0.20	159 ± 19	94 ± 12
Control 2	Male	14	66 ± 10	14 ± 3	67 ± 27	0.73 ± 0.10	126 ± 13	113 ± 13
	Female	15	70 ± 15	16 ± 4	35 ± 23	0.83 ± 0.19	157 ± 25	98 ± 10
AA + DHA Test Group	Male	15	72 ± 23	16 ± 5	80 ± 36	0.67 ± 0.13	128 ± 28	116 ± 15
	Female	15	64 ± 12	15 ± 4	35 ± 18	0.83 ± 0.17	138 ± 28	98 ± 15
AA Low Dose Test Group	Male	14	66 ± 14	15 ± 5	52 ± 30 [a]	0.63 ± 0.11	118 ± 19	107 ± 10
	Female	15	67 ± 17	16 ± 4	28 ± 11	0.70 ± 0.19	147 ± 34	100 ± 15
AA Middle Dose Test Group	Male	15	73 ± 13	16 ± 3	69 ± 31	0.67 ± 0.09	130 ± 17	116 ± 14
	Female	15	76 ± 18	18 ± 4	30 ± 14	0.76 ± 0.22	160 ± 37	99 ± 14
AA High Dose Test Group	Male	15	68 ± 16	15 ± 6	62 ± 25	0.62 ± 0.14	120 ± 23	113 ± 9
	Female	15	64 ± 13	14 ± 4	24 ± 11	0.75 ± 0.19	133 ± 25	92 ± 15

000115

TABLE VI-11 (continued)								
Group	**Sex**	**Number of Animals**	**BUN (mg/dl)**	**Creatinine (mg/dl)**	**IP (mg/dl)**	**Ca (mg/dl)**	**α_1-Globulin (%)**	**α_2-Globulin (%)**
Control 1	Male	10	17.5 ± 1.4	0.5 ± 0.1	5.7 ± 0.7	9.6 ± 0.2	22.1 ± 1.3	7.1 ± 0.5
	Female	10	19.5 ± 3.3	0.6 ± 0.1	5.0 ± 1.2	10.2 ± 0.4	16.4 ± 1.2	5.8 ± 0.6
Control 2	Male	14	17.1 ± 1.7	0.5 ± 0.1	5.9 ± 0.6	9.6 ± 0.3	21.7 ± 1.5	6.9 ± 0.6
	Female	15	19.3 ± 2.5	0.6 ± 0.1	5.1 ± 1.3	10.2 ± 0.4	16.9 ± 1.2	5.7 ± 0.5
AA + DHA Test Group	Male	15	17.2 ± 2.1	0.5 ± 0.1	6.3 ± 0.6	9.7 ± 0.3	24.1 ± 1.9 [a, d]	7.2 ± 0.4
	Female	15	19.8 ± 2.6	0.6 ± 0.1	5.5 ± 1.7	10.0 ± 0.4	17.2 ± 1.7	6.2 ± 1.5
AA Low Dose Test Group	Male	14	17.5 ± 2.4	0.5 ± 0.1	6.1 ± 0.8	9.5 ± 0.4	22.8 ± 2.0	7.1 ± 0.8
	Female	15	20.1 ± 2.2	0.6 ± 0.1	6.2 ± 1.2	10.0 ± 0.4	17.2 ± 1.5	5.7 ± 0.7
AA Middle Dose Test Group	Male	15	18.1 ± 2.4	0.5 ± 0.1	6.3 ± 0.4	9.6 ± 0.2	23.4 ± 2.5	7.6 [c] ± 0.7
	Female	15	21.3 ± 2.4	0.6 ± 0.1	5.8 ± 1.1	10.0 ± 0.5	17.7 ± 1.8	5.8 ± 0.9
AA High Dose Test Group	Male	15	18.1 ± 2.5	0.5 ± 0.1	6.5 ± 0.7 [a]	9.7 ± 0.3	23.8 ± 1.6 [c]	7.8 ± 0.5 [a, d]
	Female	15	21.1 ± 2.4	0.6 ± 0.1	5.9 ± 1.3	9.9 ± 0.3	17.7 ± 1.6	6.3 ± 1.1

TABLE VI-11 (continued)								
Group	**Sex**	**Number of Animals**	**ß-Globulin (%)**	**γ-Globulin (%)**	**Albumin (%)**	**Na (mEq/l)**	**K (mEq/l)**	**Cl (mEq/l)**
Control 1	Male	10	15.8 ± 1.1	3.9 ± 1.0	51.2 ± 1.4	146.3 ± 1.0	4.39 ± 0.17	105.9 ± 1.1
	Female	10	13.0 ± 1.3	4.5 ± 0.5	60.4 ± 1.9	145.8 ± 1.5	4.37 ± 0.38	108.5 ± 1.8
Control 2	Male	14	15.4 ± 1.2	3.9 ± 1.1	52.1 ± 2.4	147.1 ± 1.3	4.37 ± 0.29	106.3 ± 1.8
	Female	15	12.7 ± 1.5	4.7 ± 0.9	60.0 ± 2.7	145.6 ± 1.2	4.14 ± 0.24	108.8 ± 1.9
AA + DHA Test Group	Male	15	16.0 ± 1.3	4.0 ± 0.4	48.8 ± 2.8 [d]	147.3 ± 0.7	4.39 ± 0.19	106.2 ± 1.1
	Female	15	14.1 ± 1.8 [c]	5.1 ± 1.0	57.4 ± 4.8	146.1 ± 1.5	4.21 ± 0.25	109.0 ± 1.6
AA Low Dose Test Group	Male	14	15.2 ± 1.5	3.9 ± 0.6	51.1 ± 2.8	147.4 ± 0.7 [a]	4.47 ± 0.21	107.1 ± 1.3
	Female	15	13.5 ± 1.1	4.6 ± 1.3	59.0 ± 2.5	146.4 ± 1.2	4.21 ± 0.24	109.2 ± 1.6
AA Middle Dose Test Group	Male	15	16.4 ± 1.0	4.1 ± 0.7	48.5 ± 1.6 [a, d]	147.1 ± 0.9	4.41 ± 0.24	106.6 ± 1.1
	Female	15	14.0 ± 1.7	4.9 ± 0.9	57.7 ± 3.8	145.8 ± 1.0	4.16 ± 0.28	108.3 ± 1.4
AA High Dose Test Group	Male	15	16.4 ± 1.8	4.0 ± 1.0	47.9 ± 2.5 [b, d]	146.8 ± 1.2	4.45 ± 0.24	106.5 ± 1.0
	Female	15	14.9 ± 1.0 [b, d]	5.5 ± 0.8 [a]	55.5 ± 3.5 [b, d]	146.1 ± 1.2	4.21 ± 0.27	108.6 ± 1.3

Note. Values are mean ± S.D.
a: $P<0.05$, b: $P<0.01$ (significantly different from control 1)
c: $P<0.05$, d: $P<0.01$ (significantly different from control 2)

000116

TABLE VI-12
Absoluet and Relative Organ Weights of Male+ Rats

ORGAN	Control 1 (n = 10)		Control 2 (n = 14)		AA Low Dose Test Group (n = 14)		AA Middle Dose Test Group (n = 15)		AA High Dose Test Group (n = 15)		AA + DHA Test Group (n = 15)	
	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]
Brain	2.27 ± 0.10	0.44 ± 0.02	2.29 ± 0.09	0.46 ± 0.04	2.29 ± 0.07	0.46 ± 0.04	2.34 ± 0.08	0.44 ± 0.04	2.27 ± 0.11	0.44 ± 0.03	2.30 ± 0.07	0.44 ± 0.04
Pituitary	14.8 ± 1.1	2.9 ± 0.3	14.1 ± 1.6	2.8 ± 0.3	14.0 ± 1.3	2.8 ± 0.2	13.9 ± 1.5	2.6 ± 0.3	14.1 ± 1.3	2.7 ± 0.3	14.8 ± 1.1	2.8 ± 0.2
Submaxillary Gland (right)	0.42 ± 0.05	0.08 ± 0.01	0.42 ± 0.04	0 09 ± 0.01	0.40 ± 0.05	0.08 ± 0.01	0.42 ± 0.05	0.08 ± 0.01	0.42 ± 0.06	0.08 ± 0.01	0.44 ± 0.04	0.08 ± 0.01
Submaxillary Gland (left)	0.39 ± 0.04	0.07 ± 0.01	0.39 ± 0.04	0.08 ± 0.01	0.38 ± 0.04	0.08 ± 0.01	0.39 ± 0.05	0.07 ± 0.01	0.40 ± 0.04	0.08 ± 0.01	0.42 ± 0.04	0.08 ± 0.01
Thyroids	25.7 ± 3.6	5.0 ± 0.5	25.3 ± 3.3	5.1 ± 0.7	24.0 ± 3.8	4.8 ± 0.6	25.9 ± 3.8	4.9 ± 0.7	24.8 ± 4.1	4.8 ± 0.8	26.2 ± 4.8	4.9 ± 0.8
Heart	1.46 ± 0.10	0.28 ± 0.02	1.41 ± 0.18	0.28 ± 0.02	1.40 ± 0.14	0.28 ± 0.02	1.50 ± 0.17	0.28 ± 0.03	1.46 ± 0.22	0.28 ± 0.03	1.55 ± 0.38	0.29 ± 0.06
Lungs	1.46 ± 0.15	0.28 ± 0.02	1.38 ± 0.09	0.28 ± 0.02	1.39 ± 0.14	0.28 ± 0.02	1.42 ± 0.13	0.27 ± 0.02	1.38 ± 0.18	0.27 ± 0.02	1.45 ± 0.16	0.27 ± 0.02
Thymus	0.28 ± 0.08	0.05 ± 0.02	0.24 ± 0.05	0.05 ± 0.01	0.26 ± 0.08	0.05 ± 0.02	0.31 ± 0.10	0.06 ± 0.01	0.26 ± 0.07	0.05 ± 0.01	0.29 ± 0.06	0.06 ± 0.01
Liver	13.62 ± 1.58	2.63 ± 0.22	12.59 ± 1.45	2.53 ± 0.18	12.31 ± 1.43	2.47 ± 0.23	14.47 ± 1.79**	2.74 ± 0.26	13.77 ± 1.74	2.67 ± 0.19	14.31 ± 1.88**	2.68 ± 0.22
Spleen	0.85 ± 0.10	0.16 ± 0.01	0.74 ± 0.10	0.15 ± 0.01	0.77 ± 0.10	0.16 ± 0.02	0.84 ± 0.13	0.16 ± 0.03	0.78 ± 0.12	0.15 ± 0.02	0.85 ± 0.13	0.16 ± 0.02
Right Kidney	1.61 ± 0.14	0.31 ± 0.02	1.53 ± 0.12	0.31 ± 0.02	1.45 ± 0.15	0.29 ± 0.02	1.58 ± 0.16	0.30 ± 0.03	1.52 ± 0.15	0.30 ± 0.02	1.61 ± 0.17	0.30 ± 0.03
Left Kidney	1.62 ± 0.14	0.31 ± 0.02	1.52 ± 0.14	0.31 ± 0.02	1.45 ± 0.14*	0.29 ± 0.02	1.56 ± 0.16	0.30 ± 0.03	1.51 ± 0.15	0.30 ± 0.02	1.60 ± 0.17	0.30 ± 0.02
Right Adrenal	25.9 ± 2 9	5.0 ± 0.5	27.6 ± 4.7	5.6 ± 1.0	25.2 ± 2.6	5.1 ± 0.6	26.5 ± 4.3	5.0 ± 0.8	27.2 ± 3.9	5.3 ± 0.7	27.4 ± 5.1	5.2 ± 1.0
Left Adrenal	27.4 ± 3.2	5.3 ± 0.6	29.4 ± 4.7	5.9 ± 1.0	28.3 ± 2.3	5.7 ± 0.8	27.7 ± 4.1	5.3 ± 0.8	29.4 ± 4.9	5.7 ± 0.8	28.6 ± 5.2	5.4 ± 1.1
Seminal Vesicle	1.97 ± 0.31	0.38 ± 0.06	2.07 ± 0.36	0.42 ± 0.07	2.02 ± 0.24	0.41 ± 0.05	2.11 ± 0.33	0.40 ± 0.07	1.91 ± 0.21	0.37 ± 0.04	2.11 ± 0.32	0.40 ± 0.06
Prostate	0.99 ± 0.23	0.19 ± 0.05	1.07 ± 0.21	0.22 ± 0.05	1.06 ± 0.14	0.22 ± 0.04	1.13 ± 0.18	0.22 ± 0.03	1.09 ± 0.15	0.21 ± 0.04	1.19 ± 0.19*	0.23 ± 0.04
Right Testis	1.63 ± 0.08	0.32 ± 0.02	1.61 ± 0.09	0.32 ± 0.03	1.67 ± 0.14	0.34 ± 0.02	1.57 ± 0.12	0.30 ± 0.03	1.59 ± 0.25	0.31 ± 0.06	1.70 ± 0.14	0.32 ± 0.04
Left Testis	1.63 ± 0.08	0.32 ± 0.02	1.61 ± 0.11	0.33 ± 0.04	1.67 ± 0.15	0.34 ± 0.03	1.56 ± 0.15	0.30 ± 0.04	1.60 ± 0.22	0.31 ± 0.05	1.69 ± 0.16	0.32 ± 0.04

a = absolute organ weight units are grams ± SD for brain, submaxillary gland (right and left), heart, lungs, thymus, liver, spleen, kidney (right and left),seminal vesicle, prostate, testis (right and left)
absolute organ weight units are milligrams + SD for pituitary, thyroids, adrenal (right and left)

b = relative organ weight units are grams/100 grams body weight + SD for brain, submaxillary gland (right and left), heart, lungs, thymus, liver, spleen, kidney (right and left), seminal vesicle, prostate, testis (right and left)
relative organ weight units are milligrams/100 grams body weight ± SD for pituitary, thyroids, adrenal (right and left)

* P < 0.05 (significantly different from control 1)
+ P < 0.01 (significantly different from control 1)
** P < 0.05 (significantly different from control 2)
++ P < 0.01 (significantly different from control 2)

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TABLE VI-13
Absoluet and Relative Organ Weights of Female Rats

