DHASCO AND ARASCO OILS AS SOURCES OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS IN INFANT FORMULA

A Safety Assessment

TECHNICAL REPORT SERIES NO. 22

FOOD STANDARDS AUSTRALIA NEW ZEALAND
June 2003

ISBN 0 642 34532 5
ISSN 1448-3017
Published June 2003

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TABLE OF CONTENTS

SUMMARY

DHASCO and ARASCO are microbial oils rich in the long-chain polyunsaturated fatty acids (LCPUFAs) docosahexaenoic acid (DHA) and arachidonic acid (ARA), respectively. DHA and ARA are the major fatty acids present in the structural phospholipids of the human brain and retina and accumulate rapidly in foetal and infant neural tissue during the last months of gestation and the first months of postnatal life. Although both term and pre-term infants are capable of endogenous synthesis of DHA and ARA from precursor fatty acids, this capacity appears to be sub-optimal to meet the demands of the developing tissues in pre-term infants. The evidence indicates that pre-term infants in particular benefit from a dietary supply of pre-formed LCPUFAs. While breast-fed pre-term infants can obtain this dietary supply from breast milk, which naturally contains pre-formed DHA and ARA, for the formula-fed pre-term infant a dietary supply can only be obtained by supplementation of the formula. Hence, oils, such as DHASCO and ARASCO, which contain high levels of DHA and ARA, are being used to supplement a number of pre-term infant formula products and increasingly are also being used to supplement formula for term infants, although the evidence for benefit for this group is equivocal.

Intake and extent of use

DHASCO and ARASCO have been added to infant formula products in Australia and New Zealand since about 1998. In 2001, they were being added to about 17% of formulae intended for term infants up to 6 months of age, and about 87% of pre-term formulae[1]. The extracted oils are typically added to infant formula up to a maximum level of 1.25 % each of formula fat, which corresponds to a maximum level of 0.5% each of ARA and DHA. This level of supplementation equates to a maximum intake of about 70 – 85 mg each of DHASCO and ARASCO/kg bw/day.

Safety of the source organisms

DHASCO is extracted from the non-photosynthetic marine micro-algae *Crypthecodinium cohnii* and ARASCO is extracted from the common soil fungus *Mortierella alpina*. Neither *C. cohnii* nor *M. alpina* are known to be pathogenic to humans or other mammals and specific studies with the biomass from both organisms have confirmed the absence of any toxin production.

Composition of the oils

ARASCO and DHASCO are free flowing triglyceride oils with a fatty acid profile that is comparable to that of a number of other edible oils. No unusual fatty acids are present and there are no detectable (< 1.0%) cyclic or *trans* fatty acids present in either oil. The oils also contain no or only very low levels of eicosapentaenoic acid (EPA), which has been associated with reduced growth in infants. The sterol fraction of the oils constitutes about 9.5 mg/g dry weight of DHASCO and 7.9 mg/g dry weight of ARASCO (i.e., less than 1% by weight of the oil). The most common sterol in DHASCO is dinosterol, which is unique to algae and possesses an unusual chemical structure. In contrast, the sterols found in ARASCO are commonly found in plants and edible fungi, e.g., mushrooms.

[1] Figures from the Infant Formula Manufacturers' Association of Australia

The DHA and ARA-containing triacylglycerols in DHASCO and ARASCO are different to those found in breast milk. In breast milk, ARA and DHA are primarily esterified at the *sn*-2 and *sn*-3 positions, whereas in DHASCO and ARASCO they are esterified at all three positions of the triacylglycerol. Also, in contrast to breast milk, ARASCO and DHASCO contain significant amounts of triacylglycerol with two or more molecules of either DHA or ARA.

Absorption, distribution, metabolism and excretion

A number of studies, in both animals and humans, including human infants, have been conducted on the absorption, distribution, metabolism and excretion of the LCPUFAs from ARASCO and DHASCO. These studies indicate that the efficiency of intestinal absorption of ARA and DHA from ARASCO- and DHASCO-supplemented infant formula is similar to that from breast milk, this is despite some differences between breast milk and the microbial oils in positional specificities of the LCPUFAs in the triacylglycerol molecule. In the pre-term infant about 80% of ingested ARA and DHA (either from breast milk or DHASCO/ARASCO-supplemented formula) is absorbed. Efficient levels of absorption (i.e., >95%) are also seen in neonatal animal models, even at very high levels of dietary incorporation. Non-absorbed DHA and ARA are excreted via the faeces. Once absorbed, DHA and ARA are largely unavailable for oxidation, and are instead preferentially channelled into the phospholipid pool where they are rapidly incorporated into the cell membranes of the developing brain and retina. Studies with neonatal rats and pigs, as well as pre-term infants, indicate that the LCPUFAs in ARASCO and DHASCO are able to support maximal tissue accretion of ARA and DHA by the retina and other membrane phospholipids.

Toxicology studies

A number of toxicology studies have been conducted with ARASCO and DHASCO administered either singly or in combination. Acute dosing studies in rats with the oils using levels up to the maximum dose level attainable (20 g/kg body weight) yielded no adverse findings. Three short-term (4 week and 9 week) studies and three sub-chronic (13 week) studies in rats were evaluated, one of which included a full neurological and neurohistological assessment. In one of the sub-chronic studies some of the findings point to an impaired concentrating ability of the kidneys at the highest dose levels tested (4900 mg ARASCO/kg bw/day alone or in combination with 3650 mg DHASCO/kg bw/day), however, the vast majority of the treatment related findings were generally not accompanied with any associated histopathological, biochemical or haematological changes that would be indicative of toxicity at doses up to 2500 mg ARASCO/kg bw/day and 1250 mg DHASCO/kg bw/day. The most frequent changes observed (e.g. increased liver weights, decreased serum cholesterol and triglycerides) are entirely consistent with the physiological changes observed in response to the administration of high levels of LCPUFAs, irrespective of source, and are not a manifestation of toxicity specific to the administration of either ARASCO or DHASCO. A single developmental study, where ARASCO and DHASCO were administered to pregnant rats during organogenesis at dose levels up to 2500 mg ARASCO/kg bw/day and 1250 mg DHASCO/kg bw/day, likewise did not produce any treatment-related adverse developmental effects. The oils were also found to be negative in a number of bacterial and mammalian genotoxicity test systems at concentrations *in vitro* up to 5000 μg/ml, suggesting the oils are not genotoxic (both with and without metabolic activation).

Overall, there is no evidence of toxicity associated with the administration of ARASCO and DHASCO at dose levels up to 2500 mg and 1250 mg/kg bw/day, respectively. These dose levels are approximately 18 – 35 fold greater than the maximum levels being added to infant formula.

Human studies

A large number of clinical studies with pre-term and term infants have been undertaken with infant formula supplemented with DHASCO and ARASCO at levels producing ARA and DHA concentrations approximating those found in human milk. These were primarily undertaken for the purposes of establishing efficacy, however a number also examined how well the supplemented formulae were tolerated and whether its use was correlated with any adverse effects (e.g., reduced growth, changes in serological markers of spleen and liver function). These studies all indicate that formula supplemented with DHASCO and ARASCO is well tolerated by human infants and is not associated with any apparent adverse effects.

Conclusions

Neither source organism exhibits any signs of either pathogenicity or toxicity and the extracted oils do not demonstrate any consistent evidence for toxicity in animal studies or adverse effects in the studies with human infants conducted to date. This indicates there are no components of the extracted oils that raise any specific concerns and supports the conclusion that DHASCO and ARASCO are safe sources of LCPUFAs for supplementation of infant formula.

1. INTRODUCTION

DHASCO® (DHA-rich Single Cell Oil) and ARASCO® (ARA-rich Single Cell Oil) are microbial-derived triglyceride oils that are rich in the long-chain polyunsaturated fatty acids (LCPUFAs) known as docosahexaenoic acid (DHA) and arachidonic acid (ARA). The extracted oils, DHASCO® and ARASCO®, contain between 40 and 55 % DHA and ARA, respectively.

DHASCO is extracted from the algae *Crypthecodinium cohnii* and ARASCO is extracted from the fungus *Mortierella alpina*. Both oils are standardised with high oleic sunflower oil to contain 40 % by weight of DHA or ARA prior to being added to infant formula.

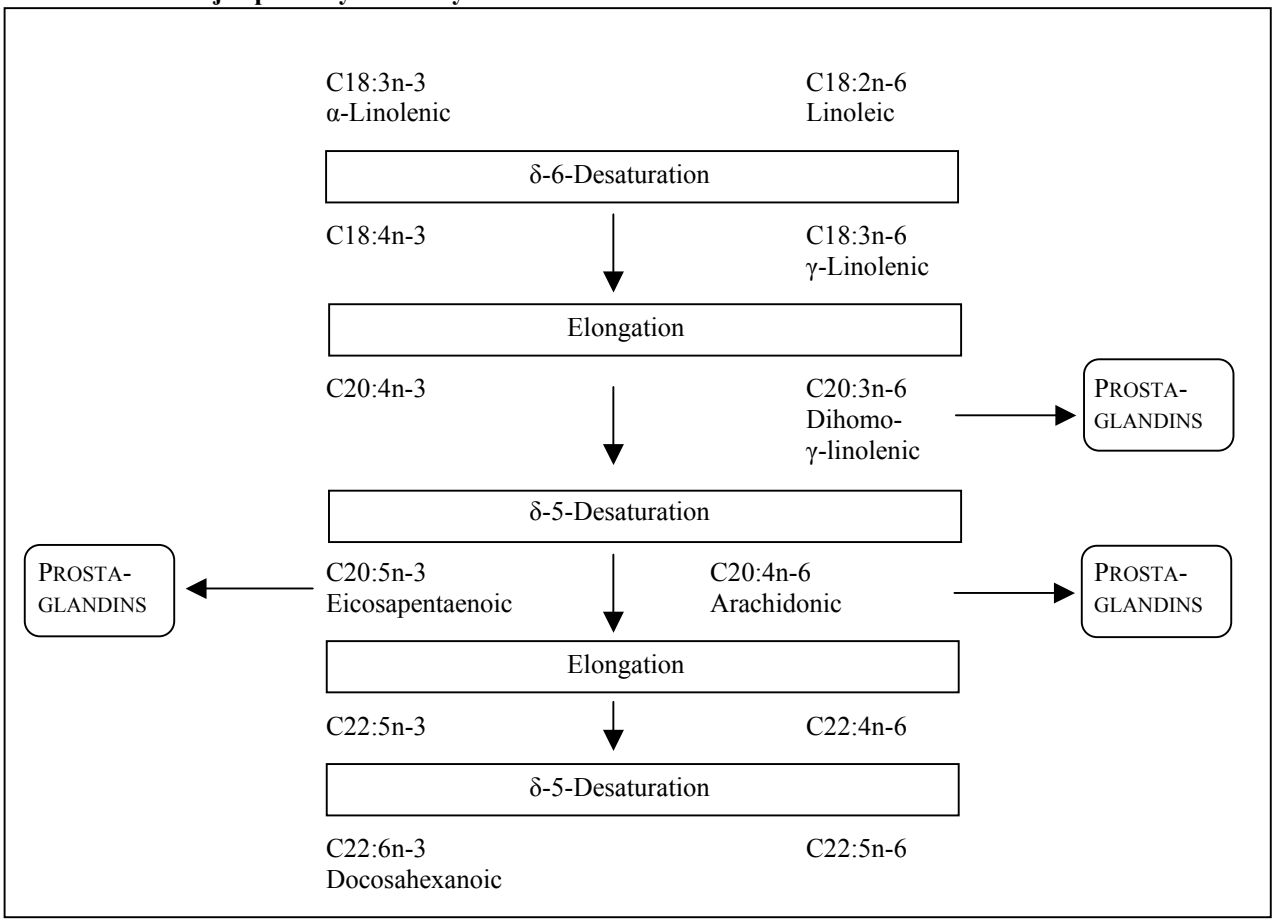
DHASCO and ARASCO have been added to infant formula products (both term and pre-term formulae) in Australia and New Zealand since about 1998 and in a number of other (primarily European) countries since about 1994. In 2001, they were being added to about 17% of formulae intended for term infants up to 6 months of age, and about 87% of pre-term formulae.

1.1 The role of LCPUFAs in early development

DHA (C22:6n-3) and ARA (C20:4n-6) are the predominant fatty acids in the structural phospholipids of the human brain and retina (Innis 1991, Martinez 1992) and accumulate rapidly in foetal and infant neural tissue during periods of most rapid growth and development, that is, during the last months of gestation and the first months of postnatal life (Martinez 1992, Makrides 1994).

Unlike term infants, pre-term infants cannot benefit from the placental LCPUFA supply during the last trimester of pregnancy. Instead, they are dependent on their own dietary supply through human milk, which contains small but significant quantities of DHA and ARA, as well as other LCPUFAs. Studies of breastfed pre-term infants have shown that the LCPUFA content in pre-term human milk provides adequate DHA and ARA to support normal neural tissue growth and development (Carlson *et al* 1986, Martinez 1992). For formula-fed pre-term infants, however, a large number of studies have shown that conventional formulae, even when it contains substantial amounts of linoleic and α-linolenic acid, which are the precursors for endogenous synthesis of ARA and DHA (see Figure 1), are unable to maintain postnatal DHA and ARA levels in plasma and erythrocyte lipids to levels observed after feeding human milk (Carlson *et al* 1986, Pita *et al* 1988, Koletzko *et al* 1989, Clandinin *et al* 1992). Although both term and pre-term infants are capable of endogenous synthesis of LCPUFAs from precursors (Salem *et al* 1996), this capacity appears to be sub-optimal and inadequate to maintain DHA and ARA at levels comparable to those found in breastfed infants (Carlson *et al* 1986, Koletzko *et al* 1989).

FIGURE 1. Major pathway for the synthesis of LCPUFAs from linoleic and α-linolenic acids.



It has been suggested that the higher tissue levels of DHA and ARA in breastfed infants is an important causative factor in the correlation between breastfeeding and better cognitive and visual function, particularly in the pre-term infant (Heird 2001). On the basis of these observations, and on the basis that breastfed infants are naturally supplied with pre-formed LCPUFAs in breast milk, it has been suggested that formula-fed pre-term infants could benefit from supplementation with LCPUFAs, particularly DHA and ARA. This had led to recommendations from various expert bodies, including the FAO/WHO (FAO 1994), for the inclusion of pre-formed LCPUFAs in infant formulae, for both term and pre-term infants. While a recently conducted study has demonstrated that pre-term infants fed a formula supplemented with ARA and DHA showed improved visual development (O'Connor *et al* 2001), the same was not seen in a similar study conducted with term infants (Auestad *et al* 2001). This suggests that term infants are better able to meet their DHA and ARA needs from essential fatty acids in their diet – either from breast milk, or from infant formula containing an appropriate fat blend providing linoleic and α-linolenic acid – the precursors of ARA and DHA, respectively.

2. SOURCE, PRODUCTION AND COMPOSITION

2.2 Sources of LCPUFAs for formula supplementation

In formulas for infants, LCPUFAs are added to the fat blend by using relatively highly unsaturated lipids. Three main sources are used: fish oil, which is mainly triacylglycerol (TAG); egg yolk lipid and phospholipids; or oils from algae and fungi (mainly TAG).

Fish oil contains large amounts of the omega-3 LCPUFAs but minimal amounts of omega-6 LCPUFAs, therefore, fish oil is typically used in combination with another LCPUFA source to supply the ARA. Some fish oils contain at least 1.5-fold as much eicosapentaenoic acid (EPA; 20:5n-3) as DHA and high EPA content has been associated with adverse effects on growth in infants (Carlson *et al* 1992, Carlson *et al* 1994, Montalto *et al* 1996). Fish oils with low EPA content are now available, although these have also been shown to have an adverse effect on the growth of pre-term infants (Carlson *et al* 1999), although a smaller effect than that observed with high-EPA fish oil occurred. It is speculated that supplementation with EPA (and/or DHA), results in feedback inhibition of the elongation and desaturation of the C18 essential fatty acids, leading to a decrease in ARA synthesis (Diersen-Schade *et al* 1999).