ORGAN	Control 1 (n = 10)		Control 2 (n = 15)		AA Low Dose Test Group (n = 15)		AA Middle Dose Test Group (n = 15)		AA High Dose Test Group (n = 15)		AA + DHA Test Group (n = 15)	
	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]	Absolute [a]	Relative [b]
Brain	2.06 ± 0.08	0.77 ± 0.07	2.00 ± 0.07	0.74 ± 0.06	2.10 ± 0.08++	0.79 ± 0.05	2.11 ± 0.08++	0.80 ± 0.07	2.05 ± 0.07	0.80 ± 0.06	2.10 ± 0.08++	0.76 ± 0.09
Pituitary	17.9 ± 3.0	6.6 ± 0.9	17.5 ± 2.9	6.5 ± 1.1	18.4 ± 2.2	6.9 ± 0.4	18.9 ± 2.4	7.1 ± 0.9	17.9 ± 3.0	7.0 ± 1.0	17.7 ± 2.5	6.4 ± 1.0
Submaxillary Gland (right)	0.24 ± 0.03	0.09 ± 0.01	0.26 ± 0.02	0.10 ± 0.01	0.24 ± 0.02	0.09 ± 0.01	0.25 ± 0.02	0.09 ± 0.01	0.25 ± 0.02	0.10 ± 0.01	0.25 ± 0.02	0.09 ± 0.01
Submaxillary Gland (left)	0.23 ± 0.03	0.09 ± 0.01	0.24 ± 0.02	0.09 ± 0.01	0.23 ± 0.03	0.09 ± 0.01	0.24 ± 0.03	0.09 ± 0.01	0.24 ± 0.03	0.09 ± 0.01	0.23 ± 0.02	0.08 ± 0.01
Thyroids	16.0 ± 1.6	6.0 ± 0.7	16.6 ± 2.5	6.1 ± 0.8	16.4 ± 1.9	6.2 ± 0.8	16.0 ± 3.1	6.0 ± 1.0	16.1 ± 2.7	6.3 ± 0.9	16.8 ± 2.0	6.1 ± 0.7
Heart	0.88 ± 0.06	0.33 ± 0.02	0.87 ± 0.08	0.32 ± 0.03	0.90 ± 0.05	0.34 ± 0.02	0.89 ± 0.09	0.34 ± 0.03	0.83 ± 0.08	0.33 ± 0.03	0.88 ± 0.09	0.32 ± 0.03
Lungs	1.14 ± 0.29	0.42 ± 0.08	1.00 ± 0.06	0.37 ± 0.03	1.09 ± 0.22	0.41 ± 0.10	1.05 ± 0.10	0.40 ± 0.04	1.02 ± 0.07	0.40 ± 0.03**	1.02 ± 0.09	0.37 ± 0.03
Thymus	0.23 ± 0.05	0.08 ± 0.02	0.22 ± 0.05	0.08 ± 0.02	0.26 ± 0.08	0.10 ± 0.03	0.24 ± 0.06	0.09 ± 0.02	0.21 ± 0.05	0.08 ± 0.02	0.23 ± 0.07	0.08 ± 0.03
Liver	6.92 ± 0.52	2.58 ± 0.14	7.10 ± 0.71	2.62 ± 0.18	7.09 ± 0.89	2.66 ± 0.22	7.16 ± 0.64	2.70 ± 0.21	7.10 ± 0.73	2.76 ± 0.19	7.31 ± 0.91	2.63 ± 0.15
Spleen	0.52 ± 0.04	0.20 ± 0.01	0.48 ± 0.6	0.18 ± 0.03	0.49 ± 0.05	0.18 ± 0.02	0.58 ± 0.06++	0.22 ± 0.02* ++	0.52 ± 0.06	0.20 ± 0.03	0.51 ± 0.08	0.19 ± 0.03
Right Kidney	0.91 ± 0.07	0.34 ± 0.03	0.91 ± 0.08	0.34 ± 0.03	0.90 ± 0.07	0.34 ± 0.03	0.94 ± 0.08	0.35 ± 0.03	0.89 ± 0.08	0.35 ± 0.03	0.92 ± 0.09	0.33 ± 0.02
Left Kidney	0.88 ± 0.06	0.33 ± 0.04	0.89 ± 0.08	0.33 ± 0.03	0.88 ± 0.07	0.33 ± 0.02	0.90 ± 0.09	0.34 ± 0.04	0.88 ± 0.08	0.34 ± 0.03	0.90 ± 0.08	0.32 ± 0 02
Right Adrenal	28.2 ± 3.5	10.5 ± 1.7	30.4 ± 3.9	11.3 ± 1.8	30.2 ± 4.7	11.3 ± 1.3	30.9 ± 3.2	11.7 ± 1.6	32.2 ± 3.3	12.6 ± 1.6*	32.7 ± 4.6*	11.8 ± 1.8
Left Adrenal	30.0 ± 4.1	11.2 ± 2.1	32.1 ± 4.1	11.9 ± 1.9	32.6 ± 4.1	12.3 ± 1.1	33.4 ± 4.3	12.6 ± 2.0	34.8 ± 3.5*	13.6 ±1.9+ **	34.0 ± 3.9	12.3 ± 1.9
Right Ovary	38.9 ± 6.8	14.6 ± 2.9	40.4 ± 7.6	15.0 ± 3.1	39.7 ± 9.2	15.1 ± 3.7	37.6 ± 8.9	14.1 ± 3.2	41.6 ± 6.4	16.3 ± 2.7	40.1 ± 6.6	14.5 ± 2.4
Left Ovary	39.6 ± 10.9	14.7 ± 3.9	38.0 ± 4.8	14.2 ± 2.5	39.9 ± 9.5	15.1 ± 3.8	36.1 ± 8.6	13.6 ± 3.1	37.6 ± 3.8	14.7 ± 1.7	39.3 ± 6.6	14.1 ± 2.0
Uterus	0.57 ± 0.08	0.21 ± 0.04	0.62 ± 0.14	0.23 ± 0.06	0.65 ± 0.24	0.25 ± 0.09	0.71 ± 0.17	0.27 ± 0.07	0.61 ± 0.14	0.24 ± 0.06	0.69 ± 0.25	0.25 ± 0.11

a = absolute organ weight units are grams ± SD for brain, submaxillary gland (right and left), heart, lungs, thymus, liver, spleen, kidney (right and left),seminal vesicle, prostate, testis (right and left)
absolute organ weight units are milligrams + SD for pituitary, thyroids, adrenal (right and left)

b = relative organ weight units are grams/100 grams body weight + SD for brain, submaxillary gland (right and left), heart, lungs, thymus, liver, spleen, kidney (right and left), seminal vesicle, prostate, testis (right and left)
relative organ weight units are milligrams/100 grams body weight ± SD for pituitary, thyroids, adrenal (right and left)

* P < 0.05 (significantly different from control 1)
+ P < 0.01 (significantly different from control 1)
** P < 0.05 (significantly different from control 2)
++ P < 0.01 (significantly different from control 2)

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C. Adult Rodent Toxicology Studies of AA- and DHA-Containing Oils from Other Sources

AA-rich oil (SUNTGA25 and 40S) and DHA-rich oil (tuna) have been tested in subchronic adult rat assays. Other sources of AA containing oil derived from *M. alpina* have been tested in rodent toxicology assays. This includes ARASCO, an AA containing oil that is produced by Martek Biosciences Corporation. Rat toxicology studies of ARASCO combined with a non-fish oil derived source of DHA have also been conducted. Tabular summaries of adult rat toxicology studies of AA-rich and DHA-rich oil from other sources are presented in Attachment 1.

In order to evaluate the animal safety data of AA-rich oil derived from *M. alpina*, it is critical to examine studies done not only with SUNTGA25 and 40S but with other AA containing oils derived from this fungal source (with and without a source of DHA). Many repeat dose rat toxicology studies have been conducted using AA containing oils from *M. alpina* alone or in combination with a DHA source (Boswell et al. 1996; Wibert et al. 1997; Hempenius et al. 1997, 2000; Wyeth 1995; Arterburn et al. 2000; Koskelo et al. 1997; Martek 1995; Burns et al. 1999; Suntory 1992a,b 1997) (Tabular summaries of these studies can be found in Attachment 1). An important consideration when interpreting results of these studies is that they differ in many important aspects with regard to study design. Dose levels, negative controls used, positive or vehicle controls used, and percent of total fat in the diet are not standardized among studies. In studies of this type, where the test material is a source not only of nutrition, but has a bioactive effect, there is a high degree of difficulty associated with interpreting results produced at high levels of exposure. The physiologic and nutritional impact of high levels of such a test article added to the diet can be difficult to distinguish from a toxicological manifestation of exposure. An added confounding factor is derived from the addition of other oils to some test material groups in order to standardize fat intake. The added oils themselves have the potential to produce nutritional disturbances at high levels of addition. Although some studies utilized a group fed a high fat diet as a point of comparison for test groups, the result of this comparison, is for all intents and purposes, comparing one test group against another test group.

It is important, therefore, to recognize that interpretation and use of the animal toxicology results in the test article risk assessment must take into account several important considerations: 1) the test article is both a source of fatty acids that could be essential for infants and is bioactive; 2) study design has a significant impact on study results; 3) understanding and differentiating between a toxic response and a physiologic response to excessive intake is critical to determining the mechanism of a response produced by AA or DHA containing oils and; 4) because the test article is a noncarcinogenic nutrient, a principal feature of the risk assessment process is the acceptance that no risk of adverse effects is expected unless a threshold dose (or intake) is exceeded. The adverse effects that may be caused by a nutrient almost certainly occur only when the threshold dose is exceeded.

Interpretation of results from animal studies conducted with AA-rich oil, alone or in combination with a DHA source, is not a straightforward matter, nevertheless, it is possible to analyze the patterns of target organ effect, dose-response and gender specific efforts indicated across studies to form conclusions regarding the safety of *M. alpina* as a source of oil for AA. In

response to GRN 000007, FDA highlighted five areas of concern from an initial review of animal toxicology data of ARASCO and DHASCO. These concerns were: potential for test article related effects on liver, spleen, kidney, hematologic parameters and reproductive parameters (Letter from FDA in response to GRAS Notice No. GRN 000007).

These issues were subsequently addressed in GRN 000041, Martek's Notification for ARASCO and DHASCO. FDA cited in their May 17, 2001 response to GRN 000041 the conclusions upon which Martek based their GRAS determination of ARASCO and DHASCO. In Martek's view, these PUFA's pose unique testing problems because it can be difficult to distinguish whether an observed effect related to the test material is a normal physiological response to the high dietary load of that particular macronutrient, a dietary deficiency that is related to the presence of a large amount of test material in the diet or a toxicological effect. In Martek's view, statistically significant effects, including increased relative liver weight, increased relative spleen weight and a change in some blood chemistries reported in some of the animal toxicology studies are not adverse toxicological findings related to ARASCO and DHASCO as sources of AA and DHA, respectively. Based on the information provided by Martek, FDA has no questions at this time regarding Martek's conclusion that ARASCO and DHASO are GRAS sources of AA and DHA, respectively.

An independent examination of repeat dose rat studies conducted on AA containing oils derived from *M. alpina* alone or in combination with DHA containing oil show increases in liver weight. These increases are similar in magnitude to those reported in rodent studies of fish oils (Rustan et al. 1992; Skuladottir et al. 1994; Murphy et al. 1997; Yaqoob et al. 1995; Mohan et al. 1991; De Craemer et al. 1994; Yamazaki et al. 1987).

In 4-week studies, ARASCO was used as the source of AA. The results of ingestion of ARASCO and ARASCO + a source of DHA on liver weight in four week rat studies are shown in Table VI-14.

TABLE VI-14 Liver Weight Effects Associated With AA-Containing Oil Ingestion Alone or With a DHA-Containing Oil From 4-Week Studies		
Dose of AA Oil (mg/kg)	**Effect on liver weight**	**Reference**
50	no effect	Boswell et al. 1996
100	no effect	Hempenius et al. 1997
600	no effect	Hempenius et al. 1997
1000	no effect	Boswell et al. 1996
2000	no effect	Hempenius et al. 1997
2500	no effect	Boswell et al. 1996
3000	no effect	Hempenius et al. 1997
Dose of AA Oil + DHA Oil (mg/kg for each component, respectively and total dose)		
1000 + 500 (total 1500)	no effect	Boswell et al. 1996
533 + 1067 (total 1600)	no effect	Wibert et al. 1997
2000 + 1000 (total of 3000)	no effect	Hempenius et al. 1997
2500 + 1250 (total of 3750)	significant effect on absolute liver weight (<20%) of males compared to high fat control; however, no significant effect on relative liver weight	Boswell et al. 1996
1500 + 3000 (total of 4500)	significant effect on relative liver weight compared to high fat control, however, no effect compared to low fat control	Wibert et al. 1997
3000 + 1500 (total of 4500)	no effect	Hempenius et al. 1997
3083 + 6167 (total of 9250)	significant effect on relative liver weight compared to high fat control, however, no effect compared to low fat control	Wibert et al. 1997

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There is no clear treatment-specific adverse effect on the liver apparent from these studies. No corresponding, consistent treatment related adverse effects were seen in clinical chemistry values. In the mid and high-dose groups from the Wibert et al. (1997) study, it is noteworthy that although liver weight increase was noted in comparison to high fat controls, the liver weights were comparable to those seen in the low fat control animals. Thus, evidence from the study suggests that the liver weight is within normal physiologic limits. In this study, both the high fat control and the high-dose AA + DHA groups had hepatic periportal vacuolation. In the Boswell et al. (1996) study, absolute, but not relative liver weight was increased, in males only. Thus, although liver weight increases were seen in these four-week studies, they appear to be occur as a response to high fat intake, not specifically oil derived from *M. alpina*.

The same analysis can be made using data from thirteen week rat studies. The effect on liver weight from ingestion of AA-containing oil alone and with DHA-containing oil is presented in Table VI-15.