Egg yolk lipid contains large amounts of cholesterol. For this reason, egg phospholipids are preferred to egg yolk lipid (Heird 2001). Although egg phospholipids contain both ARA and DHA, the proportions of the two are not necessarily the same as the proportions found in human milk. These proportions can however be modified by altering the diet of the hens (Heird 2001).

The third source of LCPUFAs for addition to infant formula is single cell organisms, principally algae and fungi. TAG containing relatively high concentrations of DHA or ARA, but without any other LCPUFAs, such as EPA, can be produced from these organisms. For this reason, these oils are preferred for addition to infant formula.

2.3 Source organisms

2.3.1 Crypthecodinium cohnii

C. cohnii is a member of the Dinophyta (dinoflagellates). This is a distinct phylum of unicellular eukaryotic micro algae comprising an estimated 2000 species (van der Hoek *et al* 1995). Most species of the Dinophyta are photosynthetic; of which a small number are known to produce a group of closely related toxins (Steidinger and Baden 1987). There are also several heterotrophic species, of which *C. cohnii* is one. None of the heterotrophic species are known toxin producers or pathogenic to either humans or other mammalian species (van der Hoek 1995). *C. cohnii* has a long history of laboratory cultivation dating back to 1908 (Kyle 1996), but has not previously been used for human food.

The *C. cohnii* strain used for the production of DHASCO is proprietary to Martek Biosciences Corporation (US Patents 5,397,591, 5,407,957 and 5,492,938). The strain originated from the University of Texas culture collection and was selected for rapid growth and high levels of production of the specific oil. The specific strain of *C. cohnii* has been deposited with the American Type Culture Collection (ATCC # 40750) under the obligations

of the US patent relating to its use. Master seed stocks of the production strain are maintained under liquid nitrogen at the ATCC.

2.3.2 Mortierella alpina

M. alpina is a member of the Phycomycetes group of fungi, which are common inhabitants of soil. Although some fungal species have been reported to produce mycotoxins, the mycotoxin-producing fungi belong to the class of Basidiomycetes, which differ from the Phycomycetes group of fungi, to which *M. alpina* belongs (Jay 1992). A number of fungal species are also human pathogens, but the vast majority of these belong to the Deuteromycetes group of fungi (Davis *et al* 1980).

The *M. alpina* strain used for the production of ARASCO originates from the ATCC (ATCC # 32222) and was selected for rapid growth and high levels of production of the specific oil. Master seed stocks of this strain are maintained cryogenically at the ATCC.

2.4 Production of DHASCO and ARASCO

2.4.1 DHASCO

DHASCO is produced from *C. cohnii* using fermentation techniques. Cultures of the organism are grown up in liquid medium in shaker flasks and are transferred to progressively larger vessels. When the culture reaches a specified cell density and fatty acid content, the cells are harvested by centrifugation and spray dried. The process for extraction of the oil is basically the same as that used in conventional vegetable oil processing plants. The oil is extracted from the biomass by blending the biomass with hexane in a continuous extraction process. The extracted oil is separated from the de-oiled solids and the clarified miscella is desolventised under vacuum and winterised to remove the more highly saturated oil fractions. The winterised oil is then refined, bleached and deodorised using standard procedures. The deodorised DHASCO is then diluted to a standard 40% DHA concentration by the addition of high oleic sunflower oil and mixed with antioxidants – tocopherols (0.025%) and ascorbyl palmitate (0.025%). The DHA-rich oil produced is free-flowing liquid, which is orange in colour as a result of carotenes co-extracting with the oil.

2.4.2 ARASCO

One specific strain of *M. alpina* was selected to produce ARASCO because it produced oil that was not only rich in ARA, but which contains no EPA or other unusual components (Kyle 1997). *M. alpina* is a psychrotrophic, non-photosynthetic organism, which requires a reduced carbon source for growth. The fermentation process for the production of ARASCO-containing biomass starts with inoculation of liquid culture medium in a shaker flask with seed stock. The growing culture is transferred to successively larger vessels based on pre-defined criteria and when the culture reaches maximum productivity it is harvested by centrifugation and then dried. The dried biomass is then subject to oil extraction similar to that described for DHASCO. The deodorised ARASCO is then diluted to a standard 40% ARA concentration by the addition of high oleic sunflower oil and mixed with antioxidants – tocopherols (0.025%) and ascorbyl palmitate (0.025%). The ARA-rich oil that is produced is free-flowing liquid oil which is slightly yellow in colour.

2.5 Composition and triglyceride structure of DHASCO and ARASCO

ARASCO and DHASCO are free flowing oils, which are predominantly triglyceride (>95%) with some diglyceride and non-saponifiable material (<5%).

2.5.1 Triglyceride structure

In breast milk, ARA and DHA are mainly in TAG, although they also occur in phospholipids in breast milk (Jensen 1989). In breast milk TAG they are primarily esterified at the *sn*-2 and *sn*-3 positions (Breckenridge 1969, Innis 1992, Martin *et al* 1993), with the *sn*-1 position being relatively deficient in these acids (Martin *et al* 1993). The ARA and DHA, however, actually only make up a very small proportion of the total fatty acids found esterified into TAG. ARA makes up 0.4% of fatty acids at the *sn*-2 position and 0.37% at the *sn*-3 position, whereas DHA makes up 0.26% of fatty acids at the *sn*-2 position and 0.13% at the *sn*-3 position (Martin *et al* 1993). The predominant fatty acids found in the breast milk TAG are oleic acid (18:1) predominantly in the *sn*-1 and *sn*-3 positions, and palmitic acid (16:0) predominantly in the *sn*-2 position.

The DHA and ARA in DHASCO and ARASCO, respectively, do not display as clear a positional specificity, with the fatty acids being found in all three positions (Myher *et al* 1996). In ARASCO, about 50% of the ARA is found in the *sn*-1 position, 30% in the *sn*-2 position and 20% in the *sn*-3 position. In DHASCO, between 40 and 50% of DHA is found in the *sn*-2 position, with about 30% in the *sn*-3 position and between 20 to 30% in the *sn*-1 position. ARASCO and DHASCO also possess the unusual feature of containing significant amounts of TAG with two or more polyunsaturated long-chain fatty acids per molecule (Myher *et al* 1996).

2.5.2 Oil composition

The composition of both oils is given in Table 1. No unusual fatty acids are present and there are no detectable (< 1.0 %) cyclic or *trans* fatty acids. Minor fatty acid components of DHASCO, listed as “other” in Table 1 generally constitute about 1% of the total fatty acid composition. Small amounts of C28:8 (n-3) has been reported in DHASCO oil (VanPelt *et al* 1999). This fatty acid is the next expected omega-3 end product of the Sprecher biochemical pathway beyond DHA and is one of the minor components of both DHASCO, as well as fish oils (Luthria *et al* 1996).

2.5.3 Sterol composition

The 1.5% by weight nonsaponifiable fraction of DHASCO and ARASCO is made up primarily of sterols, which constitute 9.5 mg /g dry weight of DHASCO, and 7.9 mg/g dry weight ARASCO. The sterol fraction of both oils have been independently analysed and the results are summarised in Table 2.

The sterols of algae are of interest because they appear to be structurally different to those of higher plants (Patterson 1991). By far the most common sterol found in *C. cohnii* is the 4α-methyl sterol, dinosterol. The next most common sterol is the 4-demethyl sterol, dehydrocholesterol. Dinosterol is unique in that it has a saturated ring system and an unusual side chain alkylation pattern.

The principle component of the sterol fraction of ARASCO is desmosterol, with smaller amounts of two 24-methyl sterols. These sterols are commonly found in plants and fungi, including edible fungi such as mushrooms (Nes and Le 1990). In addition to these common sterols, *M. alpina* strain 1S-4 has been reported to contain the sterol 24,25-methylene cholesta-5-en-3β-ol, which has not been reported previously to exist in nature (Shimizu *et al* 1992). This novel sterol, however, could not be detected in the batches of ARASCO analysed for their sterol content (Table 2).

TABLE 1. Chemical composition of ARASCO and DHASCO

ARASCO		**DHASCO**	
Fatty acids	**% total**	**Fatty acids**	**% total**
Myristic acid (14:0)	0-2	Myristic acid (14:0)	10-20
Palmitic acid (16:0)	3-15	Palmitic acid (16:0)	10-20
Palmitoleic acid (16:1)	0-2	Palmitoleic acid (16:1)	0-2
Stearic acid (18:0)	5-20	Stearic acid (18:0)	0-2
Oleic acid (18:1)	5-38	Oleic acid (18:1)	10-30
Linoleic acid (18:2)	4-15	Linoleic acid (18:2)	0-5
Linolenic acid (18:3)	1-5	Arachidic acid (20:0)	0-1
Arachidic acid (20:0)	0-1	Behenic acid (22:0)	0-1
Eicosatrienoic acid (20:3)	1-5	Docosapentaenoic acid (22:5)	0-1
Arachidonic acid (20:4)	**38-44**	**Docosahexanoic acid (22:6)**	**40 -45**
Behenic acid (22:0)	0-3	Nervonic acid (24:1)	0-2
Docosapentaenoic acid (22:5)	0-3	Others	0-3
Lignoceric acid (24:0)	0-3		
Chemical analysis		**Chemical analysis**	
DPA	<0.1%	DPA	<0.1%
EPA	<0.1 – 0.16%	EPA	<0.1%
Free fatty acid	0.10 – 0.27%	Free fatty acid	0.14 – 0.22%
Peroxide value	0.12 – 1.51 meq/kg	Peroxide value	<0.1 – 0.24 meq/kg
Volatiles	<0.01 – 0.03%	Volatiles	<0.01%
Non-saponifiables	1.18 – 1.73%	Non-saponifiables	1.36 – 1.85%
Insoluables	<0.01%	Insoluables	<0.01%
Trans fats	<1.0%	Trans fats	<1.0%
Elemental analysis	**ppm**	**Elemental analysis**	**ppm**
Arsenic	<0.5	Arsenic	<0.5
Cadmium	<0.1	Cadmium	<0.1
Chromium	<0.1	Chromium	<0.1
Copper	<0.02	Copper	<0.02
Iron	<0.02	Iron	<0.02
Lead	<0.1	Lead	<0.1
Manganese	<0.01	Manganese	<0.01
Mercury	<0.04	Mercury	<0.04
Molybdenum	<0.05	Molybdenum	<0.05
Nickel	<0.1	Nickel	<0.1
Phosphorous	<1	Phosphorous	<1
Silicon	280 – 350	Silicon	18 – 135
Sulphur	3 – 6	Sulphur	18 – 80

TABLE 2. Sterols identified in DHASCO and ARASCO

Sterol fraction		Common name	% total sterols
DHASCO:			
4α,23,24-trimethyl cholesta-22-en-3β-ol	C30:1	dinosterol	31.5
Cholesta-5,7-dien-3β-ol	C27:2	dehydrocholesterol	9.6
4α,24-dimethyl cholestan-3β-ol	C29:0		9.2
4α,23,24-trimethyl cholesta-5,22-dien-3β-ol	C30:2	dehydrodinosterol	8.2
Cholesta-7-en-3β-ol	C27:1	lathosterol	7.5
4α,24-dimethyl cholesta-22-en-3β-ol	C29:1		6.4
4α,23,24-trimethyl cholesta-22-en-3β-ol	C30:1	dinosterone	6.0
4α,23,24-dimethyl cholesta-5-en-3β-ol	C29:1		4.6
4α,23,24-trimethyl cholesta-24(28)-ene-3β-ol	C30:1		4.2
Cholesta-*x*,*x*-dien-3β-ol*	C27:2		3.6
Cholesta-5,24-dien-3β-ol	C27:2	desmosterol	2.4
Cholesta-5-en-3β-ol	C27:1	cholesterol	1.7
23 or 24-methyl cholesta-5,7-dien-3β-ol	C28:2		1.9
	C27:3		1.3
a 5,7-dien sterol	C29:2		
ARASCO:			
Cholesta-5,24-dien-3β-ol	C27:2	desmosterol	67.3
24-methyl cholesta-5,24(25 or 28)-dien-3β-ol	C28:2		14.0
24-methyl cholesta-5,25-dien-3β-ol	C28:2		12.3
	C28:2		2.1
4α,4β,14-trimethyl-8,24-dien-3β-ol	C30:2	lanosterol	1.1
Cholesta-5,25-dien-3β-ol	C27:2		2.0
24,25-methylene cholesta-5-en-3β-ol	C28:1		Not detected

* The *x* refers to unassigned double bond placement

3. DIETARY INTAKE

DHASCO and ARASCO are currently being added to infant formula at levels that provide ARA and DHA levels up to 0.5% each of formula fat. These levels are therefore consistent with those specified in Draft Standard 2.9.1 Infant Formula which prescribes a maximum level of ARA and long chain omega-3 series fatty acids of 1.0% each of formula fat. Draft Standard 2.9.1 also specifies that when added to formulas the ratio of total long chain omega 6 series fatty acids (C≥20) to total long chain omega 3 series fatty acids (C≥20) should be approximately 2.

Assuming human infants consume about 420 – 500 kJ/kg bw/day (100 to 120 kcal/kg bw/day), of which fat comprises about 50 %, an infant will consume about 210 – 250 kJ/kg bw/day of fat, or about 5.6 – 6.7 g of fat/kg body weight/day (1 g fat = 37 kJ). As the ARA and DHA in the oils are standardised to a concentration of 40%, the amount of DHASCO and ARASCO being added to formula equates to a maximum of 1.25% each of total formula fat. This level of incorporation would therefore correspond to a DHASCO and ARASCO intake of 70 – 85 mg each of DHASCO and ARASCO/kg bw/day.

4. ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION

4.1 General overview

The general physiological processes for digestion/absorption, distribution, metabolism and excretion of lipids and fatty acids are well described in the general literature (e.g., Lehninger 1982, Eckert and Randall 1983).

4.1.1 Absorption

Most of the fat ingested by humans is in the form of TAG so in this respect DHASCO and ARASCO are no different to other types of dietary fat. The ingested TAG must be hydrolysed by lipases to fatty acids and monoacylglycerols before they can be absorbed by the small intestine. Digestion of TAG actually commences in the stomach, where the churning action helps to create an emulsion (FAO 1994) and also where a small amount of lipid hydrolysis occurs mediated by both lingual lipase (secreted by glands of the tongue) and gastric lipase. In the infant, the amount of gastric hydrolysis can be quite significant with as much as 30% of ingested TAG being digested during the one- to three-hour period that fat remains in the stomach (Watkins 1985).

The further emulsification and digestion of TAG in the small intestine is facilitated by bile salts, which are secreted into the upper portion of the small intestine (duodenum). Emulsification serves to stabilise the TAG molecule and to maximise the area of oil-water interface, where lipase activity occurs (Watkins 1985). Intestinal hydrolysis of TAG is mediated by pancreatic lipase which catalyses the hydrolysis of fatty acids at the *sn*-1 and *sn*-3 positions (i.e., the outer positions) to yield free fatty acids and 2-monoacylglycerols (Tso 1985).

After hydrolysis of the ingested TAG, those fatty acids containing less than 14 carbons enter into the circulation directly via the portal vein and from there are transported to the liver, whereas larger fatty acids are taken up into intestinal cells by passive diffusion where they are re-esterified into TAG and incorporated, along with small amounts of cholesterol and phospholipid, into chylomicrons. The chylomicrons are coated with a layer of lipoproteins, are loosely contained in vesicles formed by the Golgi apparatus, and are expelled from the cell by exocytosis into the interstitial fluid of the villus. From there they enter into the lymph through the thoracic duct and are delivered into the circulation via the subclavian vein. Once in the bloodstream, chylomicrons are acted upon by vascular lipoprotein lipase, which hydrolyses the TAG, releasing individual fatty acids, which are then available for distribution, in various forms, to particular tissues. The liver clears the remnants of chylomicrons within a few hours of the ingestion of a fat-containing meal.

In children and adults, fat digestion is efficient and is nearly completed in the small intestine. In the neonate, however, secretion of pancreatic lipase is low (Norman *et al* 1972) and its levels probably do not become adequate until 4 to 6 months of age (Watkins 1975). The digestion of fat in infants is thus augmented by lingual lipase, gastric lipase and a lipase present in human breast milk (FAO 1994). Human milk lipase is a non-specific lipase that is activated by bile salt conjugates (Watkins 1985). It is stable at pH 3.5 for one hour and so can resist passage through the stomach. The enzyme hydrolyses dispersed, water-insoluble substrates (TAG, lipovitamins, and cholesterol esters) as well as water-soluble substrates (short chain and medium chain monoglycerides). When human milk fat is used as the lipid source, it is estimated that human milk lipase concentrations are sufficient to hydrolyse 30 to 40% of available TAG in two hours. Alternative enzymatic mechanisms such as these serve to maximise lipid adsorption and to circumvent the relative immaturity or inefficiency of the pancreatic, intestinal, and hepatic system. One-week-old term infants have been determined to readily absorb more than 90% of the fat from human breast milk (Widdowson 1965).

In breast milk, about half of the ARA and DHA content of TAG are found at the *sn*-2 position; the other half is esterified to the *sn*-3 position (Martin *et al* 1993). Although the

presence of some LCPUFAs, including DHA and ARA, at outer positions of the TAG is reported to induce resistance to pancreatic lipase (Bottino *et al* 1967), the non-specific lipases, such as gastric lipase, lingual lipase and human milk lipase, appear able to circumvent this resistance. Therefore, after intestinal hydrolysis of human milk TAG by the neonate, a similar proportion of ARA and DHA are absorbed as 2-monoacylglycerol and as free fatty acid (Martin *et al* 1993).

The positional differences of LCPUFAs among TAG from different sources was studied by Carnielli *et al* (1998) to determine what affect this had on their absorption by pre-term infants. The dietary intakes, faecal output and percentages of intestinal absorption of n-6 and n-3 LCPUFAs were studied in healthy pre-term infants fed exclusively pre-term breast milk, formula without LCPUFA supplementation, formula with LCPUFAs derived from phospholipids, or formula with LCPUFAs derived from DHASCO and ARASCO. The study showed that in pre-term infants fed pre-term breast milk, LCPUFAs are not absorbed completely (about 80% of ARA and DHA is absorbed) and that LCPUFAs bound to phospholipids are better absorbed (88% DHA absorbed, 85% ARA absorbed) than LCPUFAs from ARASCO- and DHASCO-supplemented formula (about 80% of ARA and DHA absorbed), or breast milk. This indicates that intestinal absorption of ARA and DHA from ARASCO- and DHASCO-supplemented infant formula is similar to that from breast milk. This was considered an important finding because, unlike in breast milk TAG, DHA and ARA in DHASCO and ARASCO do not have a strong positional specificity (see Section 2.5). On the basis of previous work by Bottino *et al* (1967) on the resistance of certain LCPUFAs of fish oils to hydrolysis by pancreatic lipase, relatively low absorption might have been expected with the LCPUFAs from DHASCO and ARASCO. However, the results of the Carnielli study indicate that LCPUFAs from these sources are absorbed as efficiently as those from breast milk.

4.1.2 Distribution

Once fatty acids are absorbed they are distributed into various lipid pools, i.e., phospholipids, TAG, sterol esters and free fatty acids, all of which have important physiological roles. The pools into which they are distributed, and their relative proportions, depend very much on the individual fatty acid concerned.

For example, studies in rats with radio-labelled linoleic acid and α-linolenic acid have shown that 50 – 60% of the label can be recovered from expired CO_2 within 24 hours (Leyton *et al* 1987), indicating that the majority of the linoleic and α-linolenic acids are oxidised to provide energy to the cells. In contrast, only 15% of administered ARA and DHA are oxidised in rats, the rest being spared from oxidation and preferentially channelled into the structural lipids, i.e., the phospholipids (Sinclair 1975, Leyton *et al* 1987). This appears to be the case also with human infants where relatively small concentrations of dietary LCPUFA have marked effects on plasma lipid composition, particularly the phospholipid pool (Koletzko *et al* 1989). A dietary LCPUFA (ARA and DHA) supply of only 1.7% with human milk and 0.5% with LCPUFA-supplemented formula led to LCPUFA values in plasma phospholipids that were 8% and 3% higher, respectively, than those of the control formula (containing no detectable ARA or DHA), indicating preferential incorporation into the phospholipid pool.

Phospholipids are the most abundant membrane lipid, where they serve primarily as structural elements of membranes and, unlike TAG, are never stored to any great extent. Phospholipids make up about a quarter of the solid matter of the brain (Farquharson *et al*

1992) and ARA and DHA are by far the most abundant fatty acids present in brain cell membranes, with particularly high concentrations in the membranes of neuronal synapses and the retina (British Nutrition Foundation 1992). During the last trimester of pregnancy, the human foetal brain experiences a rapid growth spurt where it increases in size by four to five fold (Clandinin *et al* 1980). This rapid increase in size coincides with the rapid accumulation of DHA and ARA by neural tissue (Martinez 1992, Makrides *et al* 1994).

4.1.3 Metabolism

Fatty acids are metabolised by a process known as β–oxidation, which takes place primarily in the mitochondria. Transport into the mitochondria is a carrier–dependent process using carnitine. Fatty acid molecules are degraded in the mitochondria by progressive release of two–carbon segments in the form of acetyl coenzyme A, which are then used by the citric acid cycle, producing CO_2 and NADPH, which is then oxidised to produce ATP.

It is apparent however, both from studies in rats as well as humans, including infants, that the majority of dietary ARA and DHA is unavailable for oxidation, particularly in the infant, and is instead preferentially channelled into the phospholipid pool.

4.1.4 Excretion

The lipids that are metabolised are excreted as carbon dioxide and water. Various amounts of lipid may also be excreted in the faeces and this is generally a reflection of the efficiency of intestinal absorption. In cases of malabsorption due to certain pathologies (e.g., pancreatic insufficiency, short bowel etc) lipids can be excreted in large amounts in the stools. Also, in specific studies with pre-term infants (Carnielli *et al* 1998) it appears as though between 20 – 25% of ingested LCPUFAs (either from DHASCO/ARASCO-supplemented formula or human milk) can be lost in the faeces, that is, not absorbed by the intestine. Term infants, however, exhibit more efficient absorption, readily absorbing greater than 90% of human milk fat (Widdowson 1965), therefore the proportion of ingested LCPUFAs in the faeces is likely to be considerably less than that found in pre-term infants. Studies with weanling rats (see Section 3.2 below), using DHASCO and ARASCO, indicate that less than 2% of ARA and DHA are actually excreted in the faeces, even at very high levels of diet incorporation.

4.2 Specific studies with ARASCO and DHASCO in animals and humans

A number of studies with neonatal and weanling animals were submitted, as well as a single human study using pre-term infants. The studies are listed below.

4.2.1 Animal studies

Studies evaluated:

Absorption of ARASCO and DHASCO in rats. Mason, S. and Yuhas, R. (1994**)** Wyeth-Ayerst Research. Study GTR-20407.

Tissue accretion of fatty acids in rat pups. Boyle, *et al*. (1995**)** Wyeth-Ayerst Research. Study GTR-24592.

Diets varying in n-3 and n-6 fatty acid content produce differences in phosphotidylethanolamine and phosphotidylcholine fatty acid composition during development of neuronal and glial cells. Jumpsen *et al*. (1995**)**. Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada. Study GTR-26223.

Relationship between dietary supply of long chain fatty acids and membrane composition of long and very long chain fatty acids in developing rat photoreceptors. Suh, M. *et al* (1995). Nutrition and Metabolism Research Group, University of Alberta, Canada. Study GTR-26222.

Retinal fatty acids or piglets fed microbial sources of DHA and ARA. Craig-Schmidt, M.C. *et al* (1995). Department of Nutrition and Food Science, Auburn University, Alabama, USA. GTR-26221.

Plasma and erythrocyte lipids of piglets fed formula containing microbial sources of DHA and ARA. Craig-Schmidt, M. *et al* (1995). Department of Nutrition and Food Science, Auburn University, Alabama, USA. GTR-26532.

The animal studies above examined both the absorption and tissue accretion of LCPUFAs from DHASCO and ARASCO in weanling rat, neonatal rat or neonatal pig models. The studies were all well prepared, performed and presented, although no declarations were included with any of the above studies to indicate that they have been conducted in accordance with good laboratory practice.

The absorption study with DHASCO and ARASCO in weanling rats (study (i) above) indicates that DHA and ARA are well absorbed (> 98%) when incorporated at low levels (1.7% DHASCO, 2.1% ARASCO) in a formula fat blend and at higher levels (24 % DHASCO, 29% ARASCO) with soybean oil.

The tissue accretion studies in neonatal rats indicate there is a complex interaction between n-6 and n-3 fatty acids and that even small dietary amounts of DHA and ARA can readily influence the fatty acid composition of phospholipids, reflected in the plasma, brain and retina fatty acid levels. Similar results were also obtained using the neonatal pig model.

The above studies indicate that both ARASCO and DHASCO are bioavailable and that they are able to support maximal tissue accretion of ARA and DHA by the retina and other membrane phospholipids.

4.2.2 Human studies

Study evaluated:

Bioavailability of arachidonic and docosahexanoic acids from Preemie SMA supplemented with long chain polyunsaturated fatty acids. Clandinin, M.T. *et al* (1995). Nutrition and Metabolism Research Group, Department of Food Science and Nutrition, University of Alberta, Canada. [Published as Clandinin *et al* (1997)].

Study objective

The purpose of the above study was two-fold: (i) to measure the blood lipid responses of pre-term infants fed human milk or infant formula supplemented with four different levels of ARA and DHA; and (ii) to determine the quantity of LCPUFAs in infant formula that will promote blood lipid profiles in formula-fed pre-term infants that are similar to that of human milk-fed infants.

Study conduct

The study was an open (non-blinded), sequential, prospective design. Healthy, pre-term infants whose birth weight was less than 2200 g were enrolled in the study. All study infants

were receiving 100% of their daily fluid and energy requirements enterally by 14 days of age. Infants were assigned to one of four feeding groups based on the mother's decision to breast-feed or feed infant formula to their infant. Infants were thus assigned to one of four diet groups: human milk (33 infants); pre-term formula with no added ARA and DHA (15 infants); pre-term formula with 0.4% ARA and 0.25% DHA (22 infants); and pre-term formula with 0.6% ARA and 0.45% DHA (21 infants). The different pre-term formulas varied only with respect to their ARA and DHA content. The source of ARA was ARASCO and the source of DHA was DHASCO. Between the groups, infants were matched for gestational age, postnatal age and birth weight.

Body weight was measured daily, and body length and head circumference were measured weekly. Human milk or formula intake was estimated daily. Occurrence of vomiting was used to assess study formula tolerance. Venous blood samples were obtained at approximately 12 to 14 days of age (week 0 of the study) and after an additional 4 weeks of feeding (week 4). Blood samples were analysed for total plasma and red cell membrane phospholipid fatty acid composition, complete blood count, differential white count, platelet count and serum creatinine. Routine urinalysis was done at weeks 0 and 4. Total plasma phospholipid (TPL), erythrocyte-phosphatidylcholine (RBC-PC), and erthyrocyte-phosphatidylethanolamine (RBC-PE) fatty acid compositions were also determined.

Results

There were no differences between the groups with respect to weight, length and head circumference at week 0 and week 4. There was also no difference between the groups with respect to feeding tolerance. The average daily intake of human milk or infant formula exceeded 150 ml/kg/day by 12-14 days of age.

Human milk or LCPUFA-supplemented formula feedings were associated with increases in ARA and DHA in TPL and RBC-PC relative to those fed unsupplemented formula. RBC-PE DHA levels were similar in the human milk and 0.6% ARA/0.4% DHA supplemented groups, and both were significantly different from the unsupplemented group, while RBC-PE ARA levels were not detectably different among the various groups. Supplementation with 0.6% ARA/0.4% DHA or 0.4% ARA/0.25% DHA resulted in ARA and DHA concentrations in TPL and RBC-PC that were not significantly different from each other or from the human milk-fed group.

There were no consistent effects of ARA or DHA supplementation on non-essential fatty acid concentrations in plasma or erythrocyte phospholipids regardless of the supplementation level.

No significant differences were noted in any of the haematological parameters measured. The formula fed infants all had significantly higher urine pH values at 4 weeks than the human milk-fed group but this is an expected finding related to infant formula feeding. No other differences in urine parameters were noted.

Conclusions

The DHASCO/ARASCO supplemented formula was well tolerated by the infants. Supplementation of the pre-term formula supplemented 0.6% ARA and 0.4% DHA produced ARA and DHA concentrations in TPL and erythrocyte phospholipids that match those of

human milk-fed pre-term infants. These levels of supplementation also approximate the levels of DHA and ARA found naturally in human pre-term milk and thus suggest that the LCPUFAs in DHASCO and ARASCO are as well absorbed and assimilated as those in human milk.

5. REVIEW OF TOXICOLOGY DATA

5.1 Acute studies

(i) Acute oral toxicity study of DHASCO (oil) in rats. Glaza, S.M. (1990) Hazleton Wisconsin Inc, Wisconsin, USA on behalf of Martek Corporation. Study GTR 26203. December 1990. [Published as Boswell *et al* 1996]

Test material:	DHASCO oil, described as a cloudy, viscous, amber liquid.
Test species:	Albino rats, Crl:CD®BR (Charles River Laboratories, Inc., Portage MI).
Dose:	20 g/kg body weight administered orally by gavage to 5/sex.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58.
Guidelines:	US FDA Toxicological Guidelines (Redbook I).

Study conduct

Five male and five female rats, weighing from 202 to 260g, were administered with a single dose level of 20 g/kg body weight of the test material. Food and water were available *ad libitum* throughout the study, except for approximately 17 to 20 hours before test material administration when food, but not water, was withheld. An individual dose of the undiluted test material was calculated for each animal based on its fasted body weight and administered by gavage. The test material was administered in a volume of 22.73 ml/kg body weight, based upon an average bulk density of 0.88 g/ml. Clinical signs and mortality checks were done at 1, 2.5 and 4 hours after dosing. The animals were observed daily thereafter for 14 days for clinical signs and twice daily (morning and afternoon) for mortality. Body weights were determined before test material administration (Day 0), at Day 7, and at termination of the study (Day 14). Before initiation of treatment (Day –1), at Day 7, and at termination of the experimental phase (Day 14), all animals (not fasted) were anaesthetised with ketamine and 2 ml of whole blood was collected from the retro-orbital plexus. The samples were sent frozen to the Sponsor (Martek Corporation) after termination of the study. At termination of the study, all animals were killed, subjected to gross necropsy examination and all abnormalities were recorded. After necropsy, animals were discarded and no tissues were saved. No statistical analysis was performed.

Results

No deaths were recorded during the study and all animals exhibited increased weight gain over the course of the study. Clinical signs observed were soft stools and dark stained urogenital area. All animals returned to a normal appearance within three days of test material administration. Gross necropsy examination of the animals at study termination revealed no visible lesions. The estimated LD_{50} for males and females was determined to be greater than 20 g/kg body weight.

Comment

The appearance of soft stools and stained urogenital areas are expected and normal consequences of a large single dose of a fatty substance and are thus not considered to be an adverse effect.

(ii) Acute oral toxicity study of ARASCO (oil) in rats. Glaza, S.M. (1992) Hazleton Wisconsin Inc, Wisconsin, USA. Study GTR 26204. January 1992.

Test material:	ARASCO oil, described as a yellow liquid.
Test species:	Albino rats, Crl:CD®BR (Charles River Laboratories, Inc., Portage MI).
Dose:	20g/kg body weight administered orally by gavage to 5/sex.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58.
Guidelines:	US FDA Toxicological Guidelines (Redbook I).

Study conduct

Study conduct as described for Glaza (1990) above.

Results

No deaths were recorded during the study and all animals exhibited increased weight gain over the course of the study. Clinical signs observed were oily soft stools and oily hair coat (males only). All animals returned to a normal appearance within two days of test material administration. Gross necropsy examination of the animals at study termination revealed no visible lesions. The estimated LD_{50} for males and females was determined to be greater than 20 g/kg body weight.