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TABLE VI-15 Liver Weight Effects Associated With AA-Containing Oil Ingestion Alone or With a DHA-Containing Oil From 13-Week Studies		
Dose of AA Oil (mg/kg)	**Effect on liver weight**	**Reference**
190	no effect	Hempenius et al. (2000)
312	no effect	Suntory (1997)
500	no effect	Personal Communication to Ross Products (1996)
600	no effect	Suntory (1992b)
626	no effect	Suntory (1997)
970	no effect	Hempenius et al. (2000)
1000	no effect	Koskelo et al. (1997)
1200	increased relative liver weight compared to low fat control	Suntory (1992b)
1255	no effect	Suntory (1997)
1700	no effect	Personal Communication to Ross Products (1996)
2500	no effect	Koskelo et al. (1997)
3000	increased absolute and relative liver weight compared to low fat control	Suntory (1992b)
4800	relative liver weight was increased in females only	Hempenius et al. (2000)
5000	absolute liver weight was significantly heavier compared to high fat control (relative liver weight was increased; no statistical analysis completed).	Personal Communication to Ross Products (1996)
Dose of AA Oil + DHA Oil (mg/kg for each component, respectively and total dose)	**Effect on liver weight**	**Reference**
60 + 42 (total 102)	no effect	Wyeth (1995)
298 + 156 (total 454)	no effect	Wyeth (1995)
767 + 383 (total 1150)	no effect	Burns et al. (1999)
1190 + 750 (total 1940)	no effect	Wyeth (1995)
2700 + 1350 (total 4050)	mean absolute and relative liver weights significantly heavier than high fat control. No effect on liver/brain ratio compared to controls.	Burns et al. (1999)
5700 + 2850 (total 8550)	mean absolute and relative liver weights significantly heavier than high fat control. Significantly increased liver/brain ratio only in females and not males.	Burns et al. (1999)
4940 + 3625 (total 8565)	relative liver weight was increased in females only	Hempenius et al. (2000)

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An analysis of the data indicates that levels of addition of test oil ≥ 3000 mg/kg/day for 13 weeks (either AA oil alone or in combination with DHA oil) result in consistent significant increases in liver weight. It is important to note, however, that results from these studies do not represent comparisons of consistent total dietary fat intake, nor are test groups compared to equivalent controls. An examination of the clinical chemistry and histopathology results from these studies provides additional evidence to evaluate whether increased liver weights are indicative of toxicity from either the test article or its impurities or whether they are a consequence of the metabolic overload that occurs with ingestion of such high levels of a nutritional and bioactive substance.

A study of an AA containing oil indicates that there are no effects on liver weight at doses of AA containing oil up to 1700 mg/kg but there is an increase in absolute liver weight in males and females at 5000 mg/kg, which remains higher in the males after the recovery period (Personal Communication to Ross Products 1996). This study was conducted by oral gavage. Fat intake was not controlled and thus the highest dietary fat intake was consumed by the high dose group (5 g in addition to the basal diet) and the lowest intake was consumed by the low dose group (0.5 g in addition to the basal diet). There were histomorphologic hepatic changes in male rats of the mid- and high-dose group, comprised of periportal hepatocellular cytoplasmic alteration and centrilobular hepatocellular hypertrophy. In the high dose group, effects on weight and histomorphology were accompanied by higher activities of alanine aminotransferase, asparate aminotransferase and alkaline phosphatase. No clinical chemistry changes were seen in the mid-dose.

A follow-up evaluation was done to elucidate the cause of the effects seen on liver at the mid and high doses in the previous 13-week study (Personal Communication to Ross Products 1996). A statistical evaluation on the incidence of periportal hepatocellular cytoplasmic alteration showed that the incidence of this change at the mid- and high dose was significant compared to the oil treated controls. When untreated rats are used as controls, the difference in incidence of periportal hepatocellular cytoplasmic alterations was not significant for any treated group. The incidence of centrilobular hepatocellular hypertrophy in the high dose group and in the males of the mid-dose group was significant compared to both the oil treated controls and untreated control group. Detailed microscopic re-examination of the liver slides showed that the hepatocellular alteration was not associated with storage of fat, but more closely resembled the vacuolation associated with storage of glycogen. Dietary n-3 PUFA from fish oil have been shown, *in vivo*, to inhibit glucose-6-phosphate dehydrogenase and glucose-6-phosphatase activity and increase the levels of glycogen in the liver (Chiang and Tsai 1995). AA and other unsaturated fatty acids, have also been shown, *in vitro*, to inhibit hepatic glucose-6-phosphatase activity (Mithieux et al. 1993). The periportal hepatocellular cytoplasmic alteration may reflect an increased glycogen storage, caused by an inhibition of glucose-6-phosphatase, leading to reduced glycogen breakdown. It is also important to note that the hepatocellular hypertrophy at the mid-dose was not associated with serious histopathological lesions such as single

cell necrosis, and that the effects were minimal to slight, reversible and not accompanied by increased liver weight or biochemical changes.

In the study by Burns et al. (1999), a finding of increased mean absolute and relative liver weight was seen at the mid and high dose levels compared to the high fat control. (This corresponded to a mean AA oil (ARASCO) intake of 4050 and 8550 mg/kg/day, respectively.) An analysis of the liver weight finding presented in the Wyeth GRAS Notification (GRN# 000007) indicates that although this effect was statistically significant, the mean relative liver weights were within the normal historical control ranges. All test groups were standardized for fat intake, and thus the dietary fat intake for each group (except for the low fat control) was 13.1%. No test material-related histopathology or clinical chemistry indicative of liver damage was noted in the Burns et al. (1999) study. It was noted that hepatic periportal vacuolation was seen in both the high dose test group and the high fat controls.

In the Wyeth (1995) study, AA oil (ARASCO) was given in combination with DHA and all test groups were standardized for fat intake at 5% of diet. There were no effects on liver weight, however, all groups, including the positive control (5% soybean oil) had hepatic fatty change that was reported to be slight to moderate. No clinical chemistry findings indicative of liver damage were reported. In the study by Hempenius et al. (2000), hepatocellular vacuolation was seen at the high AA and AA plus DHA dose groups. The vacuolation was also seen in the high fat control group.

Two studies evaluated the safety of SUNTGA AA-rich oils at doses from 600 - 3000 mg/kg (Suntory 1992a) and 312 - 1255 mg/kg (Suntory 1997). In the first study (Suntory 1992a), there were no consistent treatment related adverse changes in clinical chemistry. Histopatholgic examination indicated an apparent increase in the incidence of vacuolar degeneration (fatty change) in the high dose males and in mid dose and high dose females. This increase in incidence was slight for the high dose groups compared to controls. The increase was most striking in the mid dose females but does not correlate well with the increase in liver weight. The severity of the vacuolar degeneration was minimal to slight in all dose groups and would be considered to be a reversible change. There also appeared to be a very slight increase in the severity of Sudan III positive staining (indicating the presence of fats) in the high dose females. In all dose groups, the degree of positive staining was minimal to slight. In this study, it was concluded that while fatty change may have contributed slightly to the liver weight increase, the data was contradictory and inconclusive at best. In the second study (Suntory 1997), no adverse effects on liver weight were noted and there were no adverse findings indicative of liver toxicity seen in the clinical chemistry or histopathology examinations.

It is known that ingestion of high fat diets, particularly those that contain fish oil, increases liver weight and total lipid content of the livers of rats (Yaqoob et al. 1995; Høstmark et al. 1989). The increase in liver weight, however, is not completely explained by the increased lipid content of the organ. It has been speculated that increased liver weight may be due to an increase in the number of peroxisomes

(Yaqoob et al. 1995). De Craemer et al. (1994) found that the livers of mice fed a diet with 10% w/w salmon oil had a significant increase in peroxisomal β-oxidation, catalase activity and peroxisomal number. Rustan et al. (1992) also demonstrated that rats fed a 20% (w/w) fish oil diet for 3 weeks showed a significantly increased hepatic peroxisomal βoxidation. Yamazaki et al. (1987) found that compared with safflower oil enriched diet, treatment with a fish oil (15% w/w) diet produced significant increases in liver weight and specific activities in liver homogenates of peroxisomal enzymes fatty acyl-CoA oxidase and catalase. In contrast with other peroxisomal proliferating agents such as clofibrate, hepatic glutathione peroxidase and glutathione S-transferase activities are unchanged or increased with a fish oil diet. This is important since depression of these activities has been associated with decreasing hepatic clearance of certain drugs and may allow the oxidative damage hypothesized to lead to hepatocarcinogenesis seen with clofibrate in rats and mice .

The possibility that AA-rich oil may produce centrilobular hypertrophy as the result of increased cytoplasmic smooth endoplasmic reticulum caused by peroxisome proliferation was investigated in a two-week gavage study. Male rats were administered 1700 and 5000 mg/kg AA-rich oil by gavage (Personal Communication to Ross Products 1997). A 5 g/kg safflower oil control was included. There was no evidence of peroxisome proliferation at a dose level of 1700 mg/kg/day. There was a statistically significant increase in the activity of palmitoyl-CoA oxidase, an enzyme involved in peroxisomal ß-oxidation in the liver, from rats given 5000 mg/kg/day. However, the increase in activity was small (less than 2x) and reversible, as compared to that induced by known peroxisome proliferators (10 - 30x). In addition, the activity and protein level of, respectively, enoyl-CoA hydratase and CYP4A1, which are also representative for induction of peroxisome proliferation, were not affected. Furthermore, electron microscopic analysis did not show any evidence of peroxisome proliferation in livers from rats receiving 5000 mg/kg/day. Therefore, it was concluded that the periportal cytoplasmic alteration and centrilobular hypertrophy were probably not caused by peroxisome proliferation.

In conclusion, the liver weight increases seen at very high dose levels of AA-containing oil alone or in combination with DHA-containing oil (≥3000 mg/kg/day for 13 weeks) do not appear to be associated with corroborative evidence from clinical chemistry or histopathology that this finding is indicative of a serious irreversible toxic event. There was no evidence of hepatocellular hyperplasia, extensive inflammatory infiltrates, necrosis or neoplasia. The effects seen on liver weight are similar to those seen with the administration of other fatty acids at high dose levels, particularly fish oils. The liver histopathology findings in all studies of AA-rich oil (alone or in combination with DHA-rich oil) are minimal to slight and would be considered to be a reversible change. Hepatic vacuolation was seen not only in the test groups, but frequently in the high fat control animals. There was no evidence from histopathogic examination that fat accumulation accounted for the entire increase seen in liver weight. The periportal cytoplasmic alteration found in response to AA oil administration is histopathologically consistent with increased glycogen storage. Although it can not be completely excluded, evidence from a follow-up study strongly suggests that the liver

effects seen at doses up to 5000 mg/kg of AA-rich oil were not due to peroxisome proliferation. There does not appear to be any consistent treatment related effect on liver at doses levels ≤3000 mg/kg, suggesting that this is a threshold for the response.

In several studies conducted with addition of AA and DHA-rich oils, increases in spleen weight were observed (Table VI-16). The spleen is considered to be a secondary lymphoid organ. Key events that occur within the secondary lymphoid organs are: 1) specific antigen recognition in the context of the major histocompatibility complex (MHC) class II; 2) clonal expansion (proliferation) of antigen-specific cells, and; 3) differentiation of antigen-stimulated lymphocytes into effector and memory cells (Casarett and Doull 1996). When the spleen is enlarged, its ability to trap and store blood cells increases. When the spleen removes too many blood cells from the circulation, a variety of problems can develop, including anemia from a reduction in red blood cells, infection from a reduction in white blood cells and bleeding problems from too few platelets (Merck Manual 1997).

It is recognized that intake of dietary fat has a profound effect on immune response. Dietary lipids can affect the immune system by influencing substrate availability in the formation of cyclooxygenase and lipooxygenase products. These products, in turn, act as lipid mediators in the immune system. Furthermore, the cells of the immune system are dependent on cell membrane function for operations such as the secretion of lymphokines and antibodies, antigen reception, lymphocyte transformation, and contact lysis. Lipids are potentially critical components in the regulation of the immune function (FAO/WHO expert consultation on fats and oils in human nutrition (1994). Both AA and DHA have profound effects on immune function. In general, however, for healthy individuals, the FAO/WHO states that "recommendation to decrease dietary total fat intake, with the inclusion of moderate amounts of (n-6) and (n-3) PUFA with adequate antioxidant nutrients should provide for a competent immune response."

It is not unexpected, however, that very high levels of AA or DHA addition to the diet of rodents might produce effects on some immune system parameters. Effects have been seen in studies of AA-containing oil and DHA-containing oil on spleen weight. These effects are inconsistent among the 4 week studies (Table VI-16), however, at ≥1700 mg/kg in the 13- week studies (Table VI-17), consistent increases in absolute and relative spleen weight are seen.

TABLE VI-16 Spleen Weight Effects Associated With AA-Containing Oil Ingestion Alone or With a DHA-Containing Oil From 4-Week Studies		
Dose of AA Oil (mg/kg)	**Effect on spleen weight**	**Reference**
50	no effect	Boswell et al. 1996
100	no effect	Hempenius et al. 1997
600	no effect	Hempenius et al. 1997
1000	no effect	Boswell et al. 1996
2000	Absolute spleen weight increased in males only; no effect on relative weight	Hempenius et al. 1997
2500	no effect	Boswell et al. 1996
3000	Relative spleen weight increased in males only	Hempenius et al. 1997
Dose of AA Oil + DHA Oil (mg/kg for each component, respectively and total dose)		
1000 + 500 (total 1500)	no effect	Boswell et al. 1996
533 + 1067 (total 1600)	no effect	Wibert et al. 1997
2000 + 1000 (total of 3000)	no effect	Hempenius et al. 1997
2500 + 1250 (total of 3750)	no effect	Boswell et al. 1996
1500 + 3000 (total of 4500)	no effect	Wibert et al. 1997
3000 + 1500 (total of 4500)	Relative spleen weight increased in females only	Hempenius et al. 1997
3083 + 6167 (total of 9250)	no effect	Wibert et al. 1997

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TABLE VI-17 Spleen Weight Effects Associated With AA-Containing Oil Ingestion Alone or With a DHA-Containing Oil From 13-Week Studies		
Dose of AA Oil (mg/kg)	**Effect on spleen weight**	**Reference**
190	no effect	Hempenius et al. (2000)
312	no effect	Suntory (1997)
500	no effect	Personal Communication to Ross Products (1996)
600	no effect	Suntory (1992)
626	no effect	Suntory (1997)
970	no effect	Hempenius et al. (2000)
1000	increased relative spleen weights in males only	Koskelo et al. (1997)
1200	no effect	Suntory (1992)
1255	no effect	Suntory (1997)
1700	increased absolute and relative spleen weights in males and females	Personal Communication to Ross Products (1996)
2500	increased relative spleen weights in males only	Koskelo et al. (1997)
3000	no effect	Suntory (1992)
4800	relative spleen weight was increased in males and females	Hempenius et al. (2000)
5000	increased absolute and relative spleen weights in males and females	Personal Communication to Ross Products (1996)
Dose of AA Oil + DHA Oil (mg/kg for each component, respectively and total dose)	**Effect on spleen weight**	**Reference**
60 + 42 (total 102)	no effect	Wyeth (1995)
298 + 156 (total 454)	no effect	Wyeth (1995)
767 + 383 (total 1150)	no effect	Burns et al. (1999)
1190 + 750 (total 1940)	no effect	Wyeth (1995)
2700 + 1350 (total 4050)	mean absolute and relative spleen weights significantly heavier than high fat control but not low fat control. Relative spleen weight of the high fat control was higher than the low fat control.	Burns et al. (1999)
5700 + 2850 (total 8550)	mean absolute and relative spleen weights significantly heavier than high fat control but not low fat control. Relative spleen weight of the high fat control was higher than the low fat control.	Burns et al. (1999)
4940 + 3625 (total 8565)	relative spleen weight was increased in males and females	Hempenius et al. (2000)

The effect on spleen weight seen in these studies has been demonstrated with menhaden oil administration in rats. Significantly increased absolute spleen weight was seen in rats given menhaden oil at 20% (w/w) for 10 weeks (Yaqoob et al. 1995). In the Wyeth Nutritionals Response Document to FDA (December 1, 1999; Supplement to GRN 000007) a statement from Dr. Kevin Fritsche, an expert in the field of LCPs and immune effects was presented. He indicated that "In several of our feeding trials we have noted that mice and rats fed diets enriched in n-3 PUFA have had significantly heavier spleens (15 – 35%) and that these spleens contained significantly more immune cells (10-50%)." Dr. Fritsche observed that these results were the same whether the test material was sardine oil, cod liver oil, menhaden oil or flaxseed oil. These findings support the conclusion that increased spleen weight is a general response to high levels of ingestion of PUFAs in animal studies and is not necessarily a toxic phenomenon associated with a particular source.