(iii) Acute oral toxicity study of Microencapsulated Formulaid® in rats. Glaza, S.M. (1997) Corning Hazleton Inc, Wisconsin, USA. Study CHW 70101732. January 1992.

Test material:	Microencapsulated Formulaid®, Lot No. RBD28-03612 (a 2:1 mixture of ARASCO and DHASCO), described as tan granules.
Test species:	Young adult albino rats, Crl:CD®BR (Charles River Laboratories, Inc., Portage MI).
Dose:	5 g/kg body weight administered orally by gavage to 5/sex.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58 with the exception that analysis of the test material mixture for concentration, homogeneity/ solubility and stability was not conducted.

Study conduct

Five male and five female rats, weighing from 225 to 299 g, and approximately 8 to 16 weeks of age, were administered with a single dose level of 5 g/kg body weight of the test material. Food and water were available *ad libitum* throughout the study, except for approximately 17 to 20 hours before test material administration when food, but not water, was withheld. Clinical signs were done at 1, 2.5 and 4 hours after dosing and daily thereafter for 14 days. The animals were observed twice daily (morning and afternoon) for mortality. Body weights were determined before test material administration (Day 0), at Day 7, and at termination of the study (Day 14). At termination of the study, all animals were killed, subjected to gross necropsy examination and all abnormalities were recorded. After necropsy, animals were discarded and no tissues were saved. No statistical analysis was performed.

Results

No deaths were recorded during the study and all animals, with the exception of one female, exhibited increased weight gain over the course of the study. None of the animals exhibited any clinical signs during the course of the study and no lesions were observed at necropsy. The estimated LD_{50} for males and females was determined to be greater than 5 g/kg body weight.

5.2 Short-term studies

(i) 4-week oral gavage toxicity study with ARASCO, DHASCO, and Formulaid (ARASCO and DHASCO) in rats. Williams, K.D. (1994). Hazleton Wisconsin Inc., Wisconsin, USA. Study HWI 6539-100. 29 June 1994. [Published as Boswell *et al* 1996]

Test material:	ARASCO (Lot No. A011-DS-2, yellow-tan liquid), DHASCO (Lot No. DD004-WS, yellow-red liquid), and Formulaid (Lot No. F011-DS-2, a 2:1 mixture of ARASCO and DHASCO, yellow-tan liquid)
Test species:	Male and female Crl:CD®(SD)BR VAF/Plus® rats (Charles River Laboratories, Inc., Portage, Michigan)
Dose:	ARASCO: 50 (5/sex), 1000 (5/sex), 2500 mg/kg bw/day (10/sex); DHASCO: 25 (5/sex), 500 (5/sex), 1250 mg/kg bw/day (10/sex); Formulaid: 1500 (5/sex), 3750 mg/kg bw/day (10/sex). Each animal received a total of 3.75 g oil/kg bw/day, vehicle was high oleic sunflower oil.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58.

Study conduct

After 11 days acclimatisation, male and female rats were assigned at random to nine groups and were dosed according to the following:

Group	No. of animals	High oleic sunflower oil (mg/kg bw/day)	ARASCO (mg/kg bw/day)	DHASCO (mg/kg bw/day)	Formulaid (mg/kg bw/day)
1	10/sex	3750			
2	5/sex		50		
3	5/sex		1000		
4	10/sex		2500		
5	5/sex			25	
6	5/sex			500	
7	10/sex			1250	
8	5/sex				1500
9	10/sex				3750

Each animal received dose preparations containing the carrier (high oleic sunflower oil), test materials, or a combination of both at a dose volume of 4.17 ml/kg. Each animal received a total 3.75g oil/kg bw/day.

Food and water were provided *ad libitum*. Animals were observed twice daily for mortality and clinical signs and at least once each week, each animal was removed from its cage and examined for abnormalities and signs of toxicity. Individual body weight and food consumption data were collected weekly for 4 weeks. Blood samples were collected for haematology and clinical chemistry tests from 5 animals/sex/group during Week 5 of the study (i.e., prior to termination of the study). Blood samples were also collected from 5 animals/sex before treatment and during Weeks 2 and 5; serum was collected and sent to the Sponsor for possible future analyses. During Week 5, animals were anaesthetised, weighed,

exsanguinated, and necropsied. At necropsy, macroscopic observations were recorded, selected organs were weighed, and selected tissues were collected and preserved. The brain, heart, liver (representative sample), and right testis (where present) were collected from 5 animals/sex/group, frozen in liquid nitrogen and stored at -70˚C until shipped to the Sponsor. Microscopic examinations were done on tissues from 5 animals/sex/group from all high dose groups plus the high oleic sunflower oil control. Data were analysed by appropriate statistical techniques.

Results

Antemortem observations and survival: All animals survived to the end of the study. No test material-related antemortem observations were noted during the study.

Body weight and food consumption: No significant differences in body weights, cumulative body weight gain or food consumption between the treated and control groups were noted during the study.

Clinical chemistry: Some females in the mid dose ARASCO group and the high dose DHASCO group had a significantly lower total protein value than the control group. Albumin was also significantly lower for females in the mid and high dose ARASCO groups and the high dose DHASCO group. These occurrences appear to be sporadic and do not exhibit any apparent dose-response relationship. In addition, these affects are not observed in the low or high dose Formulaid groups. The males also were not similarly affected. High serum potassium levels were observed in several animals, including those in the control group, and thus do not appear to be related to the test material.

Postmortem observations: The only significant organ weight finding was higher absolute liver weights for males in the high dose Formulaid group compared to the controls and those of males in the high dose DHASCO group. The change in liver weight was also reflected in the organ-to-body weight and organ-to-brain weight ratios for males in the high dose Formulaid group. The increased liver weights were not however correlated with any histopathologic finding or clinical chemistry finding therefore is most likely to represent an adaptive change to the high concentrations of LCPUFAs in the diet.

Histopathological observations: A few histopathological changes were evident, however the incidence of the changes was similar in control and treated animals.

Conclusions

No evidence of toxicity was observed at doses of ARASCO up to 2500 mg/kg body weight/day and DHASCO up to 1250 mg/kg body weight/day, administered either individually or in combination (as Formulaid).

Comment

The high serum potassium levels observed in several animals, including those in the control group, have been attributed to an excessively deep plane of anaesthesia before blood collection because if the levels observed had been present before anaesthesia they would have seriously affected the animals (Boswell *et al* 1996).

(ii) Sub-acute (4-wk) oral toxicity study with polyunsaturated fatty acids in rats. Lina, B.A.R. (1996). TNO Nutrition and Food Research Institute, The Netherlands. Study No.1751. March 1996.

Test material:	ARASCO (Batch No. PU 506HD/KA070) and DHASCO (Batch No. 50150)
Test species:	Young male and female Wistar outbred rats (Crl:(WI)WU BR) (Charles River Wiga GmbH, Sulzfeld, Germany)
Dose:	Administered by gavage daily to 5/sex/group at the following doses: 100- 3000 mg ARASCO/kg bw/day; 50- 1500 mg DHASCO/kg bw/day; 2000 mg ARASCO/1000 mg DHASCO/kg bw/day; 3000 mg ARASCO/1500 mg DHASCO/kg bw/day.
GLP:	OECD Principles of Good Laboratory Practice.
Guidelines:	OECD Guideline for Testing of Chemicals 407 and EC Guideline 84/449/EC

Study conduct

After acclimatisation, rats were assigned to various groups proportionately by weight class by a computer randomisation program and were dosed according to the following.

Group	Treatment	Dose (mg/kg bw/day)	No. of animals
A (control)	Vehicle only	-	10/sex
B	ARASCO	100	5/sex
C	ARASCO	600	5/sex
D	ARASCO	2000	5/sex
E	ARASCO	3000	5/sex
F	DHASCO	50	5/sex
G	DHASCO	300	5/sex
H	DHASCO	1000	5/sex
I	DHASCO	1500	5/sex
J	ARASCO / DHASCO	2000/1000	5/sex
K	ARASCO / DHASCO	3000/1500	5/sex

The test substances were administered daily by gavage for 4 weeks. Each animal received dose preparations containing the vehicle (corn oil) at a constant volume of 5 ml/kg body weight. The vehicle control group received 5 ml corn oil/kg body weight only.

Food and water were provided *ad libitum*. Animals were observed twice daily for mortality and clinical signs. All abnormalities, clinical signs or reactions to treatment were recorded. The body weight of each animal was recorded at the beginning of the study (Day 0) and twice weekly thereafter. In addition, terminal body weights were recorded in order to determine the organ to body weight ratios. Food consumption was measured on a weekly basis. At necropsy, blood samples were taken from the abdominal aorta and tested for haematology parameters and clinical chemistry parameters. At necropsy, animals were killed by exsanguination under ether anaesthesia and then examined macroscopically for pathological changes. Selected organs were weighed and selected tissues (adrenals, bone marrow, brain, fatty tissue, heart, kidneys, large intestine, liver, lungs, lymph nodes, ovaries, pancreas, spleen, small intestine, stomach, testes, thyroid with parathyroids, uterus and all gross lesions) were preserved for microscopic examination. Data were analysed by appropriate statistical techniques.

Results

Antemortem observations and survival: No animals died during the study. A number of animals exhibited areas of sparsely haired skin and/or focal alopecia but this was also observed among control animals. No other abnormal clinical signs or behaviour were observed among any of the animals.

Body weight and food consumption: There were no apparent differences in food consumption between the various groups and the controls and the only statistically significant difference in mean body weights between groups was an increase in males of the DHASCO 1000 group on Day 7.

Clinical chemistry: A number of changes in clinical chemistry were observed. Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) activities were significantly increased in females of the ARASCO/DHASCO high dose group and alanine aminotransferase activity was also significantly increased in males of the DHASCO 1500 group. The changes were only slight and were well within historical control ranges.

A tendency towards decreased levels of phospholipids was observed in males of the DHASCO 1000 group, in both sexes of the DHASCO 1500 group and in both sexes of the ARA/DHA low and high dose groups. These changes reached the statistical significance in males of the DHASCO 1000 group, in both sexes of the ARASCO/DHASCO low dose group and in females of the ARASCO/DHASCO high dose group. These changes were not clearly within the range of historical control data.

In males, triglyceride levels were relatively low in the ARASCO 2000 and ARASCO 3000 groups, the DHASCO 1500 group and the ARASCO/DHASCO low dose and high dose groups, both in comparison to the controls and in comparison to the historical control data. These changes reached statistical significance in males of the ARASCO/DHASCO low dose group.

An increased creatinine level was observed in males of the ARASCO 100 group but was not seen at any of the higher dose levels.

Haematology: Values obtained for red blood cell variables and clotting potential did not show any statistically significant changes, apart from slight increases in mean corpuscular haemoglobin concentration (MCHC) in males of the ARASCO/DHASCO low dose and high dose groups. This slight increase was well within the normal range and not associated with any other changes in red blood cell variables.

There were no statistically significant changes in total white blood cell counts or in differential white blood cell counts in any group, apart from a decrease in the absolute number of lymphocytes in females of the DHASCO 50 group. This appears to be a sporadic finding, as this effect was not observed at any of the higher dose levels.

Postmortem observations: The relative weight of the spleen was significantly increased in males of the ARASCO 3000 group and in females of the ARASCO/DHASCO high dose group. The absolute weight of the spleen was increased in males of the ARASCO 2000 group but this change was not reflected in a significant increase in the relative weight of this organ. A few other organ weight changes were noted (decreased relative testes weight and increased relative liver weights in males of the DHASCO 50 group, and increased relative heart weight in females of the DHASCO 50 group and the ARASCO/DHASCO low dose group) but these changes were not observed at any of the higher doses and were thus considered to be spurious findings.

A number of gross changes were observed at necropsy however these occurred sporadically among both test and control groups and are common for animals of this strain and age. The only exception was the occurrence of local peritonitis (indicated by ascites and white deposition on the spleen – see Histopathologic observations) in one male of the DHASCO 1500 group. As this condition was not observed in any other animals it was considered to be a sporadic finding, unrelated to treatment.

Histopathologic observations: Microscopic examination did not reveal any treatment related histopathological changes. All changes observed were randomly distributed among the groups or occurred in a single animal only and are common for rats of this strain and age, except for local peritonitis (ascites with splenic capsular and serosal inflammation) observed in one DHASCO 1500 male.

Conclusion

The administration of ARASCO and DHASCO to Wistar rats at doses up to 3000 and 1500 mg/kg bw/day, respectively, for 4 weeks, either singly or in combination, was not associated with any evidence of toxicity.

Comment

The increases in ALAT and ASAT observed in some of the high dose groups were not accompanied by changes in liver weight or associated with any histopathological findings therefore they are not of toxicological significance.

The decreases in phospholipid and triglyceride levels were not always statistically significant and also did not always show a clear dose-response relationship however they appear to be definitely treatment related. These findings however are not considered to be toxic effects but rather are normal consequences of the feeding of long chain polyunsaturated fatty acids. Similar changes are also observed with the feeding of fish oils (see Appendix 1 for further discussion).

The increases in spleen weight were not accompanied by any relevant histopathological changes or change in haematology parameters and thus are not considered to be of toxicological significance (see Appendix 1).

(iii) Martek oil: Nine week oral (diet) safety study in rats. Anon (1994). Wyeth-Ayerst Research, New York, USA. Study No. 06288. 9 February 1994.

Test material:	Martek oil (Lot Nos. 17798 and 17799) containing a 1.5:1 blend of ARASCO:DHASCO produced using Martek Manufacturing Standard Operating Procedure 1.
Test species:	Male and female Charles River CD VAF rats (Charles River Laboratories, Inc., Portage, Michigan)
Dose:	129mg ARASCO + 91.9mg DHASCO (low dose), and 1044mg ARASCO + 720mg DHASCO (high dose)/kg bw/day to 15/sex/group.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58

Study conduct

After 12 days acclimatisation, male and female rats were assigned to four treatment groups (15/sex/group) and dosed via the diet according to the following:

Treatment Group	Diet/dose	% total fat component
1 (Control)	Purina certified rodent chow	-
2 (Positive control)	Soybean oil (basal diet)	100%
3 (Low)	129 mg ARASCO/kg bw/day 92 mg DHASCO/kg bw/day	2.9 % 2.0%
4 (High)	1044 mg ARASCO/kg bw/day 720 mg DHASCO/kg bw/day	23.2% 16.0%

The synthetic diets fed to Groups 2, 3 and 4 contained 5% total dietary fat and 20% protein. The total fat content of the rodent chow diet was not specified. The doses of ARASCO and DHASCO administered to Group 3 were intended to approximate the expected clinical consumption and the dose administered to Group 4 was a 8-fold excess of this amount.

The appropriate diet and water were provided *ad libitum*. All animals were observed at least twice daily for mortality. Individual body weights and group mean food consumption was recorded weekly. All animals were observed daily for changes in gross motor and behavioural activity and in appearance and were observed weekly for alterations of teeth, nose, eyes, pelage, perineum, and body orifices and to detect the onset and progression of tissue masses. Ophthalmoscopic examinations were performed on all rats 1 week prior to study initiation and during week 9. Blood samples were collected for haematology and clinical chemistry tests from 10/sex/group 1 week prior to study initiation and during weeks 4 and 9. Additional haematology parameters were also examined at termination on samples collected at necropsy during week 10. All rats surviving the 9 weeks treatment and a single animal that was killed *in extremis* received a complete necropsy. At necropsy, macroscopic observations were recorded, selected organs were weighed, and selected tissues were collected and preserved for histological examination.

Results

Antemortem observations and survival: One Group 3 male was killed *in extremis* during study week 8 due to a swollen left hind leg. No evidence of any treatment-related changes was seen in this rat. All other animals survived until the end of the study.