An evaluation of the clinical chemistry and histopathology data from the studies conducted using AA-rich oil and DHA-rich oil also corroborates that although spleen weight increases are noted, there is no histopathology indicative of a toxic or irreversible effect. Routine hematology and clinical chemistry parameters related to the immune system did not reveal evidence of an immunotoxic effect. It is postulated that spleen weight increase is an adaptive response in these animals to high doses of PUFAs that are understood to have profound biological activity within the immune system. It is also clear that there is a threshold for this response and a moderate amount of (n-6) and (n-3) PUFA are necessary for a competent immune response. Data from these studies suggest that there is a threshold for the response. In animal studies, no consistent effect on spleen weight is seen at $\leq$1700 mg/kg /day for 13- weeks.

D. Safety Study of Neonatal Piglets Administered AA-rich oil (SUNTGA40S) and DHA-rich oil (derived from tuna)

1. Introduction

Analysis of studies conducted in the adult rat revealed that the appropriateness of the animal model is critical in an evaluation of data generated from safety studies. Rats, for example, are poor models for evaluating the consequences of high fat intakes. An adult rat diet is typically 5% fat by weight (2.7% fat as percent of caloric value) as opposed to an infant diet where 50% of the calories are derived from fat. It is also acknowledged that it may not be possible to extrapolate results from adult rat studies to human infants. The differences between adult animals and neonatal animals, make it impossible to predict if a neonate would be more sensitive to the effects of oil ingestion or, if they are more sensitive, by how much.

It is therefore pivotal that the safety evaluation of a bioactive ingredient intended for infant formula be done in an animal model from which data can be extrapolated to the human infant. The objective of the study would be to assess any unique susceptibility of a neonatal animal, a surrogate model for the human neonate, to possible adverse effects of test article administration.

The newborn piglet was selected as the test species. It is a commonly used model for the study of human developmental gastrointestinal function (Moughan 1992; Miller 1987), and long chain polyunsaturated acid absorption (Chiang 1989). The responsiveness of organs such as liver, brain and bone to long chain polyunsaturated acid feeding has been demonstrated (Arbuckle 1991; de la Presa-Owens 1998; Weiler 2000). Swine milk is similar to human milk in its fat and fatty acid content (Rioux 1997; Arbuckle 1991; Weiler 2000) and the animals grow very rapidly in the first three weeks of life. The rapid growth of all organs in the piglet, which grow faster than those of the human infant, may increase the sensitivity of the piglet to potential adverse effects of AA-rich or DHA-rich oils. The newborn piglet provides the opportunity to observe the interaction between high nutrient requirements and relative metabolic immaturity in a situation analogous to the human infant.

Piglets delivered at term gestation were chosen as the appropriate animal model for this study. There is no current premature piglet animal model. Delivering animals even a few days prior to term results in respiratory immaturity that makes extrauterine survival difficult in the absence of specialized respiratory support (personal communication, Peggy Borum, Ph.D.). Other potential complications with the premature model include the need for medications (including antibiotics), related to a very immature immune system. The term piglets used in this study are provided with maternal colostrum for three days so that exogenous immunologic support (e.g., immunoglobulin) will not be required. This is provided by allowing the piglet to suckle after term delivery. An alternative, postnatal injection of gamma globulin, was rejected due to potential masking effects that this might have on any adverse effects of the test articles (personal communication, FDA meeting December 19, 2000).

An analysis of results from the adult rat toxicology studies of several AA-rich oils from *M. alpina*, with and without a source of DHA, indicates that at doses of these long-chain polyunsaturated fatty acids below 2000 mg/kg/day, no adverse effects are expected in that species. At higher doses, increased liver and spleen weights (absolute and relative) are seen. Histopathology seen in the liver was characterized by hepatic vacuolation, however, this finding was also seen in control animals from these studies that were given high fat diets. No adverse histopathology findings were associated with the increased spleen weights. The increased liver and spleen weights seen at higher doses in the adult rat model may be the result of nutritional imbalance, pharmacologic activity or impurity profile. The margin of safety between the NOAELs determined from these studies and the calculated human exposure, however, is on the order of 10-fold on a body weight basis and is appropriate for this type of ingredient.

Unlike traditional food additives, bioactive ingredients (such as AA and DHA), similar to nutrients, typically do not have a large margin of safety between the recommended level of intake and the Tolerable Upper Intake Level (UL). A summary of the calculated margins of safety (UL/Recommended Dietary Allowance (RDI) or Adequate Intake (AI)) for several nutrients indicates that for these ingredients, typical margins of safety lie between 2 and 10 (Table VI-18). This contrasts with the higher margins, i.e., 100-fold, typically generated for traditional food additives

It is therefore reasonable to choose dose levels for the piglet study that provide an exaggeration of expected human intake of AA and DHA that are within the margins of safety established for nutrients, and by extrapolation, for other bioactive ingredients. These levels of intake are not expected to cause adverse effects on organ structure or function that may occur with feeding at higher levels that are above the physiologic range. In the adult rat studies, the most consistent effect of feeding sources of AA and DHA was an increase in liver and spleen weights. Because of these results, the focus of the neonatal piglet study was to determine if there are any adverse effects on these organs from feeding AA-rich oil and DHA-rich oil at doses up to 5-fold higher than levels of ingestion by infants given formulas supplemented at the target levels.

TABLE VI-18
Food and Nutrition Board, Institute of Medicine; National Academy of Sciences
Dietary Reference Intakes (IOM 2000):
Tolerable Upper Intake Levels (UL[a]) and Recommended Intakes for Individuals (RDA or AI[b])

Life Stage Group	Calcium (g/d)		Phosphorus (g/d)		Vitamin D (μg/d)		Fluoride (mg/d		Niacin (mg/d)[c]		Folate (μg/d)[c]		Selenium μg/d)	
	UL	RDA/ AI	UL	RDA/ AI	UL	RDA/ AI	UL	RDA/ AI	UL	RDA/ AI	UL	RDA/AI	UL	RDA/AI
Infants														
0-6 mo	ND	**0.210**	ND	**0.100**	25	**5**	0.7	**0.01**	ND	**2**	ND	**65**	45	**15**
7-12 mo	ND	**0.270**	ND	**0.275**	25	**5**	0.9	**0.5**	ND	**4**	ND	**80**	60	**20**
Children														
1-3 y	2.5	**0.5**	3	**0.460**	50	**5**	1.3	**0.7**	10	**6**	300	**150**	90	**20**
4-8 y	2.5	**0.8**	3	**0.500**	50	**5**	2.2	**1**	15	**8**	400	**200**	150	**30**
Males, Females														
9-13 y	2.5	**1.3**	1.250	**1.250**	50	**5**	10	**2**	20	**12**	600	**300**	280	**40**
14-18 y	2.5	**1.3**	1.250	**1.250**	50	**5**	10	**3**	30	**14-16**	800	**400**	400	**55**
19-70 y	2.5	**1.0**	0.70	**0.70**	50	**5-10**	10	**3-4**	35	**14-16**	1000	**400**	400	**55**
>70 y	2.5	**1.2**	0.70	**0.70**	50	**15**	10	**3-4**	35	**14-16**	1000	**400**	400	**55**
Margin of Safety. UL/RDA or AI	≈ 2.5		≈ 3-6		≈ 5-10		≈ 2-5[f]		≈ 2		≈ 2		≈ 3-7	

[a] UL = The maximum level of daily nutrient intake that is likely to pose no risk of adverse effects. Unless otherwise specified, the UL represents total intake from food, water, and supplements. Due to lack of suitable data, ULs could not be established for thiamin, riboflavin, vitamin B_{12}, pantothenic acid, biotin, or any carotenoids. In the absence of ULs, extra caution may be warranted in consuming levels above recommended intakes.
[b] This table presents Recommended Dietary Allowances (RDAs) in **bold type** and Adequate Intakes (AI) in ordinary type followed by an asterisk (*). RDAs and AIs may both be used as goals for individual intake. RDAs are set to meet the needs of almost all (97 to 98 percent) individuals as in a group. For healthy breastfed infants, the AI is the mean intake. The AI for other life-stage and gender groups is believed to cover needs of all individuals in the group, but lack of data or uncertainty in the data prevent being able to specify with confidence the percentage of individuals covered by this intake.
[c] The ULs for magnesium represents intake from a pharmacological agent only and do not include intake from food and water.
[d] The ULs for niacin, folate, and vitamin E apply to synthetic forms obtained from supplements, fortified foods, or a combination of the two.
[e] ND = Not determinable due to lack of data of adverse effects in this age group and concern with regard to lack of ability to handle excess amounts. Source of intake should be from food only to prevent high levels of intake.
[f] With the exception of infants 0-6 mo.

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2. Materials and Methods

Test Materials

The test items were supplied by the Sponsor as ready to feed formula similar in composition to human infant formula, meeting the estimated nutrient requirements for pigs (National Research Council 1998 Nutrient Requirement of Swine, 10th pd. National Academy Press, Washington, DC pp 9-1 IS). The composition of the formulas and fatty acid composition of the formulas is presented in tables VI-19 and VI-20. The test items were supplied labeled with alphanumeric codes such that the treatments were blinded until completion of the experimental work. There were no differences in formulations, such as smell, that allowed identification of the individual treatments. Following completion of the experimental work the decoding of the study was supplied by the Sponsor. The alphanumeric codes and their relation to the treatments are included in Table VI-21. The test items were supplied in 32 fl. oz. metal cans and were stored in a clean dry secure environment at least 8 cm from the ground. The concentration of the test substances, oils and caloric content of the test items are included in Table VI-21.

Animals and Husbandry

Forty-eight, 3-day-old piglets (24 male and 24 female) were used in the study. In order to obtain these piglets, 10 pregnant sows were used (Landrace/Large White crosses' were obtained from Pigs Supplies and Services, P.O. Box 463, Aylesbury Bucks, HP22 5ZX). The sows were brought into the experimental unit 4 weeks before they were due to farrow. Initially they were group housed with straw for bedding. Five days before they were due to farrow the sows were transferred to individual farrowing crates. The farrowing crates contained shaving and rubber matting for bedding. Heat lamps provided additional heating for piglets.

The sows were fed a daily ration of concentrated diet that did not contain any fish meal or fish oils (AF Masterbreed SS Pencils, AF Feeds, Cumbria House, Stalker Road, Gilwilly Trading Estate, Penrith, Cumbria, CAI 1 913G). Water from the domestic mains supply was available *ad libitum*. The water used by Inveresk Research is analysed by the local water authority at 6 month intervals for dissolved materials, heavy metals, pesticide residues, pH, nitrates and nitrites.

In order to ensure that the sows all farrowed around the same time some sows were induced by injection of Lutalyse (2 ml Dinoprost 5 mg/ml). All piglets were weighed within ca 24 h of birth and identified by individual ear tags. At ca 1 day old the piglets had their teeth clipped, to prevent injury, according to normal husbandry practice and at 3 days old, each piglet was given an intramuscular iron dextran injection (1 ml Leodex 200 mg/ml). Prior to acceptance into the study, each piglet was subjected to veterinary examination to ensure that it was suitable for the study. Piglets were deemed suitable for the study based on body weight

and included only one male and one female from each litter in each group, with the heaviest available piglets being used for the study. They were randomly assigned to one of 4 groups and given study number according to the treatment groups included in Table VI-19. The body weights of the piglets were also recorded on Study Day 1, at least weekly throughout the study, and then immediately prior to sacrifice.

Piglets selected for inclusion in the study were group housed by treatment group in pens with wood shavings as bedding and heat lamps to provide additional heat. Shavings were changed at least once a day. Domestic mains tap water was available *ad libitum*. The animals were maintained on a 12 h light/12h dark lighting schedule with additional continuous light from heat lamps. The daily range in room temperature and humidity was recorded throughout the acclimatization and study periods.

Dose Levels

Dose levels were chosen on the basis of an exaggeration of the estimated mean intake of AA and DHA by infants consuming formula containing target levels of AA-rich oil and DHA-rich oil. This exaggeration was intended to test tolerance of the neonatal piglet to levels of consumption of AA, DHA and AA + DHA that are, respectively: 5-fold, 5-fold and 3 fold + 3-fold higher than the targeted estimated intakes of these long-chain polyunsaturated fatty acids in infants. The highest estimated mean intake of AA and DHA by infants was found in the preterm infant clinical trial of Similac® Special Care®. At term corrected age, these infants consumed mean amounts of approximately 32 mg/kg/day AA and approximately 20 mg/kg/day DHA. (These intakes decreased over time with the lowest intakes of AA and DHA (12 and 3 mg/kg/day, respectively) occurring at 12-months in the term infant study of Similac® with Iron.)

At a 5-fold exaggeration of the estimated mean AA intake, the target AA dose for piglets was 160 mg/kg/day. At a 5-fold exaggeration of the estimated mean DHA intake, the target DHA dose or piglets was 100 mg/kg/day. At a 3-fold exaggeration of the estimated mean AA + DHA intakes, the target AA + DHA dose for piglets was 96 + 60 mg/kg/day, respectively (total 156 mg/kg/day AA + DHA).