Body weight and food consumption: No differences were observed in body weight gain between groups throughout the study and food consumption of rats fed ARASCO and DHASCO (Group 3 and Group 4 rats) were comparable to those fed the high fat (soybean oil) control diet (Group 2 rats). Slight decreases in food consumption, with concomitant decreases in body weight gain, were observed during the first week of the study in female rats in Groups 2, 3 and 4 and in male rats in Group 3 and 4, compared to the rats in Group 1 (rodent chow diet). Sporadic decreases in food consumption, but without any corresponding changes in body weight gain, continued to occur throughout the study in male and female rats in Groups 2, 3 and 4. These differences are most likely be attributed to differences in diet composition of Groups 2, 3 and 4, compared to Group 1, although details of the specific diet formulations were not provided in the study report.

Clinical observations: No treatment-related differences were observed following physical and ophthalmologic examination.

Haematology: During week 4, slight decreases in haematocrit values occurred in male Group 3 and 4 rats and in female Group 4 rats, compared to Group 1 and 2 rats. Slight decreases in reticulocyte counts were also evident in male and female rats in Groups 2, 3 and 4 at weeks 4 and 9. The magnitude of this change was greatest in Group 3 and 4 rats. These changes appear to be treatment (i.e. DHASCO/ARASCO) related, however they do not clearly correlate with any specific histopathologic changes therefore they may not be toxicologically significant.

Clinical chemistry: Variations in several clinical chemistry parameters were observed. These included slight to moderate increases in cholesterol and the HDL fraction and decreases of the same magnitude in triglyceride values in male and female rats in Groups 2, 3 and 4 throughout the treatment. Some fluctuations also occurred in the LDL fraction, with slight decreases noted in Group 3 male rats and Group 4 male and female rats at week 4, and slight decreases in this fraction seen in Group 2 females during week 4 and 9, with Group 2 males also similarly affected at week 9 only. Changes in Groups 3 and 4 were generally equivalent to or less severe than changes observed in Group 2. As these effects were also noted in the high fat control (Group 2) rats, they do not appear to be test-material related, and are more likely attributed to the fat load in the diet fed to Groups 2, 3 and 4.

Slight decreases in potassium values occurred at week 4 in male and female rats in Groups 2, 3 and 4. At week 9, similar decreases were still evident in male Group 3 and 4 rats, as well as in female Group 2 rats. Individual female rats in Groups 2, 3 and 4 also exhibited slight increases in blood urea nitrogen values during weeks 4 and 9. Because the changes in potassium the blood urea nitrogen values were also noted in Group 2 rats, they do not appear to be treatment related.

Postmortem observations: Mean absolute and adjusted female ovarian weights were mildly increased in Group 4 rats, and mean absolute and adjusted male testicular weights were slightly increased in Group 4 rats, compared to Group 2 rats. Mean absolute and adjusted liver weights were slightly increased in Group 4 female rats compared to Group 2 rats. Mean absolute and adjusted brain weights were slightly decreased in Group 3 and 4 female rats, compared to Group 2 rats. These changes appear to be treatment related although the magnitude of the changes is quite small and, with the exception of hepatic fatty change, they also do not correlate with any specific histopathologic finding therefore they may have no biological or toxicological significance. Organ-to-brain weight ratios are not reported.

Gross pathologic lesions consisted of radial streaks in the kidneys from one Group 4 male, two Group 3 and two Group 4 females and hepatic discoloration in one Group 2 female and one Group 3 female. These lesions appear to correlate with some of the histopathologic findings (see below). The remaining gross pathologic lesions encountered in the tissues were consistent with spontaneous lesions encountered in control animals.

Histopathologic observations: Histopathologic lesions observed consisted of increased incidence of tubular mineralisation, tubular basophilia and hepatic fatty change in females from Group 2, 3 and 4 and an increased incidence of eosinophilic gastritis and gastric gland mucification in both males and females from Groups 2, 3, and 4. The renal histopathological findings appear to correlate with the slight increases in blood urea nitrogen values observed in females of Groups 2, 3 and 4 and the renal tubular mineralisation was also found to correlate well with the occurrence of radial streaks in the kidneys of Group 3 and 4 animals. The hepatic fatty change correlated with the occurrence of hepatic discoloration observed in

one Group 2 and one Group 3 female. There is no clear indication from the data that these effects are treatment related as they were also frequently observed in Group 2 animals. The only effects that might be treatment related are eosinophilic gastritis and gastric gland mucification, the incidence of which appears to be slightly increased in Group 4 males, compared to Group 2 males. The incidence and severity (slight, mild, moderate, marked) of these lesions are summarised in the following table.

	GROUP (FEMALES)							
	1		**2**		**3**		**4**	
EFFECT	**Tot.**	**Severity**	**Tot.**	**Severity**	**Tot.**	**Severity**	**Tot.**	**Severity**
Tubular mineralisation	**6**	6,0,0,0	**14**	5,5,4,0	**14**	1,5,8,0	**15**	3,6,4,2
Tubular basophilia	**0**		**8**	4,3,1,0	**11**	6,3,2,0	**12**	3,7,2,0
Hepatic fatty change	**0**		**9**	7,1,1,0	**11**	8,2,1,0	**14**	7,5,1,0
Eosinophilic gastritis	**0**		**9**	6,3,0,0	**5**	4,1,0,0	**5**	1,3,1,0
Gastric gland mucification	**0**		**8**	0,5,3,0	**4**	2,2,0,0	**7**	1,4,2,0
	GROUP (MALES)							
	1		**2**		**3**		**4**	
EFFECT	**Tot.**	**Severity**	**Tot.**	**Severity**	**Tot.**	**Severity**	**Tot.**	**Severity**
Tubular mineralisation	**2**	2,0,0,0	**1**	1,0,0,0	**3**	3,0,0,0	**1**	0,1,0,0
Tubular basophilia	**5**	4,1,0,0	**2**	2,0,0,0	**4**	2,1,1,0	**7**	4,3,0,0
Hepatic fatty change	**0**		**2**	2,0,0,0	**1**	1,0,0,0	**3**	3,0,0,0
Eosinophilic gastritis	**0**		**6**	2,4,0,0	**4**	2,2,0,0	**12**	6,5,1,0
Gastric gland mucification	**0**		**9**	3,5,1,0	**3**	2,1,0,0	**15**	1,6,8,0

Conclusion

A number of changes were observed, many of which occurred in both the high fat control group (Group 2), as well as the low and high dose ARASCO/DHASCO groups (Groups 3 and 4), therefore they could not be specifically attributed to ARASCO and DHASCO administration.

Effects that may be related to DHASCO and ARASCO administration were slightly decreased haematocrit values and reticulocyte counts, slightly increased ovarian and testicular weights in female and males, respectively, slightly increased liver weights in females, slightly decreased brain weights in females, and an increased incidence of eosinophilic gastritis and gastric gland mucification in males. The magnitude of these changes, however, was quite small and likely to be within historical control ranges, and therefore, these effects do not appear to have any toxicological significance.

Therefore, doses of ARASCO up to 1044 mg/kg bw/day and of DHASCO up to 720 mg/kg bw/day administered for a period of 9 weeks to rats do not appear to be associated with any toxicologically significant effects.

Comments

The performing laboratory attributed the majority of the effects seen to the synthetic diet being fed to Groups 2, 3 and 4. They reported that the renal lesions are similar to those in previous reports from studies feeding synthetic diets to rats and are thought to result from improper calcium to phosphorous rations. The hepatic fatty change was considered minor and reversible and was interpreted to result from high levels of carbohydrates in the synthetic diets. Similar lesions in the stomach to those described in this study have also apparently been reported in previous studies with rats fed synthetic diets. Apart from the fat and protein

content of the synthetic diet fed to Groups 2, 3 and 4, no other information was provided in the report regarding the diet formulation.

5.3 Sub-chronic studies

(i) Subchronic (3-month) combined neurotoxicity and toxicity study of ARASCO and DHASCO in the rat via oral gavage. Boswell, K. (1995). Pharmaco LSR, New Jersey, USA. Study No. 94-2352. 17 August 1995.

Test material:	ARASCO (Lot No. A013-DS) and DHASCO (Lot No. D015-DS)
Test species:	Male and female CD® (Sprague-Dawley derived) (Charles River Laboratories, Inc., Portage, Michigan)
Dose:	1000 and 2500 mg ARASCO/kg bw/day and 500 and 1250 mg DHASCO/kg bw/day to 20/sex/group by gavage. Each animal received a total of 3ml oil/kg bw/day; vehicle was high oleic sunflower oil.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58.

Study conduct

Animals were randomly assigned to six groups of 20 animals per sex and were acclimatised for approximately two weeks prior to dosing by gavage according to the following:

Treatment group	Test material	Dose (mg/kg bw/day)
1 (untreated control)	-	0
2 (vehicle control)	High oleic sunflower oil	0
3 (low)	ARASCO	1000
4 (high)	ARASCO	2500
5 (low)	DHASCO	500
6 (high)	DHASCO	1250

Control Group 1 received standard laboratory diet only. The dose volume for the oils was 3 ml/kg. Animals were observed twice daily for mortality and gross signs of toxicological effects. In addition, animals were given detailed physical examinations for signs of local or systemic toxicity and tissue masses twice pre-treatment and weekly thereafter. Ophthalmoscopic examinations were performed prior to treatment and at termination of the study. Body weight measurements were taken twice prior to treatment and weekly thereafter during treatment and also at termination. Food consumption was measured weekly, beginning one week prior to treatment. In addition to above, 10/sex/group were examined pretest, Week 5, 9 and 13 for motor activity, signs of autonomic function (ranking and degree of lacrimation and salivation, presence or absence of piloerection and exophthalmos, count of urination and defecation), description, incidence and severity of any convulsions, tremors or abnormal movements, reactions to general stimuli, posture and gait evaluations, forelimb and hindlimb grip strength, landing foot splay, and ranking or righting ability.

Blood was collected by venipuncture of the orbital sinus from 10/sex/group, selected at random, at termination of treatment and was tested for haematology parameters and clinical chemistry parameters. Organ weights were measured and histology was performed. Neuropathology was also performed on tissues from 5/sex/group at necropsy. The tissues examined were brain (forebrain, cerebral cortex, hippocampus, basal ganglia, midbrain, cerebellum and pons, medulla), spinal cord (cervical, thoracic and lumbar – cross and longitudinal sections), sciatic, tibial and sural nerves (cross and longitudinal sections). Data were analysed by appropriate statistical techniques.

Results

Antemortem observations and survival: One male and one female from the high dose ARASCO group and one male from the low dose DHASCO group died during the study. The death of the male from the high dose ARASCO group was due to gavage error. The cause of death of the other two animals could not be established on either macroscopic or microscopic examination. As there were no morphological changes and clinical signs of toxicity, the deaths of one animal each in the low dose DHASCO and the high dose ARASCO groups were not attributed to the test material. All other animals survived until the end of the study.

The majority of animals were free of any unusual signs throughout the study. The abnormalities that did occur did so sporadically in individual animals. All animals received ocular examinations pre-test and at termination of the study. There was no indication of dose or compound related ocular disease and none of the ocular abnormalities observed could be attributed to the test material.

Body weight and food consumption: Mean body weights and body weight gains of ARASCO and DHASCO-treated groups were comparable or slightly lower than body weights of animals in the vehicle control group throughout the study. All values were within 5% of concurrent control values. Mean food consumption of the vehicle control group were lower than those of the untreated control group due most likely to the fat content of the vehicle (high oleic sunflower oil). Mean food consumption values for the ARASCO and DHASCO-treated groups were comparable to that of the vehicle control group.

Haematology: There was no indication of any effect on mean haematology values at termination of the study. A few statistically significant differences between the control and treated groups were observed (e.g., increased white blood cell count in high dose ARASCO males and females, decreased mean corpuscular volume in high dose ARASCO males, and increased prothrombin time in high dose DHASCO females) but were not considered to be toxicologically significant.

Clinical chemistry: Mean alkaline phosphatase values were elevated for males and females in the low and high dose DHASCO groups compared to the control mean values. Differences were generally statistically significant but were not dose-related. No other changes in serum enzymes were seen and no morphological changes were seen upon microscopic examination. These differences may represent metabolic changes associated with DHASCO administration but do not appear to be toxic effects. All other parameters evaluated were comparable between the control and treated groups.

Neurological observations: Administration of ARASCO and DHASCO was not associated with changes in motor activity for either sex during the periods tested and also did not affect the overall neurological condition of the animals as measured by a battery of functional assessments.

Postmortem observations: A number of statistically significant differences in organ weights between control and treated groups were seen. These included: increased liver weights in high dose ARASCO females; increased kidney weights in high dose DHASCO males and females; and increased spleen weights in high dose ARASCO males. However, these differences were generally slight and were also not reflected in the organ-to-body or organ-to-brain weight ratios. Therefore, no consistent pattern of changes, indicative of an effect of

either test material, was seen. There were also no histopathologic findings that were considered to be treatment related or that correlated with any of the organ weight findings.

Histopathologic observations: No histopathologic findings appear to be treatment related. They occurred with comparable incidence and severities in rats from the treatment and control groups (e.g., an increased incidence of chronic progressive nephropathy in males of the vehicle control and high dose DHASCO and ARASCO groups, increased incidence of lymphocytic infiltration in the liver of males and females from the vehicle control and high dose ARASCO and DHASCO groups) or they occurred sporadically and have been seen in rats of similar strain and age previously used in the testing laboratory.

Conclusion

None of the changes observed appear to be toxicological effects related to the administration of either DHASCO or ARASCO. All the changes observed were slight and were not indicative of a consistent pattern of effects. They are most likely to be metabolic or adaptive changes to the administration of high dose of LCPUFAs. Therefore it can be concluded that the administration of 2500 mg ARASCO/kg bw/day or 1250 mg DHASCO/kg bw/day to rats for three months is not associated with any toxicologically significant effects.

(ii) Martek oil: thirteen week oral (diet) safety study in rats. Wren, J.M. (1995). Hazleton Wisconsin Inc. Study No. 9430-102. 11 August 1995.

Test material:	Martek Oil (Lot Nos. unspecified) produced using Martek Manufacturing Standard Operating Procedure 2.
Test species:	Male and female Charles River CD VAF rats (Charles River Canada, Quebec, Canada).
Dose:	88.2 mg ARASCO + 58.3mg DHASCO (low dose), 441 mg ARASCO + 291.5 mg DHASCO (mid dose), 1764 mg ARASCO + 1166 mg DHASCO (high dose)/kg bw/day to 20/sex/group. Vehicle was soybean oil.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58.

Study conduct

After 18 days acclimatisation, male and female rats were assigned to six groups (20/sex/group) and were dosed with a total of 50g/kg fat (5%) in their diet according to the following:

Group	Description	Fat blend	Estimated dose
1	Vehicle control	100% soybean oil	-
2	Low dose	2.0% ARASCO, 1.3% DHASCO, 96.7% soybean oil	90 mg ARASCO/kg bw/day, 59 mg DHASCO/kg bw/day
3	Mid dose	10% ARASCO, 6.5% DHASCO, 83.5% soybean oil	450 mg ARASCO/kg bw/day, 293 mg DHASCO/kg bw/day
4	High dose	40% ARASCO, 26% DHASCO, 34% soybean oil	1800 mg ARASCO/kg bw/day, 1170 mg DHASCO/kg bw/day
5	Untreated control	-	

The untreated control group received standard laboratory diet only. The low dose treatment group was given ARASCO and DHASCO at the proposed clinical concentration. The middle and high dose groups received 5 fold and 20 fold multiples of the proposed clinical concentration. Drinking water and the appropriate diets (prepared fresh daily) were available *ad libitum*. Animals were observed twice daily for mortality and at least once per day for general clinical observations including general appearance and behaviour. Body weight was

measured once per week (twice pre-test) and food consumption was recorded weekly. Detailed clinical observations were made weekly and ophthalmologic examinations were performed once pre-test and immediately prior to termination.

Blood was collected from 10/sex/group prior to commencement of the study and during weeks 4 and 13 and was tested for haematology parameters and clinical chemistry parameters. Additional blood samples were taken from the last 5/sex/group in order to supply adequate plasma for fatty acid analysis.

Necropsies were performed during week 14 and included external examination with gross evaluation of tissues from every animal. Organ weights were measured and histology was performed. Additional liver sections from all rats were stained with Oil Red O and examined. Data were analysed by appropriate statistical techniques.