Dose Preparation

Piglets were fed the dose formulation from standard commercially available lamb feeding bottles (500 ml) and nipples (Non-Vac Lamb Feeder Bottles, NetTex, Priestwood, Harvel, Meopham, Kent, DA13 ODA, UK). The bottles were sterilized prior to each use with commercially available Universal Steam Sterilizers (Product Code 44226, Lindam Ltd, Harrogate, HG2 8PA, UK). Bottles were labeled with animal numbers and group numbers; therefore, each bottle was assigned to one individual animal and was only used for one type of formulation. Bottles were filled with an appropriate volume of formulation and

were stored at ca-2-8°C prior to use. Bottles were used within 24 h of preparation and within ca 6 hours of removal from the refrigerator.

Approximately 2 h before feeding the bottles were placed in an incubator set to ca 40°C. The temperature of the bottles was monitored to ensure that they had heated up sufficiently (ca 40°C). Bottles were returned to the incubator between feeds. A stability study using triplicate samples of each of the piglet formulas indicated that the levels of AA and DHA remained unchanged under the conditions used in this piglet feeding study.

Dose Administration

Initially piglets were offered formulation every ca 2 h. The piglets were fed by group and each piglet was fed from the piglet's own bottle. Bottles were weighed immediately before and after each feed occasion and total amount of formulation consumed by each piglet each day was calculated.

Initially, some piglets were reluctant to drink and had to become accustomed to drinking from bottles. To achieve this, piglets were individually removed from the pen and the nipple of the bottle placed in their mouths. The piglets were carefully monitored during the first few days of the study to ensure that they did not become dehydrated. Piglets that had not suckled for several hours were fed a small amount of formulation (ca 5 ml) by administration over the back of their throat using disposable plastic syringes. Any formulation fed by syringe was removed from the bottle appropriate for that piglet and therefore is included in the food consumption data.

Once piglets had started to suckle they were allowed to feed until they lost interest in the food. On study Days 9-20 the frequency of feeding was reduced to every ca 3 h. Piglets were fasted overnight prior to sacrifice.

Blood Sampling

Blood samples were collected immediately prior to sacrifice for measurement of hematology and clinical chemistry parameters on Study Day 20.

Blood samples were collected following sedation to light general anesthesia by intramuscular injection of ketamine and midazolam. Blood samples were collected either by venipuncture of the anterior vena cava or by intracardiac puncture. The on study animals were blood sampled and sacrificed in a random order. Each blood sample (ca 10 ml) was split among the following tubes for clinical chemistry and hematology: 0.5 ml EDTA; 1 ml citrate and; 2 ml lithium heparin. The remainder of each blood sample was collected in an EDTA tube and separated into plasma and red blood cells by centrifugation at 3000 *g* for 10 mins. The plasma and red blood cells were dispatched to an analytical laboratory for analysis under a separate protocol.

Hematology and clinical chemistry parameters measured (with abbreviations and units) are listed below:

Parameters	Units
Hemoglobin: (Hb)	$g.dl^{-1}$
Total Red Blood Cell Count (RBC)	$x\ 10^{12}.l^{-1}$
Hematocrit (Hct)	$l.l^{-1}$
Mean Cell Volume: (MCV)	fl
Mean Cell Hemoglobin: (MCH)	pg
Mean Cell Hemoglobin: (MCHC)	$g.dl^{-1}$
Total White Blood Cell Count (WBC)	$x\ 10^{9}.l^{-1}$
Platelet Count: (Plat)	$x\ 10^{9}.l^{-1}$
Prothrombin Time: (PT)	s
Activated Partial Thromboplastin Time (APTT)	s
Urea: (Urea)	$mmol.l^{-1}$
Glucose: (Glu)	$mmol.l^{-1}$
Aspartate Amino-transferase: (AST)	$iu.l^{-1}$
Alanine Aminotransferase: (ALT)	$iu.l^{-1}$
Lactate Dehydrogenase: (LDH)	$iu.l^{-1}$
Sodium: (Na)	$mmol.l^{-1}$
Potassium: (K)	$mmol.l^{-1}$
Chloride: (CI)	$mmol.l^{-1}$
Total Protein: (TP)	$g.l^{-1}$
Albumin: (Alb)	$g.l^{-1}$
Albumin/Globulin Ratio (AG-R)	
Alkaline Phosphatase:(AP)	$iu.l^{-1}$
Creatinine: (Crea)	$\mu mol.l^{-1}$
Triglycerides: (Trig)	$mmol.l^{-1}$
Total Bilirubin: (T.Bil)	$\mu mol.l^{-1}$
Gamma Glutamyl Transferase: (GGT)	$iu.l^{-1}$
HDL Cholesterol: (HDL)	$mmol.l^{-1}$
LDL Cholesterol: (LDL)	$g.l^{-1}$

Sacrifice

The on study animals were sacrificed on Study Day 20 after 19 days of feeding the test formulations. Immediately following the collection of the blood samples each animal was humanely sacrificed by overdose of sodium pentobarbitone followed by exsanguination.

Each carcass was subjected to a gross post mortem examination and the weights of the adrenals, brain, kidneys, liver, spleen, testes; thyroid, thymus and parathyroid (when available) were recorded. Any gross lesions were examined histologically. Samples of the following tissues were taken and fixed and stained for histological investigations:

Adrenals	Forebrain
Midbrain	Cerebellum
Ileum	Kidneys
Liver	Spleen
Testes	Thyroid
Thymus	Parathyroid (when available)
Lymph nodes	

Samples of the following tissues were retained in neutral buffered 10% formalin (with the exception of the testes, which were retained in Bouin.'s fluid, and the eyes, which were retained in Davidson's fluid) for further analysis if required:

Adrenals	Aorta
Bone	Bone marrow
Forebrain	Midbrain
Cerebellum	Cecum
Colon	Corpus and cervix uteri
Duodenum	Epididymus
Esophagus	Eyes;
Gall Bladder	Heart
Ileum	Jejunum
Kidneys	Liver
Lung (with main-stem bronchi)	Ovaries and fallopian tubes
Mammary glands	Pituitary
Pancreas	Rectum
Prostate	Sciatic nerve
Salivary gland	Skeletal muscle
Seminal vesicle	Spinal cord (2 locations)
Skin	Stomach
Spleen	Thymus
Testes	Trachea
Thyroid	Vagina
Urinary Bladder	Parathyroid (when available)

Statistical Analysis

In comparing the mean responses of each endpoint of interest[2] across the four experimental groups employed in this study (i.e., control and treatment groups 1, 2, and 3), the objectives of the statistical analyses were to: 1) determine if these means are significantly different from each other due to some overall treatment effect; and 2) if there is a significant overall treatment effect, determine which of the three mean treatment responses are significantly different from the control. A well-recognized parametric statistical technique for achieving this first objective is to perform an analysis of variance ("ANOVA"). If the ANOVA

[2] The endpoints of interest include: body weight, absolute and relative (percent of body weight) organ weights, food consumption, hematology, and clinical chemistries.

shows a significant treatment effect ($p < 0.05$), then to meet the second objective, the next step is to employ some kind of multiple comparison procedure, such as Dunnett's Test. Dunnett's Test is a multiple comparison procedure that specifically compares each treatment group mean with the control mean in a pairwise fashion, while maintaining the overall experimentwise error rate at some prespecified level (e.g., 0.05). Because we are only interested in pairwise comparisons of treatment means versus the control mean, multiple comparison procedures that involve all pairwise comparisons (i.e., comparisons among the treatment means in addition to comparisons of treatment versus control) are not relevant for this analysis. Therefore, ANOVA followed by Dunnett's test is the analysis used to evaluate the data from this study.

Statistical analysis of relative organ weight is consistent with previously conducted toxicological studies of AA- and DHA-containing oils. Adult rat toxicology studies available on AA-containing and DHA-containing oils have all reported analysis of organ weights as percentage of body weight (Hempenius et al. 1997, 2000; Koskelo et al. 1997; Suntory 1992, 1997; Wyeth 1995; Burns et al. 1999; Boswell et al. 1996; Wibert et al. 1997). Finally, a neonatal piglet study of AA-containing and DHA-containing oils also reported analysis of organ weights as percentage of body weight (de la Presa-Owens et al. 1998).

3. Results

Administration of Test Materials

The piglets were fed the test materials for 19 consecutive days. The piglets all learned to suckle from the feeding bottles and drank until they lost interest in the food. The original protocol specified feeding of test material for 21 consecutive days. Piglets consumed more formulation than expected, however, and the volume of formulation available for the study was not sufficient for the full 21 days of feeding. Due to restrictions on movement of animal products, it was not possible to supply additional formulation in time and the study was terminated after 19 consecutive days of feeding.

Clinical Observations

No abnormal test article related clinical observations were noted; the health status of the animals receiving AA-rich and DHA-rich oils was comparable to that of control animals.

Body Weights

Group mean body weights for males and females are summarized in Tables VI-22 and VI-23, respectively. Body weights increased in all groups during the course of the study. There were no statistically significant differences in body weight between the control animals and those receiving the test formulations.

Food Consumption

Group mean daily food consumption for males and females is summarized in Tables VI-24 and VI-25, respectively. Food consumption increased in all groups

throughout the study. The daily food consumption dropped briefly at days 9-10 due to the change in feeding frequency from every ca 2 h to every ca 3 h. There were no significant differences in daily food consumption between the control animals and those receiving test formulations with the exception of day 10 when the Group 4 (5X AA) males drank statistically significantly more formula than the control males. This is not thought to have any adverse consequence or biological significance.

Daily caloric intake during the treatment period per kg body weight of the piglets was calculated; results are presented in Tables VI-26 and VI-27 for males and females respectively. Mean caloric intakes (kcal/kg/day) for males and females respectively were: Control (240; 254); 5X DHA (240; 254); 3X AA + 3X DHA (240; 255); 5X AA (256; 258). These intakes were not different between treated and control groups.

Test Article Intake

Intake during the treatment period of AA-rich and DHA-rich oils and of AA and DHA, per kg body weight, was calculated based on the body weight of the piglets, amount of formula consumed, concentration of oils in the formulas and the percent of AA and DHA of the test oils. Results are presented in Tables VI-28 to VI-35.

The mean intake of AA-rich oil, DHA-rich oil and AA-rich + DHA-rich oils (mg/kg/day) for male piglets was 618, 653 and 365 + 387, respectively; for female piglets, mean intake (mg/kg/day) was 623, 691 and 388 + 412, respectively.

The mean intake (males and females combined) of AA was approximately 250 mg/kg/day, an 8-fold exaggeration of the estimated mean human preterm infant AA intake. The mean intake (males and females combined) of DHA was approximately 136 mg/kg/day, a 7-fold exaggeration of estimated human infant DHA intake. Mean intake (males and females combined) of AA + DHA was approximately 236 mg/kg/day, a 4-fold exaggeration of the estimated human preterm infant combined AA + DHA intake.

Clinical Chemistry and Hematology

The mean values for clinical chemistry and hematology parameters for males and females are shown in Tables VI-36 to VI-39. No statistically significant differences were seen in any of the hematology parameters in either sex.

There was a statistically significant reduction in triglyceride concentration of the males in Groups 2 (5X DHA) and 4 (5X AA) in comparison to controls; this finding was not seen in the females, nor in Group 3 (3X AA + 3X DHA) animals. There was a statistically significant reduction in total bilirubin concentration of Group 4 males (5X AA) compared to controls; this was not seen in the females. In females, there was a statistically significant reduction in the globulin concentration of Group 2 (5X DHA) compared to controls; this was not seen in the males. The specific clinical chemistry changes were seen only in one sex (either male or female but not both) and did not result in any adverse health consequence to the animals. These changes were not considered to be biologically significant.

Organ Weights

Group mean absolute organ weights are summarized in Tables VI-40 and VI-41 for males and females, respectively. Males of group 2 (5X DHA) and group 4 (5X AA) had statistically significantly heavier absolute spleen weights than controls. Absolute spleen weights from males of group 3 (3X AA + 3X DHA) were not statistically significantly different from controls. Absolute spleen weights from females of all treated groups were not statistically significantly different from controls. There were no other significant differences in absolute organ weights in any treated group compared to controls.

Group mean relative organ weights are summarized in Tables VI-42 and VI-43 for males and females, respectively. No significant differences in relative organ weights from male piglets were found between any test article group compared to controls. Statistically significantly higher relative thyroid gland weights were seen from females of group 3 (3X AA + 3X DHA) compared to controls. Females of group 4 (5X DHA) had statistically significantly lower relative kidney weights compared to controls. There were no other significant differences in relative organ weights in any treated group of females compared to controls.

Necropsy Findings

Histopathology results are summarized in Table VI-44. There were a number of findings at necropsy and histopathology that were apparent in all groups and not considered to be related to the test articles. Interstitial nephritis in the kidney was recorded in animals from all groups (including control). It was a focal or localized, minimal or mild, mainly chronic change and was determined by the study pathologist to likely be the result of old infections/inflammatory embolism. Incidence of this lesion was not corrleated with organ weight variations and was not considered to related to test article administration. There were no adverse histopathologic findings in the spleens, livers or thyroid glands of animals from any test group compared to controls. No other adverse histopathology findings were noted in any other organ from the test of control groups.

4. Discussion

Administration of AA-rich oil, DHA-rich oil and AA-rich oil + DHA-rich oil to neonatal piglets did not produce any adverse test article related effects under the conditions of this study.

There were no test article related effects indicative of an adverse health consequence to the animals seen in the clinical signs, body weights, food consumption, clinical chemistry, hematology, organ weights or gross and histopathology.

Several statistically significant effects on absolute organ weights were observed. Absolute spleen weights in males from groups 2 (5X DHA) and 4 (5X AA) were elevated compared to control. The elevated absolute spleen weights in these groups were seen in males and not in females, and relative spleen weights were not statistically significantly

elevated. There was no corroborative evidence of toxicity seen in an examination of the hematology and no pathologic reason for the increase in spleen weight was noted with histopathologic examination. The increase in absolute spleen weight could have been due to the retention of blood within the spleen. This may be a spurious effect associated with the use of pentobarbitone used at sacrifice (Jubb et al. 1993). Therefore, the increase in absolute spleen weight is not considered to be an adverse effect.

The relative kidney weights of the females from group 4 (5X DHA) were statistically significantly lower compared to controls, however, there was no corroborative evidence of toxicity seen in an examination of the clinical chemistry and no pathologic reason for the decrease in kidney weight was noted with histopathologic examination. Kidney weights from males of this group were not statistically significantly different from controls. This statistically significant finding is not considered to be an adverse effect.

The only statistically significant effect on organ weight seen in group 3 (3X AA + 3X DHA) was an increase in relative thyroid gland weight of the females; no significant effect was seen on this organ in the males. This effect was not seen in either group 2 (5X DHA) or group 4 (5X AA) and there was no evidence of toxicity seen in the histopathologic examination. This statistically significant finding is not considered to be an adverse effect.

In this study, actual mean intake of AA was 250 mg/kg/day, an 8-fold exaggeration of estimated mean AA intake for preterm infants ingesting SSC at term corrected age (estimated from Study AG38). The exaggeration exposure to AA was 21-fold that of term infants consuming SWI at 12 months in the term infant study (Study AF92). For DHA, actual mean intake was 136 mg/kg/day, 7-fold exaggeration of estimated mean DHA intake for preterm infants ingesting SSC at term corrected age (estimated from Study AG38). The exaggeration of exposure to DHA was 45-fold that of term infants consuming SWI at 12 months in the term infant study (Study AF92). The actual mean intake of AA + DHA was 236 mg/kg/day, a 4-fold exaggeration of the mean AA + DHA intake for preterm infants ingesting SSC at term corrected age (estimated from Study AG38). Exaggeration of exposure for term infants consuming SWI at 12 months in the term infant Study (AF92) was 16-fold.

TABLE VI-19

Composition of Piglet Formulas (per liter)

Study Group	Control	5X-AA	5X-DHA	3X-AA + 3X-DHA
Protein, g	**59.1**	**59.5**	**59.5**	**59.2**
Fat, g	**56.0**	**57.9**	**58.3**	**58.6**
Carbohydrate[1] g	**65.9**	**69.3**	**68.3**	**68.1**
Energy[2] kcal	**1004**	**1036**	**1036**	**1037**
Minerals				
Calcium, mg	**2670**	**2441**	**2440**	**2545**
Phosphorus, mg	**1742**	**1575**	**1575**	**1586**
Magnesium, mg	**183**	**184**	**183**	**184**
Sodium, mg	**832**	**824**	**834**	**837**
Potassium, mg	**2618**	**2545**	**2534**	**2545**
Chloride, mg	**1341**	**1369**	**1344**	**1340**
Iron, mg	**18.3**	**27.6**	**20.1**	**20.8**
Maganese, µg	**931**	**1025**	**993**	**1008**
Iodine, mg	0.42	0.42	0.42	0.42
Zinc, mg	**30.7**	**33.1**	**33.0**	**33.4**
Copper, mg	1.15	1.15	1.15	1.15
Selenium, µg	**118**	**120**	**124**	**124**
Vitamins				
Vitamin A, IU	**1346**	**1294**	**1376**	**1325**
Vitamin D, IU	263	263	263	263
Vitamin E, IU	**17.2**	**22.8**	**23.1**	**22.6**
Vitamin KI, µg	72.9	67.4	70.8	68.0
Vitamin C, mg	**164**	**163**	**153**	**174**
Thiamin (B1), mg	**2.07**	**2.32**	**2.26**	**2.25**
Riboflavin (B2), mg	1.07	1.07	1.07	1.07
Pyridoxine (B6), mg	**0.793**	**0.870**	**0.864**	**0.858**
Vitamin B12, µg	6.44	6.44	6.44	6.44
Niacin, mg	15.0	15.0	15.0	15.0
Folic acid, µg	319	319	319	319
Pantothenic acid, mg	7.62	7.62	7.62	7.62
Biotin, µg	88.0	88.0	88.0	88.0
Choline total, mg	300	300	300	300
m-Inositol, mg	50.3	50.3	50.3	50.3
L-carnitine, mg	16.0	16.0	16.0	16.0
Taurine, mg	68.8	68.8	68.8	68.8

Values shown in bold are from analysis of the clinical products. All other values were protected from the premix fortification levels and analytical results of marker nutrients, except for iodine, choline and carnitine, which are shown as target levels.

[1]Calculated by difference; based on analytical results for total solids, ash, protein, and fat.

[2]Energy = (g fat)(9 kcal/g fat) + (g protein)(4 kcal protein) + (g carbohydrate) (4 kcal/g carbohydrate).

TABLE VI-20

Fatty Acid Composition of Neonatal Piglet Formulas, g/100 g total fatty acids

Study Group		Control	5X-AA	5X-DHA	3X-AA 3X-DHA
Fatty Acid:					
Butyric	4:0	0	0	0	0
Caproic	6:0	0.26	0.30	0.23	0.29
Caprylic	8:0	3.13	3.03	3.02	3.04
Capric	10:0	2.30	2.24	2.23	2.24
Lauric	12:0	15.73	15.58	15.46	15.60
Myristic	14:0	6.35	6.19	6.29	6.28
Palmitic	16:0	8.75	8.70	9.02	8.92
Palmitoleic	16:1 n-7	0.11	0.11	0.30	0.23
Hexadecadienoic	16:2	0	0	0.03	0.01
Hexadecatrienoic	16:3	0.05	0.04	0.08	0.07
Hexadecatetraenoic	16:4	0.03	0.03	0.01	0.03
Margaric	17:0	0.07	0.08	0.11	0.10
Stearic	18:0	3.82	3.91	3.81	3.89
Oleic	18:1 n-9	35.45	35.39	36.53	35.62
Linoleic	18:2 n-6	20.43	18.71	18.15	17.99
Alpha-Linolenic	18:3 n-3	2.19	1.89	1.85	1.81
Gamma-Linolenic	18:3 n-6	0.02	0.13	0.03	0.10
Stearidonic	18:4 n-3	0.06	0.05	0.11	0.08
Arachidic	20:0	0.28	0.30	0.28	0.29
Eicosenoic	20:1 n-9	0.18	0.20	0.26	0.24
Eicosadienoic	20:2 n-6	0	0.06	0.04	0.06
Homogamma-Linolenic	20:3 n-6	0	0.16	0.01	0.10
Eicosatetraenoic	20:4 n-3	0	0.04	0.04	0.02
Arachidonic	20:4 n-6	0	1.80	0.09	1.16
Eicosapentaenoic	20:5 n-3	0	0.01	0.33	0.22
Heneicosapentaenoic	21:5	0	0	0	0
Behenic	22:0	0.23	0.35	0.23	0.32
Erucic	22:1 n-9	0.01	0.01	0.02	0.05
Docosatetraenoic	22:4 n-6	0	0.03	0	0.01
N-3-Docosapentaenoic	22:5 n-3	0	0	0.07	0.05
N-6-Docosapentaenoic	22:5 n-6	0	0	0.04	0.03
Docosahexaenoic	22:6 n-3	0	0	1.04	0.64
Lignoceric	24:0	0.11	0.51	0.10	0.35
Nervonic	24:1 n-9	0.07	0.09	0.11	0.10

TABLE VI-21 Treatments, Groups and Contents of Formulations Used on Study						
Group	**Animal Numbers**	**Formulation Fed**	**Treatment[a]**	**Concentration of AA and DHA in Formulation (mg/g formula)**	**Concentration of AA-rich oil and DHA-rich oil in Formulation (mg/g formula)**	**Caloric Content of Formulation (kcal/g)**
1	1-6 (M) 7-12 (F)	P00-FA1	Control	0	0	0.962
2	13-18 (M) 19-24 (F)	P00-MA1	5X DHA	0.546 DHA	2.700 (DHA-rich oil)	0.993
3	25-30 (M) 31-36 (F)	P00-RT1	3X AA + 3X DHA	0.616 AA 0.337 DHA	1.510 (AA-rich oil) 1.601 (DHA-rich oil)	0.993
4	37-42 (M) 43-48 (F)	P00-TR1	5X AA	0.961 AA	2.401 (AA-rich oil)	0.994

[a] DHA = docosahexaenoic acid from DHA-rich oil from tuna (Mochida); AA = arachidonic acid from AA-rich oil from *M. alpina* (SUNTGA40S)

000145

TABLE VI-22
Body Weights (kg)
Group Mean Values: Males

Group/Treatment		Pretrial (Day)*	Treatment Period (Days)			
		-3	1	8	15	20
1	Number	6	6	6	6	6
Control	Mean	1.763	2.176	3.894	6.247	7.636
P00-FA1	SD	0.456	0.679	1.069	1.507	2.033
2	Number	6	6	6	6	6
5X DHA	Mean	1.848	2.274	3.998	6.410	7.731
P00-MA1	SD	0.434	0.585	0.817	1.279	1.420
3	Number	6	6	6	6	6
3X AA + 3X DHA	Mean	1.802	2.226	3.962	6.377	7.746
P00-RT1	SD	0.294	0.415	0.735	1.112	1.255
4	Number	6	6	6	6	6
5X AA	Mean	1.695	2.094	3.948	6.536	8.006
P00-TR1	SD	0.287	0.330	0.533	0.851	1.009

No statistically significant effects (ANOVA + Dunnett's test)
* Weight on first day of life

000146

TABLE VI-23 Body Weights (kg) Group Mean Values: Females						
Group/Treatment		**Pretrial (Day)***	**Treatment Period (Days)**			
		-3	1	8	15	20
1	Number	6	6	6	6	6
Control	Mean	1.877	2.326	4.109	6.906	8.342
P00-FA1	SD	0.392	0.519	0.958	1.134	1.318
2	Number	6	6	6	6	6
5X DHA	Mean	1.758	2.111	3.894	6.267	7.608
P00-MA1	SD	0.395	0.493	0.770	1.183	1.513
3	Number	6	6	6	6	6
3X AA, + 3X DHA	Mean	1.612	1.945	3.584	5.853	7.045
P00-RT1	SD	0.326	0.445	0.897	1.233	1.359
4	Number	6	6	6	6	6
5X AA	Mean	1.570	1.923	3.635	6.133	7.421
P00-TR1	SD	0.192	0.392	0.590	0.923	1.115

No statistically significant effects (ANOVA + Dunnett's test)
* Weight on first day of life

000147

TABLE VI-24
Food Consumption (g/animal/day)(1.043 g/ml formula)
Group Mean Values: Males

Group/Treatment		Treatment Period (Days)									
		1	2	3	4	5	6	7	8	9	10
1	Number	6	6	6	6	6	6	6	6	6	6
Control	Mean	224	514	689	734	875	993	1107	1208	1120	923
P00-FA1	SD	90	263	227	240	267	281	298	424	545	256
2	Number	6	6	6	6	6	6	6	6	6	6
5X DHA	Mean	265	590	639	792	906	1009	1085	1213	1130	1142
P00-MA1	SD	95	82	156	203	209	198	257	273	281	187
3	Number	6	6	6	6	6	6	6	6	6	6
3X AA + 3X DHA	Mean	322	536	683	791	890	955	1034	1178	1135	1049
P00-RT1	SD	99	83	157	215	180	190	205	182	198	271
4	Number	6	6	6	6	6	6	6	6	6	6
5X AA	Mean	301	551	721	825	927	961	1083	1181	1213	1319*
P00-TR1	SD	128	137	100	130	206	205	132	172	228	149

Significantly different from the Control: * P<0.05, ** P<0.01, *** P<0.001(ANOVA + Dunnett's test)

000148

TABLE VI-24 (continued)
Food Consumption (g/animal/day) (1.043 g/ml formula)
Group Mean Values: Males

Group/Treatment		Treatment Period (Days)								
		11	12	13	14	15	16	17	18	19
1	Number	6	6	6	6	6	6	6	6	6
Control	Mean	1061	1115	1350	1580	1620	1682	1491	1747	1052
P00-FA1	SD	299	214	353	466	481	567	877	720	574
2	Number	6	6	6	6	6	6	6	6	6
5X DHA	Mean	1127	1177	1355	1232	1272	1406	1542	1645	1097
P00-MA1	SD	202	405	427	350	434	388	252	364	285
3	Number	6	6	6	6	6	6	6	6	6
3X AA + 3X DHA	Mean	1110	1255	1268	1243	1385	1470	1487	1590	1056
P00-RT1	SD	317	396	307	382	375	334	297	255	286
4	Number	6	6	6	6	6	6	6	6	6
5X AA	Mean	1246	1328	1356	1351	1387	1551	1571	1702	1124
P00-TR1	SD	266	246	311	269	161	160	383	348	206

Significantly different from the Control: * P<0.05, ** P<0.01, *** P<0.001 (ANOVA + Dunnett's test)

000149

TABLE VI-25
Food Consumption (g/animal/day)
Group Mean Values: Females

Group/Treatment		Treatment Period (Days)									
		1	2	3	4	5	6	7	8	9	10
1	Number	6	6	6	6	6	6	6	6	6	6
Control	Mean	338	664	738	861	950	1078	1162	1360	1197	1234
P00-FA1	SD	147	226	315	259	350	360	302	325	435	264
2	Number	6	6	6	6	6	6	6	6	6	6
5X DHA	Mean	339	654	717	832	939	967	1060	1159	1206	1142
P00-MA1	SD	105	136	144	209	230	231	240	292	299	330
3	Number	6	6	6	6	6	6	6	6	6	6
3X AA + 3X DHA	Mean	280	534	657	759	847	957	1035	1157	1161	1076
P00-RT1	SD	109	157	203	237	253	345	258	323	375	193
4	Number	6	6	6	6	6	6	6	6	6	6
5X AA	Mean	378	573	682	760	852	985	966	1163	1117	1067
P00-TR1	SD	108	98	147	115	185	211	282	232	346	398

Significantly different from the Control: * P<0.05, **P<0.01, ***P<0.001 (ANOVA + Dunnett's test)

000150

TABLE VI-25 (continued)
Food Consumption (g/animal/day)
Group Mean Values: Females

Group/Treatment		Treatment Period (Days)								
		11	12	13	14	15	16	17	18	19
1	Number	6	6	6	6	6	6	6	6	6
Control	Mean	1349	1448	1495	1638	1854	1840	1825	1668	1216
P00-FA1	SD	207	157	155	214	229	182	343	485	329
2	Number	6	6	6	6	6	6	6	6	6
5X DHA	Mean	1190	1269	1277	1316	1339*	1484	1637	1698	1113
P00-MA1	SD	255	352	311	372	361	505	460	531	304
3	Number	6	6	6	6	6	6	6	6	6
3X AA + 3X DHA	Mean	987*	1121	1145	1224	1386	1442	1512	1566	1022
P00-RT1	SD	138	274	374	294	324	444	497	438	315
4	Number	6	6	6	6	6	6	6	6	6
5X AA	Mean	1205	1224	1286	1326	1526	1560	1559	1602	1091
P00-TR1	SD	330	338	203	380	373	379	439	407	224