Results

Antemortem observations and survival: All animals survived until the end of the treatment period. No changes were evident in clinical observations or in ophthalmologic examinations between the groups.

Body weight and food consumption: No changes in body weight or food consumption was observed between the groups.

Haematology: During week 4, slightly decreased total red blood cell count and haematocrit values and slightly increased platelet and reticulocyte values occurred in Groups 2, 3 and 4, compared to Group 1. These changes were not considered biologically relevant because of the magnitude and transitory nature of the changes and their lack of correlation with specific histopathological changes.

Clinical chemistry: Slightly decreased cholesterol (week 4 only in Group 4 males), LDL (week 4 and 13 in Group 4 males and females) and triglyceride values (week 4 only in Group 3 and 4 males, week 4 and 13 in Group 4 females) were seen throughout treatment and are likely to reflect secondary metabolic changes associated with the consumption of high levels of LCPUFAs, rather than toxicological changes. Slightly increased blood urea nitrogen values occurred in individual female rats in Groups 1 – 4 and generally correlated with the histopathological renal findings in these groups.

Postmortem observations: Organ weight changes consisted of slightly higher absolute mean kidney weight in male rats, mildly higher absolute and adjusted mean thyroid weights in female rats, and slightly higher adjusted mean liver weight in female rats in Group 4 (high dose DHASCO + ARASCO), compared to Group 1 (vehicle control). These observations did not correlate with any histopathological findings and therefore do not appear to be toxicologically significant. Gross pathological observations included radial streaks in the kidneys in low numbers of female rats in Groups 1, 2, 3 and 4, which correlated with the occurrence of renal tubular mineralisation.

Histopathologic observations: A number of histopathological lesions were observed and consisted of increased incidences of renal tubular mineralisation and renal tubular basophilia (females only), hepatic fatty change, eosinophilic gastritis, gastric gland mucification, and eosinophilic chief cells in male and female rats. These lesions were found to occur in

treatment as well as vehicle control groups and were thus attributed to the synthetic diet, which was not fed to the untreated control group.

Conclusions

No treatment-related toxicological effects were observed in the study at doses of ARASCO up to 1800 mg/kg bw/day combined with doses of DHASCO up to 1170 mg/kg bw/day.

(iii) Sub-chronic (13-week) oral toxicity study, preceded by an *in utero* phase, with polyunsaturated fatty acids in rats. Lina, B.A.R. and Waalkens-Berendsen, D.H. (1997). TNO Nutrition and Food Research Institute, The Netherlands. Study No.450588. May 1997 [Published as Hempenius *et al* 2000]

Test material:	ARASCO (Batch No. PU 512 HG 1) and DHASCO (Batch No. 50150), both having the appearance of clear, light yellow oil.
Test species:	Male and female Wistar outbred rats (Crl:(WI)WU BR) (Charles River Wiga GmbH, Sulzfeld, Germany).
Dose:	3 g – 75 g ARA oil/kg diet, 75 g ARA oil + 55 g DHA oil/diet. Vehicle was corn oil.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58.

Study conduct

From the start of the pre-mating period (F0 rats), throughout mating, gestation and lactation, until termination of treatment of the F1 animals, the test substances were administered at a constant concentration in the diet. A standard cereal based rodent diet was used and the total level of fat in each test diet and in the corn oil control diet was kept constant by adding the appropriate amounts of corn oil. The various levels of diet incorporation are indicated in the table below.

Group	Treatment	Treatment level (g/kg diet)	Corn oil level (g/kg diet)	No. of F1 rats/sex
A (control)	Rodent diet	-	-	10
B (control)	Corn oil	0	130	20
C (low dose ARA)	ARASCO	3	127	20
D (mid dose ARA)	ARASCO	15	115	20
E (high dose ARA)	ARASCO	75	55	20
F (high dose ARA/DHA)	ARASCO /DHASCO	75 + 55	0	20

Parental animals (F0) received the above test diets and control diets from 4 weeks prior to mating, and treatment was continued throughout mating, gestation, lactation and weaning of the F1 pups. Subsequently the sub-chronic study was started and the above test and control diets were fed to randomly selected male and female F1 rats for a period of 13 weeks. Feed and drinking water were provided *ad libitum*.

In utero phase: For the F0 animals, the general condition and behaviour of animals were checked daily and all abnormalities were recorded. The body weight of each animal was recorded at the commencement of the study and weekly thereafter until the parental rats were discarded. Mated females were weighed on Days 0, 7, 14 and 21 of gestation and on Days 1, 4, 7, 14 and 21 of lactation. Food consumption was measured on a weekly basis, during the pre-mating period in both males and females. Food consumption of mated females was recorded during pregnancy on Days 7, 14, 21 and during lactation on Days 4, 7, 14 and 21.

A number of observations were made with respect to fertility and reproductive performance for each group. These included: number of females placed with males; pre-coital time,

number of successful copulations; number of males that became sires; number of pregnant females; number of females surviving delivery; number of females with live born; number of females with stillborn pups; duration of gestation; number of pups delivered number of pups lost; number of litters lost; mating index (no. of females mated/no. of females place with males X 100); male fertility index (no. of sires/no. of males placed with females X 100); female fertility index (no. pregnant females/no. females mated X 100); gestation index (no. females with live foetuses/no. of pregnant females X 100); live birth index (no. live pups/total no. pups born X 100).

For the pups the following observations were made: daily viability checks; observation of appears of pups on Days 1, 4, 7, 14 and 21 of lactation; the number of live pups per litter on Days 1, 4, 7, 14 and 21 of lactation; the number of pups per sex on Days 1, 4, 7, 14 and 21; the number of male pups at Day 1 and 21; the sex ratio at Day 1 and 21; the weight of the litters as a whole on Days 1, 4, 7 and 14 post partum; the weight of individual pups was recorded on Day 21.

Sub-chronic study with the F1 animals: all animals were checked daily for clinical signs and any abnormalities recorded. Ophthalmoscopic observations were made prior to the start of the study and towards the end of the treatment period (on Day 84) in all rats on the corn-oil control group (B), the ARA high dose group (E) and the ARA/DHA group (F). Body weights of each animal were recorded at the start of the study (Day 0) and weekly thereafter, including at necropsy. Food consumption was measured weekly. In addition to these observations, a functional evaluation battery of observations and tests selected to detect signs of neurological, behavioural and physiological dysfunctions were undertaken in Week 1 and Week 12 of the study in 10 animals/sex of each group. These observations, in combination with histopathological examination of tissue samples representative of major areas of brain, spinal cord and peripheral nerves, were used to assess neurotoxicity.

For each test group, the intake of ARASCO and/or DHASCO/kg bw/day, as calculated on the basis of food intake, body weight and nominal dietary incorporation of the test substance is provided below.

Prior to necropsy, blood samples were taken from the abdominal aorta of 10 rats/sex/group and were tested for various haematology parameters and clinical chemistry parameters. Blood glucose was measured in blood taken from the tip of the tail shortly before the termination of the study (Day 88) and was taken from the same animals from which blood was taken just prior to necropsy.

On Day 87 – 88 of the study, the same 10 rats/sex/group that were used for haematology were deprived of water for 24 hours and of food during the last 16 hours of this period. The rats were kept in metabolism cages and urine was collected. The concentration ability of the kidneys was investigated by measuring urinary volume and density in individual samples. The same urine was also subjected to urinalysis as follows: appearance; glucose; pH; occult blood; ketones; protein; bilirubin; urobilinogen; and microscopy of sediment.

At necropsy the animals were examined macroscopically for pathological changes. A full necropsy was also performed on a single male rat belonging to the ARA high dose group (E162) that was killed unscheduled on day 67. Selected organs were examined and weighed and selected tissues were preserved for microscopic examination. Histopathological examination was performed for all animals of the corn-oil control group (B) and the ARA

high dose group (E). The kidneys, liver, lungs, small intestines, Peyer's patches, mesenteric lymph nodes and gross lesions were also examined microscopically in all rats of the ARA low and mid dose groups and the ARA/DHA high dose group. Histopathological examination was not conducted in rats of the rodent diet group (A), except for examination of brain, spinal cord, small intestines, Peyer's patches, and mesenteric lymph nodes in both sexes and of the liver in females. Data were analysed by appropriate statistical techniques.

Results for the in utero phase

All F0 animals survived until the end of the treatment period. No changes were evident in clinical observations between groups. Body weight gain in F0 females was lower than in the corn oil control during the pre-mating and mating period in the ARA/DHA high dose group, and during the first week of the gestation period in the ARA high dose group and the ARA/DHA high dose group. At the end of the lactation period however parental body weights were comparable in all groups. Mean body weights of F0 males were comparable in all groups.

There were no treatment related differences in fertility or reproductive performance among the ARA groups, the ARA/DHA high dose group and the corn oil control. All of the reproduction variables measured were normal for rats of this strain and none of the pregnant females died.

There were no treatment related differences in the general condition of pups, viability, sex ratio or number of pups per litter. Pup weight gain in the ARA/DHA high dose group was lower than in corn oil controls from Day 7 of lactation.

Results for sub-chronic study

Antemortem observations and survival: General condition and behaviour were not adversely affected by treatment in any of the groups and the functional observation battery and motor activity assessment did not reveal any unusual findings. Alopecic areas were frequently observed but the incidence of this finding was similar in test and control groups. One male rat of the ARA high dose group (E162) was killed on Day 67 of the study because of conditional decline. Microscopic examination revealed severe pyelonephritis. Similar findings were not observed in any of the other rats therefore the death of this rat was not considered to be treatment related. Ophthalmoscopic examination did not reveal any treatment related changes. The few changes that were observed are a common finding in rats of this strain and age.

Body weight and food consumption: At the start of the study mean body weight in the ARA/DHA high dose groups tended to be lower than in the corn oil controls but the differences were not statistically significant. There were no dose-related differences in body weight gain between the test groups and the corn oil controls. Males of the rodent diet group showed statistically significantly increased body weights as compared to the corn oil controls throughout the study. A similar tendency was observed in females of this group in the first few weeks of the study. Mean food consumption did not show any consistent differences between the test groups and the corn oil controls. The food consumption data, along with the body weight data, were used to calculate the dietary intake of ARASCO and DHASCO, which is presented below.

	Mean dietary intake (mg/kg bw/day)				
	ARA low ARASCO	ARA mid ARASCO	ARA high ARASCO	ARA/DHA high ARASCO	DHASCO
Males (average over 13 weeks)	190	958	4738	4883	3581
Females (average over 13 weeks)	192	984	4860	4997	3665

Haematology: Haematocrit was slightly decreased in males of the ARA/DHA high dose groups and mean corpuscular haemoglobin concentration was slightly increased in males of the ARA high dose and the ARA/DHA high dose group as compared to the corn oil controls. No other differences were observed.

Clinical chemistry: A number of differences in clinical chemistry parameters were observed between the test groups and the corn oil control group. These consisted of: decreased alkaline phosphatase activity in males and females of the ARA high dose group; decreased cholesterol in females of the ARA high dose group and in both sexes of the ARA/DHA high dose group; decreased triglycerides and phospholipids in males and females of the ARA high dose group and the ARA/DHA high dose group; increased creatinine concentration in males of the ARA high dose group and the ARA/DHA high dose group; increased urea concentration in males of the ARA/DHA high dose group. These changes however do not appear to be treatment related as similar changes were also seen in the rodent diet control group, compared to the corn oil group.

The renal concentration test showed an increased volume and a decreased density in the ARA high dose group in both sexes and in the ARA/DHA high dose group in males as compared to the corn oil controls. Urinary volume was also higher in males and females of the rodent diet control group, but was not accompanied by a decrease in density. There were no differences in semi-quantitative observations in the urine or in the microscopy of the urinary sediment among groups.

Postmortem observations: A number of differences in organ weights between the corn oil controls and the ARA high dose group or the ARA/DHA high dose group were evident. These comprised: increased absolute and relative spleen weights in both sexes of the ARA high dose group and the ARA/DHA high dose group (the increase in relative weight was not statistically significant in males of the ARA high dose group); increased absolute and relative liver in females of the ARA high dose group and the ARA/DHA high dose group (the increase in absolute liver weight was not statistically significant in the ARA high dose group); increased absolute and relative adrenal weight in females of the ARA/DHA high dose group; increased absolute and relative testes weights in males of the ARA high dose group (the increase in relative weight was not statistically significant); and increased absolute thymus weight in males of the ARA/DHA high dose group, although this was not reflected in the relative weight of this organ. In females of the rodent diet group (A), the weights of the kidneys and liver were increased compared to the corn oil controls (B). Other significant changes in organ weights in the rodent diet group were ascribed to the higher terminal body weights in this group.

Macroscopic examination at necropsy did not reveal any treatment related findings. The abnormalities observed are all common findings in this strain of rat and occurred sporadically

in both control and treatment groups. A number of male animals exhibited a pale liver however no dose response was evident and there was an equal incidence of this finding in males of the corn oil control group, therefore this change was not considered treatment related.

Observations in the male rat of the ARA high dose group (E162) that was killed on Day 67 of the study included unilateral hydronephrosis, dilatation of ureter and urinary bladder and bladder calculi.

Histopathologic observations: Microscopic examination revealed a number of changes, a number of which appear to be treatment related. The mesenteric lymph nodes of most males and several females of the ARA high dose group and the ARA/DHA high dose group contained focal aggregates of oil droplets. Oil droplets were also present in the tips of the villi of the small intestine of many animals of the ARA and ARA/DHA high dose groups. This histopathological change was not present in any animal of the other groups, except for one male in the corn oil control group. In addition, lipogranulomas were observed in the mesenteric lymph nodes in a number of rats in these groups. These changes were not present in any animals of any of the other groups. Oil droplets were also observed in the Peyer's patches of the small intestine of several rats in all groups including the corn oil controls, but not in the rodent diet control group. The incidence did not differ significantly between the test groups and the corn oil control group therefore this finding does not appear to be treatment related.

Several males and females of the corn oil group and the ARA high dose group exhibit vacuoles in the brain, especially in the white matter of the cerebellum, and in the spinal cord. The vacuoles did not contain any fat. These findings were not observed in the rodent diet control group. As the vacuoles occurred in both the ARA high dose group and in the corn oil controls, and their incidence was lower in the ARA high dose group, they do not appear to be treatment related.

In females, a dose-dependent increase in hepatocellular vacuolation in the liver was observed and reached statistical significance in the ARA high dose and ARA/DHA high dose groups. Hepatocellular vacuolation was not present in any female of the rodent diet control group. In males, vacuolation was also present in the liver of about one third of the males of all ARA groups and the corn oil control group but was absent in the ARA/DHA high dose group. The incidence of mononuclear cell infiltrate in the liver was slightly increased in males of the ARA/DHA high dose group.

In all test groups as well as the corn oil controls, several males showed increased hyaline droplet nephropathy. This change is commonly found in male rats and its incidence is reported to vary considerably (Hempenius *et al* 2000). In this study, the incidence was statistically significantly increased in the ARA high dose and ARA/DHA high dose groups. Signs of cell damage and regenerative features did not accompany these changes.

All other histopathological changes are common findings in rats of this strain and age and were equally distributed among the various groups or occurred in one or a few animals only, therefore they could not be related to the treatment. Microscopic examination of the male rat killed on Day 67 (E162) revealed the presence of severe pyelonephritis.

Discussion and conclusion

The administration of ARA-oil or DHA-oil to parental (F0) rats did not affect the health, fertility, reproductive performance or pup characteristics. The only change observed was growth retardation in parental females of the ARA and/or ARA/DHA high dose groups during the pre-mating, mating and gestation period, accompanied by a decrease in pup weight in the ARA/DHA high dose group. These lower pup weights were not however reflected in significant effects on body weights of F1 rats in the sub-chronic study.

The slight increase in mean corpuscular haemoglobin concentration in males of the ARA and ARA/DHA high dose groups may be treatment related. Similar changes were also observed in males in a 4-week study following administration of high levels of ARASCO + DHASCO (see (ii) in Section 4.2). These changes were only slight and, apart from a slight decrease in haematocrit values in males of the ARA/DHA high dose group, are not accompanied by any other changes in red blood cell parameters. For this reason, the increase in mean corpuscular haemoglobin concentration has doubtful toxicological significance.

The decreases in cholesterol, triglycerides and phospholipid concentrations in the plasma of rats of the ARA and/or ARA/DHA high dose groups are a well-documented and normal consequence of the incorporation of high levels of long chain polyunsaturated fatty acids in the diet. These changes are not considered to have any toxicological significance.

The increased volume and decreased density of urine observed in the renal concentration test in the ARA and ARA/DHA high dose groups may point to an impaired concentrating ability of the kidneys. Other findings that may be associated with this finding were increased plasma creatinine concentration and increased hyaline droplet nephropathy in males of the ARA and ARA/DHA high dose groups. In a similar study, using doses of ARASCO of 5900 mg/kg bw/day and DHASCO of 3000 mg/kg bw/day administered in combination, no such findings were reported (Burns *et al* 1999), although a renal concentration test appears not to have been performed.

The increases in spleen weight (both sexes) and in liver weight (females) in the ARA and ARA/DHA high dose groups appear to be treatment related and have been noted in a number of other short term and sub-chronic studies (see Section 4.2 and other sub-chronic studies above). This appears to be a recurrent finding associated with the feeding of diets high in long chain polyunsaturated fatty acids (refer to Appendix 1 for further discussion). Such effects are generally not regarded as toxic *per se* as they are typically not accompanied by biochemical or morphological changes that would be indicative of toxicity. In this particular study the increased liver weight in females of the ARA and ARA/DHA high dose groups was accompanied with an increased incidence of hepatocellular vacuolation and is a finding that has been observed in similar studies (Duthie *et al* 1988, Burns *et al* 1999). In Burns *et al* (1999), where even higher doses of ARASCO and DHASCO were used (up to a total of 8900 mg/kg bw/day), hepatic vacuolation was found to occur in both the high fat control as well as the high dose groups. It is speculated that the finding of hepatic vacuolation in animals fed high levels of LCPUFAs indicates that the fat level of the diets is close to that which may impede normal physiological functions in rats. While this finding may be regarded as an adverse effect associated with a diet high in LCPUFAs, it does not appear to be an adverse effect specific to either ARASCO or DHASCO.

The presence of oil droplets in the mesenteric lymph nodes and in the intestinal villi in the ARA and ARA/DHA high dose groups, as well as the appearance of lipogranulomas, is clearly treatment related, as these lesions did not occur in any other groups. These findings however do not appear to be associated with any adverse physiological effects, as determined from the absence of significant abnormalities such as inflammation. These findings are probably related to the absorption of high levels of certain fats from the intestine and their passage into the lacteals and mesenteric lymph vessels and are regarded as a harmless finding (Hempenius *et al* 2000).

In conclusion, the administration of 4738 – 4997 mg ARASCO/kg bw/day alone or in combination with 3581 – 3665 mg DHASCO/kg bw/day to rats for a period of 3 months is associated with a number of treatment related changes. Some of these findings point to an impaired concentrating ability of the kidneys at the highest dose levels tested, however, the vast majority of these changes appear to be a physiological adaptation to high dietary levels of LCPUFAs and not a manifestation of toxicity specific to the administration of either ARASCO or DHASCO. No treatment related changes were observed at the mid dose level of ARASCO. This dose level is equivalent to an intake of 958 mg ARASCO/kg bw/day.

5.4 Chronic studies

No chronic studies were submitted.

5.5 Reproduction studies

No reproduction studies were submitted.

5.6 Developmental studies

(i) Developmental toxicity study with ARASCO and DHASCO in rats. Henwood, S.M. (1995). Hazleton Wisconsin, Inc., Wisconsin, USA. Study No. HWI 6539-103. 16 August 1995. [Published as Arterburn *et al* 2000]

Test material:	ARASCO (Lot No. A013-DS) and DHASCO (Lot No. D015-DS)
Test species:	Female Crl:CD® (SD) BR VAF/Plus® rats (Charles River Laboratories, Inc., Portage, Michigan)
Dose:	1000 and 2500 mg ARASCO/kg bw/day and 500 and 1250 mg DHASCO/kg bw/day to 25 mated females/group by gavage. Each animal received a total of 2.5g oil/kg bw/day; vehicle was high oleic sunflower oil.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58

Study conduct

Mated female rats were assigned at random to five groups of 25 animals/group and were dosed by gavage according to the following:

Group	ARASCO (mg/kg)	DHASCO (mg/kg)	High oleic sunflower oil (mg/kg)
1 (control)	-	-	2500
2 (low)	1000	-	1500
3 (high)	2500	-	-
4 (low)	-	500	2000
5 (high)	-	1250	1250

Doses were administered in a total volume of 2.78 ml/kg bw on days 6 through 15 of gestation. Animals in Group 1 received the carrier according to the same dosing regimen as the test groups.

Food and water were provided *ad libitum*. Animals were observed twice daily for mortality and moribundity and for indications of toxic effects. Detailed clinical observations were made and body weights recorded on days 0 and 6 through 20 of gestation. Individual food consumption data were recorded during days 0 to 6, 6 to 9, 9 to 12, 12 to 16, and 16 to 20 of gestation. Necropsies were done on day 20 of gestation. Uteri with visible implantations were excised, weighed and the number and placement of implantation sites, live and dead foetuses, early and late resorptions, and any abnormalities were recorded. Each live foetus was sexed, weighed and examined for external abnormalities. Approximately one half of all live foetuses from each litter were processed and examined for soft tissue development and the remaining foetuses were eviscerated, processed and examined for skeletal abnormalities. The maternal necropsy included examination of the external surface of the body, all orifices, the cranial cavity, external surfaces of the brain and spinal cord, nasal cavity, and thoracic, abdominal and pelvic cavities and viscera. Uteri with no visible implantations were excised and stained for detection of implantations and confirmation of pregnancy status. Selected maternal tissues were collected and held for possible histological examination.

Results

Antemortem observations and survival: All animals survived to gestation day 20 and no animals had any significant clinical signs or symptoms that were test material related.

Body weights and food consumption: There were no effects on mean body weights, body weight changes, gravid uterine weights, and food consumption. The mean food consumption by the low-dose DHASCO group was higher than the control group during the first 6 days of gestation, but was not statistically significant thereafter. These animals had not received any test material during this interval so the difference in food consumption was not related to the treatment.

Postmortem observations: There were no test material-related necropsy findings in the females. No significant differences were evident in mean pre-implantation loss, post-implantation loss, percent live foetuses (male, female and total), resorptions (early, late and total) or sex ratio for any test material-treated groups.

Foetal observations: There were no significant differences in covariate-adjusted mean foetal body weights. A number of foetal external, soft tissue and skeletal abnormalities were present but they occurred in both control and treated groups in a non dose-related pattern and thus do not appear to be treatment related. These included a cleft palate and lip in one control foetus, ablepharia in one Group 2 foetus, and an absent tail and anal atresia in one group 5 foetus.

Several soft tissue variations in development were also observed. The incidence of under-developed renal papilla was increased in several of the treatment groups and was significantly higher in the low DHASCO group (Group 4) compared to the control group (Group 1). This incidence of this effect was not however dose-related as the high DHASCO and ARASCO groups had fewer incidences than the low dose groups. Several foetal and litter incidences, mostly in the DHASCO groups, were outside the laboratories historical control ranges for this

effect, but all values fell within regional historical control ranges. The foetal and litter incidence of dilated renal pelvis was also significantly higher in the low ARASCO and a low DHASCO groups compared to the control but were not dose related. These renal effects tended to be clustered within specific litters and the differences in frequencies in the low dose groups could be largely attributed to two litters in each group where 60 – 100% of the examined foetuses were affected. Both dilated renal pelvis and under-developed renal papilla represent variations in development, usually caused by slight delays, and because they have no persistent effects, they are not considered to be toxicologically significant (Arterburn *et al* 2000). Foetal skeletal abnormalities were present in both control and treated groups and their incidence was not dose-dependent.

Conclusion

Administration of ARASCO and DHASCO to pregnant rats during organogenesis at dose levels up to 2500 mg ARASCO/k bw/day and 1250 mg DHASCO/kg bw/day did not produce any adverse developmental effects that could be related to the treatment.

5.7 Genotoxicity

Studies evaluated:

(i) Mutagenicity test on RBD-ARASCO in the Salmonella/mammalian-microsome reverse mutation assay (Ames test). Lawlor, T.E. (1994) Hazleton Washington, Inc, Virginia, USA. Study 16015-0-401. 23 February 1994.

(ii) Mutagenicity test on RBD-DHASCO in the Salmonella/mammalian-microsome reverse mutation assay (Ames test). Lawlor, T.E. (1994) Hazleton Washington, Inc., Virginia, USA. Study 16016-0-401. 23 February 1994.

(iii) Mutagenicity test on RBD-ARASCO in the L5178Y TK+/- mouse lymphoma forward mutation assay. Cifone, M.A. (1994) Hazleton Washington Inc., Virginia, USA. Study 16140-0-431. 17 June 1994.

(iv) Mutagenicity test on RBD-DHASCO in the L5178Y TK+/- mouse lymphoma forward mutation assay. Cifone, M.A. (1994) Hazleton Washington Inc., Virginia, USA. Study 16141-0-431. 17 June 1994.

(v) Mutagenicity test on RBD-ARASCO measuring chromosomal aberrations in Chinese hamster ovary (CHO) cells. Murli, H. (1994) Hazleton Washington Inc., Virginia, USA. Study 16140-0-437. 23 May 1994.

(vi) Mutagenicity test on RBD-DHASCO measuring chromosomal aberrations in Chinese hamster ovary (CHO) cells. Murli, H. (1994) Hazleton Washington Inc., Virginia, USA. Study 16141-0-437. 15 June 1994.

The studies were all well prepared, performed and presented. All of the studies described were conducted in compliance with the Good Laboratory Practice regulations as set forth in the US Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160), and the OECD's Principles of Good laboratory Practice C (81) 30 (Final) Annex 2, issued 1979 – 1980. Studies were designed with appropriate positive and negative control test substances and appropriate criteria were defined for positive and negative outcomes. Appropriate preliminary studies were done to determine the solubility of the test material and to assess the dose range for the mutagenicity tests. The preparation of S9 mix for metabolic activation is described, and the procedures were appropriate. There were no deviations from the defined protocols for any of the studies. The main features and findings of each study are summarised in the table below.

Test	Test material	Concentration	Test object	Result
AMES	ARASCO oil	100 – 5000µg/plate (+/- S9 activation)	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	-ve
AMES	DHASCO oil	100 – 5000µg/plate (+/- S9 activation)	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	-ve
Forward mutation assay	ARASCO oil	125 - 4990µg/ml (+/- S9 activation)	Mouse lymphoma L5178Y cell line TK+/-	-ve
Forward mutation assay	DHASCO oil	125 - 5000µg/ml (+/- S9 activation)	Mouse lymphoma L5178Y cell line TK+/-	-ve
Chromosomal aberrations	ARASCO oil	501 - 5010µg/ml (+/- S9 activation)	Chinese hamster ovary (CHO) cells	-ve
Chromosomal aberrations	DHASCO oil	500 - 5000µg/ml (+/- S9 activation)	Chinese hamster ovary (CHO) cells	-ve

Conclusion

DHASCO and ARASCO were found to be negative in a battery of genotoxicity test systems at doses *in vitro* up to 5000 µg/ml, both with and without metabolic activation. This suggests that DHASCO and ARASCO are both non-genotoxic.

5.8 Other studies

(i) Analysis of dinoflagellate extract and spray-dried biomass for the presence of brevetoxins. Anon (1995). Chiral Corporation, Florida, USA. Study No. GTR-26219. June 1995.

A series of studies were done on *Crypthecodinium cohnii* extract (DHASCO oil and spray-dried biomass) for the presence of dinoflagellate toxins. The main features of each study is summarised in the table below.

Test	Test specificity	Sensitivity
Intraperitoneal mouse bioassay	Non-specific for lethal substances	1-4µg/mouse for saxitoxin, 20µg/mouse for brevetoxin. Not sufficiently precise for ciguatoxin or okadaic acid.
Radioimmunoassay (RIA)	Brevetoxin and Ciguatoxin	2.0ng/ml (2 ppb)
Synaptosome binding assay	Site 5 toxins, including brevetoxin and ciguatoxin structures	1.0ng/ml (1 ppb)
9-anthryldiazomethane (ADAM) esterification fluorescence HPLC (F-HPLC)	Okadaic acid and dinophysistoxin 1	140ng/g sample (140 ppb)
Microtitre plate protein phosphatase inhibition assay	Okadaic acid and dinophysistoxin 1	2µg/g sample (2 ppm)
HPLC	Saxitoxin and derivatives	1 – 10pg/100mg sample (1 – 10 ppb)
ELISA	Saxitoxin	0.03ng/g sample
Capillary electrophoresis	Saxitoxin and derivatives	1µg/g sample (1 ppm)

Study conduct and results

Intraperitoneal mouse bioassay: Extracts were made of 2.0 g of DHASCO or spray-dried biomass using methanol and petroleum ether and the dried methanol phase was resuspended in ethanol for analysis. Samples were suspended in phosphate buffered saline (pH 7.4) and were injected i.p. into Swiss white mice (16 – 20 g each). Animals were dosed with 0, 500, 1000 and 2000 mg equivalents of either DHASCO or spray-dried biomass.

No animal exhibited any visible signs of toxicity. The LD_{50} of this material was calculated to be > 100 g/kg bw.

Synaptosome binding assay: Extracts were made of DHASCO and spray-dried biomass using methanol and petroleum ether and the dried methanol phase was resuspended in ethanol for analysis. Volumes of 10 and 1 µl were used for the synaptosome binding assay. Prepared samples were analysed in triplicate for their ability to displace [^{3}H]-PbTx-3 from its binding site.

There was no displacement with either the 10 µl or 1 µl sample of the oil, and for the spray-dried biomass extract there was displacement equivalent to 1.17 nM for both the 10 µl and 1 µl samples.

The performing laboratory commented that a displacement value of 1.17 nM is very close to the detection limit. Also, for the 1 µl of extract the displacement at individual points was 5063, 5394 and 2411. If the 2411 point is discarded, there is no displacement. The laboratory regarded this as a negative (at the limits of sensitivity).

ADAM esterification fluorescence HPLC (F-HPLC): A 0.9811 g sample of DHASCO was extracted using methanol and petroleum ether and the methanol phase was then dried and weighed out using acetone, yielding 13.308 mg. This material was then tested for okadaic acid (OA) and dinophysistoxin 1 (DTX-1) using ADAM F-HPLC. For analysis of the spray-dried biomass, a 0.5021 g sample was homogenised in methanol and then extracted with petroleum ether. The methanol phase was dried and then weighed using acetone, yielding 0.602 mg. This material was then tested for OA and DTX-1.

Both samples were negative therefore the levels of OA and DTX-1 were below 140 ng/g sample.

p-nitrophenyl phosphate (PNPP)/protein phosphatase 1 (PP1) inhibition assay: The DHASCO and the spray-dried biomass were prepared in the same way as for the ADAM F-HPLC analysis. The PNPP/PP1 inhibition assay is a receptor-based assay for polyether toxins and at the time the test was conducted was still considered to be experimental.

Both samples were found to be negative with no inhibition occuring therefore the levels of OA and DTX-1 can be said to be below 2 µg/g sample

HPLC: The HPLC was conducted according to published procedures for the HPLC analysis of shellfish toxins (Sullivan 1990). A 1 g sample of DHASCO was prepared for analysis by extracting in acetic acid. The aqueous phase was removed and dried and resuspended in acetic acid to a final volume of 1ml. A 1 g sample of the spray-dried biomass was similarly

extracted in acetic acid, with the aqueous phase being removed, cleaned up on a column, dried and resuspended in acetic acid to a final volume of 3ml, which was then filtered (0.45 μm pore) prior to analysis.

The DHASCO sample exhibited no peaks in the HPLC analysis. The biomass sample exhibited two large peaks early in the chromatogram and a few broad peaks during the later half of the gradient. On further analysis these peaks were found to be artefacts due to fluorescent material in the biomass sample, rather from the presence of saxitoxin or its analogues.