Significantly different from the Control: * P<0.05, **P<0.01, ***P<0.001 (ANOVA + Dunnett's test)

0001551

TABLE VI-26
Daily Caloric Intake per kg Body Weight (kcal/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	119.1	226.7	277.5	265.7	291.4	303.3	306.5	296.7	178.8	187.3
2 5X DHA P00-MA1	123.2	261.6	250.6	285.1	301.9	302.0	294.8	302.2	214.8	279.2
3 3X AA + 3X DHA P00-RT1	146.3	228.5	271.2	284.7	296.7	287.3	286.8	298.2	264.6	201.8
4 5X AA P00-TR1	141.5	251.2	302.1	311.1	312.7	292.0	304.5	298.9	276.2	290.2

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001152

TABLE VI-26 (continued)
Daily Caloric Intake per kg Body Weight (kcal/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	226.0	242.1	260.9	247.3	239.1	192.7	215.8	145.2	239.8
2 5X DHA P00-MA1	NA	195.6	233.7	201.6	196.9	206.6	217.4	212.7	133.1	240.0
3 3X AA + 3X DHA P00-RT1	NA	241.9	222.7	203.8	213.9	218.0	204.8	207.2	122.4	239.9
4 5X AA P00-TR1	NA	243.2	236.0	220.4	212.5	227.5	208.9	217.8	155.1	255.7

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

000453

TABLE VI-27
Daily Caloric Intake per kg Body Weight (kcal/kg/day)
Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	139.5	264.1	263.3	289.4	288.5	301.4	297.2	319.3	216.1	237.6
2 5X DHA P00-MA1	162.7	285.0	290.7	309.7	302.9	297.3	297.4	294.2	267.1	243.7
3 3X AA + 3X DHA P00-RT1	140.3	258.6	288.5	307.6	317.9	319.8	317.4	320.4	270.6	246.3
4 5X AA P00-TR1	203.5	271.9	296.3	301.9	309.8	317.3	281.3	316.6	242.7	263.2

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001154

TABLE VI-27 (continued)
Daily Caloric Intake per kg Body Weight (kcal/kg/day)
Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	271.1	243.1	247.9	260.0	258.2	222.8	195.3	133.9	253.8
2 5X DHA P00-MA1	NA	236.6	230.0	220.6	212.6	219.8	227.4	220.3	138.9	254.0
3 3X AA + 3X DHA P00-RT1	NA	208.9	216.4	224.4	234.6	229.5	222.7	217.4	139.9	255.4
4 5X AA P00-TR1	NA	200.1	239.3	228.0	244.5	235.3	219.8	216.3	131.0	258.1

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001155

TABLE VI-28
Daily AA-Rich Oil Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	0	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	0	0	0	0	0	0	0	0	0	0
3 3X AA + 3X DHA P00-RT1	222.5	347.5	412.4	432.9	451.2	436.9	436.1	453.5	402.4	306.8
4 5X AA P00-TR1	341.8	606.7	729.8	751.4	755.4	705.4	735.6	721.9	667.3	700.9

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

000456

TABLE VI-28 (continued)
Daily AA-Rich Oil Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	NA	0	0	0	0	0	0	0	0	0
3 3X AA + 3X DHA P00-RT1	NA	367.9	338.7	310.0	325.3	331.6	311.5	315.1	186.1	364.8
4 5X AA P00-TR1	NA	587.5	570.1	532.3	513.3	549.6	504.6	526.1	374.7	617.6

No statistically significant effects(ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to staggering of the study
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001157

TABLE VI-29
Daily AA-Rich Oil Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	0	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	0	0	0	0	0	0	0	0	0	0
3 3X AA + 3X DHA P00-RT1	213.3	393.3	438.7	467.7	483.5	486.3	482.6	487.3	411.5	374.5
4 5X AA P00-TR1	491.6	656.9	715.7	729.3	748.3	766.4	679.4	764.7	586.2	635.7

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001458

TABLE VI-29 (continued)
Daily AA-Rich Oil Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	NA	0	0	0	0	0	0	0	0	0
3 3X AA + 3X DHA P00-RT1	NA	317.6	329.0	341.2	356.7	349.0	338.6	330.7	212.7	388.3
4 5X AA P00-TR1	NA	483.2	578.0	550.7	590.6	568.5	531.0	522.4	316.4	623.4

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001159

TABLE VI-30
Daily DHA-Rich Oil Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	0	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	335.0	711.2	681.3	775.3	821.0	821.2	801.5	821.7	584.2	759.1
3 3X AA + 3X DHA P00-RT1	235.9	368.4	437.3	459.0	478.4	463.2	462.4	480.9	426.7	325.3
4 5X AA P00-TR1	0	0	0	0	0	0	0	0	0	0

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001460

TABLE VI-30 (continued)
Daily DHA-Rich Oil Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	NA	532.0	635.4	548.3	535.4	561.8	591.0	578.3	362.0	652.6
3 3X AA + 3X DHA P00-RT1	NA	390.0	359.1	328.7	344.9	351.5	330.3	334.1	197.3	386.8
4 5X AA P00-TR1	NA	0	0	0	0	0	0	0	0	0

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

000161

TABLE VI-31
Daily DHA-Rich Oil Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	0	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	442.4	775.0	790.4	842.0	823.5	808.3	808.6	800.1	726.2	662.7
3 3X AA + 3X DHA P00-RT1	226.2	417.0	465.1	495.9	512.6	515.6	511.7	516.6	436.3	397.1
4 5X AA P00-TR1	0	0	0	0	0	0	0	0	0	0

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001462

TABLE VI-31 (continued)
Daily DHA-Rich Oil Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	NA	643.4	625.4	600.0	578.0	597.6	618.2	598.9	377.8	690.6
3 3X AA + 3X DHA P00-RT1	NA	336.7	348.8	361.7	378.2	370.0	359.0	350.6	225.5	411.7
4 5X AA P00-TR1	NA	0	0	0	0	0	0	0	0	0

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001163

TABLE VI-32
Daily AA Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	0	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	0	0	0	0	0	0	0	0	0	0
3 3X AA + 3X DHA P00-RT1	90.8	141.7	168.3	176.6	184.0	178.2	177.9	185.0	164.2	125.2
4 5X AA P00-TR1	136.8	242.8	292.1	300.7	302.4	282.3	294.4	288.9	267.1	280.5

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001164

TABLE VI-32 (continued)
Daily AA Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	NA	0	0	0	0	0	0	0	0	0
3 3X AA + 3X DHA P00-RT1	NA	150.1	138.2	126.5	132.7	135.3	127.1	128.6	75.9	148.8
4 5X AA P00-TR1	NA	235.2	228.2	213.1	205.5	220.0	202.0	210.6	150.0	247.2

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

000465

TABLE VI-33
Daily AA Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	0	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	0	0	0	0	0	0	0	0	0	0
3 3X AA + 3X DHA P00-RT1	87.0	160.4	179.0	190.8	197.2	198.4	196.9	198.8	167.9	152.8
4 5X AA P00-TR1	196.8	262.9	286.5	291.9	299.5	306.8	271.9	306.1	234.6	254.4

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

000166

TABLE VI-33 (continued)
Daily AA Intake per kg Body Weight (mg/kg/day)
(Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	NA	0	0	0	0	0	0	0	0	0
3 3X AA + 3X DHA P00-RT1	NA	129.6	134.2	139.2	145.5	142.4	138.1	134.9	86.8	158.4
4 5X AA P00-TR1	NA	193.4	231.3	220.4	236.4	227.5	212.5	209.1	126.6	249.5

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001167

TABLE VI-34
Daily DHA Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	0	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	67.7	143.8	137.8	156.8	166.0	166.1	162.1	166.2	118.1	153.5
3 3X AA + 3X DHA P00-RT1	49.7	77.5	92.0	96.6	100.7	97.5	97.3	101.2	89.8	68.5
4 5X AA P00-TR1	0	0	0	0	0	0	0	0	0	0

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001168

TABLE VI-34 (continued)
Daily DHA Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Males

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	NA	107.6	128.5	110.9	108.3	113.6	119.5	116.9	73.2	132.0
3 3X AA + 3X DHA P00-RT1	NA	82.1	75.6	69.2	72.6	74.0	69.5	70.3	41.5	81.4
4 5X AA P00-TR1	NA	0	0	0	0	0	0	0	0	0

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

000169

TABLE VI-35
Daily DHA Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									
	1	2	3	4	5	6	7	8	9[1]	10[1]
1 Control POO-FA1	0	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	89.5	156.7	159.8	170.3	166.5	163.5	163.5	161.8	146.9	134.0
3 3X AA +3X DHA P00-RT1	47.6	87.8	97.9	104.4	107.9	108.5	107.7	108.8	91.8	83.6
4 5X AA P00-TR1	0	0	0	0	0	0	0	0	0	0

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

0001720

TABLE VI-35 (continued)
Daily DHA Intake per kg Body Weight (mg/kg/day)
Group Mean Values: Females

Group/Treatment	Treatment Period (Days)									Mean[2]
	11	12[1]	13	14	15	16	17	18	19[1]	
1 Control POO-FA1	NA	0	0	0	0	0	0	0	0	0
2 5X DHA P00-MA1	NA	130.1	126.5	121.3	116.9	120.8	125.0	121.1	76.4	139.7
3 3X AA + 3X DHA P00-RT1	NA	70.9	73.4	76.1	79.6	77.9	75.6	73.8	47.5	86.7
4 5X AA P00-TR1	NA	0	0	0	0	0	0	0	0	0

No statistically significant effects (ANOVA + Dunnett's test)
[1] Mean value based on data from part of each group due to change in feeding interval frequency
[2] Overall mean excludes Day 19 when animals were fasted overnight

000171

TABLE VI-36
Clinical Chemistry: Day 20
Group Mean Values: [a] Males

Group/ Treatment		Urea	Glu	AST	ALT	AP	LDH	Na	K	Cl	TP	Alb	Glob	AG-R	Chol	Crea	T.Bi	Trig	GGT	LDL
1	Number	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Control	Mean	3.8	5.99	74	23	1304	1056	144	4.4	100	49	33	16	2.23	2.3	76	11.8	0.59	42	0.84
P00-FA1	SD	0.9	1.39	64	5	430	190	2	0.9	2	5	4	5	0.6	0.3	3	4.6	0.08	7	0.26
2	Number	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
5X DHA	Mean	3.4	6.49	119	25	1280	1061	145	4.3	101	48	33	14	2.4	2.1	73	7.5	0.40*	42	0.73
P00-MA1	SD	1.0	1.02	159	5	216	148	1	0.6	2	2	3	2	0.4	0.2	9	3.4	0.14	20	0.17
3	Number	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
3X AA +	Mean	3.2	6.10	61	25	1390	1025	144	4.4	100	49	31	18	1.8	2.3	77	7.5	0.47	46	0.80
3X DHA	SD	0.5	0.61	31	4	281	182	0	0.6	1	3	2	3	0.3	0.3	5	2.6	0.13	7	0.19
P00-RT1																				
4	Number	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
5X AA	Mean	3.5	6.96	39	21	1229	810	143	4.3	98	48	31	17	2.0	2.3	71	4.8**	0.36**	43	0.88
P00-TR1	SD	1.1	0.71	12	2	307	171	1	0.2	2	3	3	4	0.5	0.2	3	1.0	0.05	27	0.21

[a] abbreviations and units are defined in the Materials and Methods
Significantly different from the Control: * P<0.05, **P<0.01, ***P<0.001 (ANOVA + Dunnett's test)

0001172

TABLE VI-37
Clinical Chemistry: Day 20
Group Mean Values [a] : Females

Group/ Treatment		Urea	Glu	AST	ALT	AP	LDH	Na	K	Cl	TP	Alb	Glob	AG-R	Chol	Crea	T.Bi	Trig	GGT	LDL
1	Number	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Control	Mean SD	4.7	6.52	118	24	1126	1163	144	4.8	100	54	36	18	2.1	2.6	81	12.1	0.71	39	1.01
P00-FA1		1.1	0.69	181	10	286	450	2	1.9	2	4	4	3	0.6	0.3	4	6.0	0.16	18	0.21
2	Number	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
5X DHA	Mean	5.1	6.04	87	27	1396	983	145	4.1	100	51	37	15*	2.6	2.4	79	8.9	0.57.	35	0.79
P00-MA1	SD	1.9	0.81	72	4	381	178	1	0.4	2	4	4	2	0.4	0.4	7	1.9	25	12	0.42
3	Number	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
3X AA+3X DHA	Mean	4.7	6.01	51	25	1424	867	143	4.2	100	51	36	15	2.4	2.6	76	9.5	0.64	35	1.01
P00-RT1	SD	1.3	0 74	48	3	486	112	2	0.4	2	2	3	2	0.5	0.4	6	4.1	0.15	7	0.36
4	Number	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
5X AA	Mean	4.2	6.50	43	21	1223	867	144	4.3	100	53	35	18	2.0	2.8	77	8.8	0.62	40	1.01
P00-TR1	SD	1.4	1.10	31	3	326	154	1	0.4	1	4	3	2	0.3	0.2	7	3.8	0.22	14	0.11

[a] abbreviations and units are defined in the Materials and Methods
Significantly different from the Control: * P<0.05, **P<0.01, ***P<0.001 (ANOVA + Dunnett's test)

0001173

TABLE VI-38
Hematology: Day 20
Group Mean Values [a] : Males

Group/Treatment		Hb	RBC	Hct	MCH	MCV	MCHC	WBC	Plat	PT	APTT
1	Number	5	5	5	5	5	5	5	5	4	4
Control	Mean	10.9	5.26	0.322	20.8	61.1	34.0	6.23	529	10	16
P00-FA1	SD	1.1	0.54	0.034	0.8	1.2	0.8	0.89	109	1	2
2	Number	6	6	6	6	6	6	6	6	6	6
5X DHA	Mean	11.2	5.48	0.329	20.5	60.5	33.9	6.20	425	10	15
P00-MA1	SD	0.6	0.57	0.018	1.5	4.5	0.5	1.18	215	1	3
3	Number	6	6	6	6	6	6	6	6	6	6
3X AA + 3X DHA	Mean	11.5	5.66	0.335	20.3	59.3	34.2	8.16	598	10	15
P00-RT1	SD	0.7	0.38	0.020	0.8	2.9	0.4	2.23	169	1	2
4	Number	6	6	6	6	6	6	6	6	5	5
5X AA	Mean	10.8	5.38	0.316	20.2	59.0	34.2	7.94	522	9	15
P00-TR1	SD	0.7	0.45	0.020	1.1	2.9	0.3	3.83	61	0	1

[a] abbreviations and units are defined in the Materials and Methods
No statistically significant effects (ANOVA + Dunnett's test)

000174

TABLE VI-39
Hematology: Day 20
Group Mean Values [a]: Females

Group/Treatment		Hb	RBC	Hct	MCH	MCV	MCHC	WBC	Plat	PT	APTT
1	Number	6	6	6	6	6	6	6	6	5	5
Control	Mean	11.9	5.58	0.349	21.3	62.6	34.0	8.13	517	9	17
P00-FA1	SD	.1.2	0.58	0.034	0.8	2.8	0.7	2.59	185	2	3
2	Number	6	6	6	6	6	6	6	6	6	6
5X DHA	Mean	11.8	5.50	0.349	21.5	63.6	33.9	5.41	529	10	15
P00-MA1	SD	0.9	0.48	0.028	0.8	2.6	0.5	1.55	69	0	2
3	Number	6	6	6	6	6	6	6	6	6	6
3X AA + 3X DHA	Mean	11.5	5.46	0.337	21.0	61.8	34.1	5.72	479	10	16
P00-RT1	SD	0.5	0.30	0.016	1.3	3.8	0.4	3.78	242	1	5
4	Number	6	6	6	6	6	6	6	6	6	6
5X AA	Mean	12.0	5.72	0.354	21.1	62.0	34.0	5.90	566	10	17
P00-TR1	SD	0.6	0.16	0.021	1.0	3.4	0.6	1.37	109	0	3

[a] abbreviations and units are defined in the Materials and methods
No statistically significant effects (ANOVA + Dunnett's test)

0001175

TABLE VI-40
Absolute Organ Weights (g)
Group Mean Values: Males

Group/Treatment		Body Weight (kg)	Adrenal Glands	Brain	Kidneys	Liver	Spleen	Testes	Thymus	Thyroid
1	Number	6	6	6	6	6	6	6	6	5
Control	Mean	7.4	1.018	50.58	55.19	227.50	22.85	10.81	32.56	0.652
P00-FA1	SD	2.0	0.127	3.63	15.71	56.09	4.84	2.19	10.81	0.197
2	Number	6	6	6	6	6	6	6	6	6
5X DHA	Mean	7.5	0.846	52.08	54.15	248.55	32.63*	10.53	31.86	0.795
P00-MA1	SD	1.3	0.092	3.21	8.48	39.84	11.16	1.90	11.10	0.170
3	Number	6	6	6	6	6	6	6	6	6
3X AA + 3X DHA	Mean	7.5	0.878	51.67	50.84	241.00	27.05	11.79	28.00	0.734
P00-RT1	SD	1.2	0.148	4.35	8.32	44.78	7.01	2.83	7.10	0.127
4	Number	6	6	6	6	6	6	6	6	6
5X AA	Mean	7.8	0.872	51.04	57.70	252.78	35.93•	10.07	33.50	0.846
P00-TR1	SD	1.0	0.232	5.07	10.27	24.83	2.88	2.45	7.25	0.136

Significantly different from the Control: * P<0.05, ** P<0.01, *** P<0.001 (ANOVA + Dunnett's test)

000176

TABLE VI-41
Absolute Organ Weights (g)
Group Mean Values: Females

Group/Treatment		Body Weight (kg)	Adrenal Glands	Brain	Kidneys	Liver	Spleen	Thymus	Thyroid
1	Number	6	6	6	6	6	6	6	6
Control	Mean	8.0	0.773	53.45	63.38	260.40	24.80	34.76	0.668
P00-FA1	SD	1.3	0.108	4.21	8.36	59.29	4.53	9.91	0.154
2	Number	6	6	6	6	6	6	6	6
5X DHA	Mean	7.4	0.856	49.45	54.74	239.61	25.96	33.48	0.676
P00-MA1	SD	1.5	0.142	2.70	14.93	59.13	6.11	6.97	0.105
3	Number	6	6	6	6	6	6	6	6
3X AA +3X DHA	Mean	6.8	0.797	50.62	50.11	221.42	24.12	26.09	0.716
P00-RT1	SD	1.3	0.136	3.57	11.37	48.97	4.54	8.73	0.102
4	Number	6	6	6	6	6	6	6	6
5X AA	Mean	7.2	0.880	50.31	50.91	240.16	29.19	39.01	0.720
P00-TR1	SD	1.1	0.098	4.46	9.14	37.04	12.00	7.50	0.106

No statistically significant effects (ANOVA + Dunnett's test)

000177

TABLE VI-42
Relative Organ Weights (% Body Weight)
Group Mean Values: Males

Group/Treatment		Body Weight (kg)	Adrenal Glands	Brain	Kidneys	Liver	Spleen	Testes	Thymus	Thyroid
1	Number	6	6	6	6	6	6	6	6	5
Control	Mean	7.4	0.0145	0.719	0.750	3.111	0.331	0.153	0.439	0.0096
P00-FA1	SD	2.0	0.0035	0.144	0.033	0.237	0.123	0.041	0.071	0.0020
2	Number	6	6	6	6	6	6	6	6	6
5X DHA	Mean	7.5	0.0116	0.714	0.733	3.372	0.429	0.143	0.425	0.0107
P00-MA1	SD	1.3	0.0018	0.111	0.082	0.525	0.109	0.023	0.122	0.0013
3	Number	6	6	6	6	6	6	6	6	6
3X AA + 3X DHA	Mean	7.5	0.0119	0.701	0.682	3.218	0.363	0.159	0.375	0.0099
P00-RT1	SD	1.2	0.0024	0.078	0.054	0.198	0.073	0.038	0.073	0.0016
4	Number	6	6	6	6	6	6	6	6	6
5X AA	Mean	7.8	0.0114	0.660	0.738	3.262	0.467	0.129	0.430	0.0108
P00-TR1	SD	1.0	0.0037	0.065	0.046	0.223	0.061	0.023	0.067	0.0008

No statistically significant effects (ANOVA + Dunnett's test)

000178

TABLE VI-43
Relative Organ Weights (% Body Weight)
Group Mean Values: Females

Group/Treatment		Body Weight (kg)	Adrenal Glands	Brain	Kidneys	Liver	Spleen	Thymus	Thyroid
1	Number	6	6	6	6	6	6	6	6
Control	Mean	8.0	0.0099	0.682	0.797	3.268	0.313	0.431	0.0083
P00-FA1	SD	1.3	0.0023	0.120	0.051	0.604	0.048	0.081	0.0014
2	Number	6	6	6	6	6	6	6	6
5X DHA	Mean	7.4	0.0118	0.693	0.737	3.242	0.357	0.459	0.0095
P00-MA1	SD	1.5	0.0012	0.125	0.070	0.234	0.065	0.063	0.0021
3	Number	6	6	6	6	6	6	6	6
3X AA + 3X DHA	Mean	6.8	0.0120	0.765	0.735	3.253	0.359	0.384	0.0107*
P00-RT1	SD	1.3	0.0025	0.126	0.046	0.173	0.051	0.119	0.0014
4	Number	6	6	6	6	6	6	6	6
5X AA	Mean	7.2	0.0123	0.706	0.704*	3.334	0.399	0.539	0.0100
P00-TR1	SD	1.1	0.0010	0.081	0.050	0.176	0.130	0.045	0.0007

Significantly different from the Control: * P<0.05, ** P<0.01, *** P<0.001 (ANOVA + Dunnett's test)

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Table VI-44 Summary of Histological Findings

HISTOLOGICAL FINDINGS	GROUP/TREATMENT TOTALS							
	MALES				FEMALES			
	1 Control	2 5X DHA	3 3X AA + 3X DHA	4 5X AA	1 Control	2 5X DHA	3 3X AA + 3X DHA	4 5X AA
RESPIRATORY SYSTEM								
Trachea					(1)			
No abnormality detected					1			
Lung	(2)		(2)	(1)	(2)	(1)		
No abnormality detected	1		0	0	1	0		
Alveolar macrophage accumulation, diffuse	0		1	0	1	0		
Agonal congestion/hemorrhage	1		0	1	1	0		
Inflammation, interstitial	1		2	1	1	1		
HAEMOPOIETIC SYSTEM								
Lymph Node (Bronchial)	(1)	(1)	(3)				(3)	
Erythrophagocytosis	1	1	3				3	
Lymph Node (Mesenteric)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	5	6	6	6	6	6	6	6
Pigment deposits	1	0	0	0	0	0	0	0
Lymph Node (Mediastinal)	(1)					(1)		
Inflammatory cell infiltration	1					0		
Erythrophagocytosis	0					1		
Lymph Node (Inguinal)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	
Pigment deposits	1	1	1	1	1	1	1	
Lymph Node (Lumbar)	(1)	(1)	(2)	(1)	(1)	(1)	(1)	(2)
Pigment deposits	1	1	2	1	1	0	1	2
Erythrophagocytosis	0	0	0	0	0	1	0	0
Lymph Node (Popliteal)			(1)		(1)			
Pigment deposits			1		1			
Inflammatory cell infiltration			1		0			
Lymph Node						(1)		
Erythrophagocytosis						1		
Spleen	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	6	6	6	6	6	6	6	6
Thymus	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	6	6	5	6	6	6	6	6
Remnant, cystic	0	0	1	0	0	0	0	0
CARDIOVASCULAR SYSTEM								
Heart	(2)	(1)					(1)	
No abnormality detected	1	0					0	
Valvular haematocyst	0	0					1	
Agonal congestion/hemorrhage	1	0					0	
Pericarditis, purulent	1	0					0	
Hemorrhage, pericardial, localized	0	1					0	
ENDOCRINE SYSTEM								
Thyroid Gland	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	6	5	6	6	6	6	6	6
Follicular distension	0	1	0	0	0	0	0	0
Parathyroid Gland	(1)							
No abnormality detected	1							

Figures in brackets represent the number of animals from which this tissue was examined microscopically.
The absence of a numeral indicates that the lesion specified was not identified.

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Table 44 Summary of Histological Findings

HISTOLOGICAL FINDINGS	GROUP/TREATMENT TOTALS							
	MALES				FEMALES			
	1 Control	2 5X DHA	3 3X AA + 3X DHA	4 5X AA	1 Control	2 5X DHA	3 3X AA + 3X DHA	4 5X AA
Adrenal Gland	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	6	6	5	6	6	6	6	6
Focus, pale cell, cortex	0	0	1	0	0	0	0	0
GENITAL SYSTEM								
Testis	(6)	(6)	(6)	(6)				
No abnormality detected	5	6	6	5				
Immature	6	6	6	6				
Inflammation, acute	1	0	0	1				
URINARY SYSTEM								
Kidney	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	5	2	2	3	5	2	3	3
Tubular regeneration, focal	0	0	0	1	0	0	0	1
Interstitial nephritis	1	4	3	2	1	4	3	2
Capsular fibrosis, focal	0	0	1	0	0	0	0	0
Agonal congestion/hemorrhage	0	0	0	2	0	0	0	0
Urinary Bladder			(2)					
No abnormality detected			2					
Agonal congestion/hemorrhage			1					
ALIMENTARY SYSTEM								
Stomach		(1)						(1)
No abnormality detected		1						0
Gastritis, lymphocytic, diffuse		0						1
Ileum	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	6	6	6	6	6	6	6	6
Rectum			(1)			(1)		
No abnormality detected			0			1		
Proctitis			1			0		
Intestines			(1)		(1)			
No abnormality detected			1		0			
Agonal congestion/hemorrhage			1		1			
Liver	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	1	2	4	2	3	4	2	3
Eosinophilic cell focus	0	0	0	0	0	1	0	0
Capsular fibrosis	2	0	0	1	0	0	0	1
Agonal congestion/hemorrhage	0	0	0	0	1	0	1	0
Glycogen vaculation	1	1	1	1	0	0	1	1
Inflammation, capsular, acute	1	0	0	1	0	0	0	0
Inflammation, focal/multifocal	3	3	1	2	3	1	4	1
Cyst, capsular	0	0	0	1	0	0	0	0
Gall Bladder	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	6	6	6	6	6	6	6	6
INTEGUMENTARY SYSTEM								
Skin and Subcutis		(2)	(2)				(1)	(1)
No abnormality detected		0	0				1	0
Dermatitis		2	2				0	1
NERVOUS SYSTEM								
Brain	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No abnormality detected	6	6	6	6	6	6	6	6
MUSCULO SKELETAL SYSTEM								
Foot/Leg			(1)					
Cellulitis, acute			1					
TISSUES NOT INCLUDED WITHIN BODY SYSTEMS								

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Table 44 Summary of Histological Findings								
HISTOLOGICAL FINDINGS	GROUP/TREATMENT TOTALS							
	MALES				FEMALES			
	1 Control	2 5X DHA	3 3X AA + 3X DHA	4 5X AA	1 Control	2 5X DHA	3 3X AA + 3X DHA	4 5X AA
Tail					(1)			
Inflammation, ulcerative					1			

Figures in parenthesis represent the number of animals from which this tissue was examined microscopically because of planned inspection or a question of a variation from the expected apearance.
The absence of a numeral indicates that histopathologic analysis was not done for that organ.

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E. Additional Neonatal Animal Studies of AA- and DHA-Containing Oils From Several Sources

In a study of neonatal piglets, groups of animals were fed formulas containing 0.8% AA (% total fatty acids; using SUNTGA25 as the source of AA) or 0.3% DHA (% total fatty acids; DHA-rich oil from tuna as the source of DHA) (de la Presa-Owens et al.1998). After 18 days, no significant differences were seen in absolute or relative liver weights in the AA-rich oil supplemented formula-fed group compared to control. A statistically significant reduction in relative liver weight, compared to control formula, was seen in the group fed DHA-rich oil supplemented formula. No significant differences were seen in the test groups when absolute or relative liver weight was compared to the sow's milk reference group.

Two additional studies in neonatal piglets given AA and DHA oils (DHASCO and ARASCO) have been reported in the literature (Huang 2000; Weiler 2000). Huang (2000) utilized feeding of up to 3.5% AA and 1.7% DHA in the formula for 29 days. Weiler used 0.5% AA + 0.1% DHA in the formula for 14 days. No adverse effects were reported on liver weights with administration of these long-chain polyunsaturated fatty acids, although data was not given. In another study, authors reported that in neonatal rats given DHASCO and ARASCO for 13 days (Ward 1998) there were no increases in liver weights (data not given). A 3 x 3 factorial design in this study fed AA at 0, 0.4, and 2.4% and DHA at 0, 0.4, and 2.4% of formula for 13 days.