ELISA for saxitoxin: The analysis was done using a commercial saxitoxin testing kit (R-Biopharm GmbH, Germany). Extracts of DHASCO and the spray-dried biomass were prepared as described for the HPLC testing. Samples and standards were analysed in duplicate and two dilutions of each sample were analysed – a 200 and a 2000 times dilution. The samples were analysed according to the test kit instructions and appropriate controls were included.

The level of saxitoxin in both samples was below the detection limit (0.03 ng/g sample)

Capillary electrophoresis: An extract of the spray-dried biomass was prepared as described for the HPLC testing. DHASCO was not analysed because definitive negative results for saxitoxin were obtained from both HPLC and ELISA. Detection was by on-column UV absorbance at 200 nm.

The capillary electrophoresis results confirmed the absence of saxitoxin and its derivatives in the biomass sample; therefore there is no indication of any saxitoxin or its derivatives in either sample.

Conclusion

All tests were negative (at the limit of detection) and therefore DHASCO can be considered non-toxic at the levels tested.

(ii) Acute oral toxicity study of fungal biomass in rats. Glaza, S.M. (1997). Covance Laboratories Inc, Wisconsin, USA. Study No.70403367. July 1997.

Test material:	*Mortierella alpina* biomass (Lot No. 6700000019), a tan powder.
Test species:	Young adult albino rats, Crl:CD® (SD)BR (Charles River Laboratories, Inc., Portage MI).
Dose:	5 g/kg body weight administered orally by gavage to 5/sex.
GLP:	US Code of Federal Regulations for Non-clinical Laboratory Studies, 21 CFR 58.

Study conduct

The test material was mixed with distilled water to a concentration of 0.25 g/ml and administered as a single oral dose of 5 g/kg body weight by gavage to five female and five male rats. Food and water were available *ad libitum* throughout the study, except for approximately 17 to 20 hours before test material administration when food, but not water, was withheld. Clinical observations were conducted at 1, 2.5 and 4 hours after test material administration and daily thereafter for 14 days. Mortality checks were conducted twice a day for 13 days after dosing. Body weights were determined at Day 0, Day 7 and at termination

of the study. At the termination of the study all animals were subjected to an abbreviated gross necropsy examination and any abnormalities were recorded. No tissues were saved.

Results

No deaths were recorded during the study and all animals exhibited body weight gain throughout the study with the exception of two females, which exhibited insignificant weight loss of 4 – 9 g during the second week of the study. All animals appeared normal throughout the study with the exception of one female and four males, which exhibited soft stools on the day of treatment. Three of the males also exhibited dark stained urogenital areas. All animals returned to normal appearance by Day 2 after treatment. No gross lesions were observed at necropsy. The estimated LD_{50} for males and females was determined to be greater than 5 g/kg body weight.

6. CLINICAL STUDIES

A large number of clinical studies with pre-term and term infants have been undertaken with infant formula supplemented with DHASCO and ARASCO. These were primarily undertaken for the purposes of establishing efficacy, however many also examined how well the supplemented formulae were tolerated and whether its use was correlated with any adverse effects, especially on growth. These studies all indicate that formula supplemented with DHASCO and ARASCO is well tolerated and is not associated with any apparent adverse effects on growth or development of the infants. The salient features of these studies are summarised in Table 3.

TABLE 3. Clinical studies with DHASCO and ARASCO in pre-term and term infants

Author Location (Sponsor)	Dose	Duration	Outcome
Pre-term infants:			
Carnielli *et al* 1994 Europe (Numico)	SF with 0.75% ARA from ARASCO + 0.6% DHA from DHASCO	Not stated	Plasma PL of SF group similar to HM group; no significant difference between groups in growth.
Clandinin *et al* 1997 Children's Health Centre, Canada (Wyeth)	SF with (i) 0.32% ARA from ARASCO + 0.24% DHA from DHASCO, (ii) 0.49% ARA from ARASCO + 0.35% DHA from DHASCO, (iii) SF with 1.1% ARA from ARASCO + 0.76% DHA from DHASCO.	4 – 6 weeks	No difference in growth or clinical parameters between formula groups. Plasma PL of low and medium dose groups similar to that of HM group. The plasma PL of the high dose group was higher than in the HM group.
Foreman-van Drongelen *et al* 1996 The Netherlands (Numico)	SF with 0.6% ARA from ARASCO + 0.4% DHA from DHASCO	From full GI feeds to 40 weeks postconceptual age	No difference between groups in growth or clinical events. Plasma PL and RBC of SF group higher than CF group.
Gross *et al* 1997 Vanderhoof *et al* 1999 Multi-centre trial – USA & Canada (Wyeth)	SF with 0.6% ARA from ARASCO + 0.4% DHA from DHASCO	From full GI feeds to 40 weeks postconceptual age	No difference in growth, serum chemistries or GI symptoms between formula groups. Plasma PL of SF group similar to HM group.
Hansen *et al* 1997, Diersen-Schade *et al* 1999 Multi-centre trial _ North America (Mead Johnson)	DHA SF with 0.34% DHA from DHASCO, DHA/ARA SF with 0.6% ARA from ARASCO + 0.33% DHA from DHASCO	Approximately 28 days	No adverse events observed. Growth in the DHA/ARA SF group was better than the CF group; no difference in visual acuity between groups.
Full term infants:			
Birch *et al* 1998 Retina Foundation of the Southwest, Dallas, USA (Mead Johnson)	SF with 0.35% DHA from DHASCO, SF with 0.35% DHA from DHASCO + 0.72% ARA from ARASCO	4 months	All groups had similar growth rates and tolerated all diets well.
Carlson *et al* 1999 Multi-centre trial – USA & Canada (Mead Johnson)	SF with 0.3% DHA from fish oil + 0.6% ARA from ARASCO, SF with 0.3% DHA from DHASCO + 0.6% ARA from ARASCO	Not stated	The SF had no adverse effects on growth or development. Infants fed the DHA/ARA SF gained weight more rapidly and weighed more than the CF group through to 12 months of age.
Gibson *et al* 1997 Flinders Medical Centre, Australia (Wyeth Nutritionals)	SF with (i) 0.2% ARA/0.2% DHA, (ii) 0.32% ARA/0.2% DHA, (iii) 0.4% ARA/0.25% DHA	6 weeks	The mid and high dose groups had plasma ARA and DHA levels similar to those in the HM group while supporting normal growth during the first 6 weeks of life.

Abbreviations: CF, control formula; SF, supplemented formula; HM, human milk; PL, phospholipid; RBC, red blood cell

7. CONCLUSIONS

No adverse findings were observed in acute studies with rats up to dose levels of 20 g/kg body weight. In one sub-chronic study some of the findings point to an impaired concentrating ability of the kidneys at the highest dose levels tested (4900 mg ARASCO/kg bw/day alone or in combination with 3650 mg DHASCO/kg bw/day), however, the vast majority of the treatment related findings observed in sub-chronic studies were generally not accompanied with any changes that would be indicative of toxicity at doses up to 2500 mg ARASCO/kg bw/day and 1250 mg DHASCO/kg bw/day. The treatment related changes observed (e.g. increased liver weights, decreased serum cholesterol and triglycerides) are entirely consistent with the physiological changes observed in response to the administration of high levels of LCPUFAs, irrespective of source, and are not a manifestation of toxicity specific to the administration of either ARASCO or DHASCO. No treatment-related adverse developmental effects were observed and the oils were also found to be negative in a number of bacterial and mammalian genotoxicity test systems.

The clinical studies with pre-term and term infants were primarily undertaken for the purposes of establishing efficacy, however a number also examined how well the DHASCO and ARASCO supplemented formulae were tolerated and whether its use was correlated with any adverse effects (e.g., reduced growth, changes in serological markers of spleen and liver function). These studies all indicate that formula supplemented with DHASCO and ARASCO is well tolerated by human infants and is not associated with any apparent adverse effects.

Overall, there is no evidence of toxicity associated with the administration of ARASCO and DHASCO at dose levels up to 2500 mg and 1250 mg/kg bw/day, respectively. These dose levels are approximately 18 – 35 fold greater than the maximum levels being added to infant formula. These results support the conclusion that DHASCO and ARASCO are safe sources of LCPUFAs for supplementation of infant formula.

APPENDIX 1

DISCUSSION OF RECURRENT FINDINGS

A number of recurrent findings (e.g., increased liver weights) were observed in both the short term (4- and 9-week) and sub-chronic studies evaluated above. These findings are also reported to occur in a number of other short term and sub-chronic studies undertaken with DHASCO and ARASCO: these studies have not been specifically assessed for this evaluation.

The performing laboratories who have undertaken the studies have not considered any of the recurrent findings to be of toxicological significance, however because their occurrence might be considered an important finding an Expert Panel was convened by Martek Biosciences (the manufacturer of DHASCO and ARASCO) to undertake a simultaneous evaluation of all the short term and sub-chronic studies conducted to date on ARASCO and DHASCO (thirteen in total) in an attempt to gain a better understanding of the relevance and consistency of these findings. The outcome of the Expert Panel review is summarised below[2].

Liver and spleen weights

One of the most common recurring findings in both the short term and sub-chronic studies evaluated above (and also in the other studies not specifically assessed for this evaluation) is a statistically significant increase in relative liver weights at the highest doses of ARASCO or DHASCO, or ARASCO/DHASCO blends. This finding however is reported to not be consistently observed across all studies, although it was consistently observed in all the short term and sub-chronic studies assessed for this evaluation. Importantly, however, the increases in liver weights observed were generally not accompanied by changes in liver histopathology or abnormally high levels of liver enzymes in the serum.

A simultaneous evaluation by the Expert Panel of the liver-related clinical chemistries in all studies did not reveal any consistent dose-dependent effects. All the studies contained both low fat and high fat controls, although the choice of control fat source varied (corn oil, soybean oil, canola oil, and high oleic sunflower oil). The high fat control was necessary to distinguish physiological responses to a high fat diet from specific test material-related phenomena. The total fat load in these studies were generally two to three times the normal level found in standard rat chow. In addition, synthetic diets were used in some studies, while others used standard rat chow, some groups mixed the oils directly into the diet and others provided the oils by gavage at a specific dose based on animal weight. As a result the trials represented a broad spectrum of designs.

Although some of the studies reported a statistically significant increase in liver weights, relative to body weights compared to the high fat controls, none of the mean relative liver weights were found to be outside the historical control range. A slight to moderate accumulation of lipid was also noted in some, but not all, of the high dose treatment groups but the incidence of this finding was not different than the high fat control group and was attributed to the high fat diet or the use of synthetic diets with high fat and carbohydrate, as

[2] As reported in the submission by the Infant Formula Manufacturers Association of Australia, the New Zealand Infant Formulas Marketers' Association and Martek Biosciences Corporation to Proposal P93: Review of Infant Formula.

has been previously reported in the literature (Clapp *et al* 1982, Shah *et al* 1986, Hoek *et al* 1988, Mars *et al* 1988). No other histopathological changes (e.g. necrosis) were observed consistently in any of the groups and there were no consistent changes in clinical chemistry that would suggest toxicity. Some studies reported a decrease in albumin levels and/or total protein levels but this finding was not consistent across studies nor did the changes parallel increases in liver weights within or across studies.

The Expert Panel undertook a comprehensive survey of the published literature which revealed that significant increases in relative liver weights from high doses of LCPUFAs is well established in rats, mice, guinea pigs, and rabbits. Most of these studies used various fish oils. Regardless of the source of the LCPUFA, a recurrent finding of the studies was a consistent 20 – 40% increase in relative liver weights in response to the feeding of test fish oils at levels of 3 – 5% of the diet as LCPUFA.

When the Expert Panel compared studies done with ARASCO and DHASCO to the studies with fish oil referred to above in most cases the doses of ARASCO and DHASCO used were lower than those for the fish oil and there were no significant increases in relative liver weights at these low levels. When the doses of ARASCO and DHASCO were similar to those reported for fish oils, the liver responses to the diets were also similar. Thus, the Expert Panel concluded that the increased liver weights seen in some studies where very high levels of DHASCO and ARASCO were used is consistent with a well-established effect of the LCPUFAs themselves and is not due to some unknown component unique to the oils.

There are several hypotheses in the literature to explain the effect of high doses of LCPUFAs, regardless of source, on liver weights. Polyunsaturated fatty acids are well known to down-regulate lipogenesis (fat biosynthesis) thereby slightly decreasing the total body weight without affecting lean body mass. This is apparently often difficult to detect in the growing animal and in fact significant changes in growth were not seen in any of the studies with DHASCO and ARASCO. If there was a reduction in total body fat as a result of LCPUFAs in the diet then other organs should also show an increase relative to body weight. Organ to organ weight ratios are therefore generally accepted to be a better measure of specific changes in an organ under these circumstances.

When the liver to brain ratios is examined in the studies there is no longer an observable effect of dose on liver weights in twelve out of the thirteen studies conducted to date. The hypothesis that the change in relative liver weights is due to a reduced lipogenesis and body fat content would be consistent with the lack of histological or clinical chemical evidence for any liver toxicity. Literature reports also note that LCPUFAs are generally metabolised in the liver and the increased liver size in response to high doses of LCPUFAs simply represents a natural hypertrophy of this organ to handle the increased metabolic load imposed upon it by the high doses of LCPUFAs.

The other major recurrent finding was with the spleen. Like the liver, relative spleen weights were increased in only some of the studies and the increased spleen weights were found only in the high dose groups. The spleen weight changes were all within the historical normal values and there were no consistent dose-related responses. Furthermore there were no significant changes in any of the studies when comparing spleen/brain weight ratios. Because there is no associated histopathology or alterations in clinical chemistry the Expert Panel concluded that these findings are not adverse effects. In many of the studies with fish oil the

authors also reported an increase in relative spleen weights in addition to increases in relative liver weights.

The Expert Panel reported that clinical studies further demonstrate that the modest increases in liver and spleen weight are of no toxicological significance. A large multi-centre study using ARASCO and DHASCO in pre-term infant formula showed no effects on growth or any serological marker of liver or spleen function (Vanderhoof *et al* 1999). These clinical studies showed no differences between formula-fed groups (with and without DHASCO and ARASCO) for liver function markers such as serum protein, albumin, ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALK P (alkaline phosphatase), bilirubin, BUN (blood urea nitrogen) or other routine analysis. Nor did these studies show any differences in markers for spleen function such as haemoglobin, mean cell volume, haematocrit, basophils, eosinophils, white blood cells, lymphocytes, monocytes, neutrophils, platelets or red blood cells.

The Expert Panel thus concluded that the administration of high doses of ARASCO, DHASCO or fish oil (more than 2 g/kg/day) to rats in a sub-chronic fashion can modestly increase liver and spleen weights relative to body weight. This effect largely takes place within a few weeks of administration of the high levels of LCPUFAs. Regardless of the source of LCPUFAs the magnitude of the response is similar when using similar levels of LCPUFAs and consistent with other reports in the literature for a wide variety of different fish oils and animal models. Thus, the relative liver and spleen weight changes appear to be a generalised LCPUFA effect and are not specific to either DHASCO or ARASCO.

Blood chemistry

As with liver and spleen weights, some of the studies also noted statistically significant changes in certain blood parameters measured. A review of these data by the Expert Panel revealed that although there are a few reported statistically significant effects, these effects are not dose-related, they are not seen consistently across comparable studies and the observations are not consistently observed in both sexes. Due to these and other factors, the Expert Panel concluded that these observations were not of toxicological significance.

The only blood chemistry markers in the thirteen rat studies that reached statistical significance were cholesterol and triglycerides. Significant reductions in cholesterol levels have been seen in two studies and a reduction of serum triglycerides was noted in the highest dose groups of three studies. This observation of cholesterol lowering is not unexpected because cholesterol lowering by fish oil is a well-observed phenomenon (Harris 1989). A reduction in serum triglycerides is also consistent with literature reports on the effect of high dose LCPUFA supplementation (especially fish oil) and is attributed to the LCPUFA not the test materials DHASCO or ARASCO.

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