

**NEW DIETARY INGREDIENT NOTIFICATION
FOR OMEGA-3 PHYTOSTEROL ESTERS**

**Enzymotec Ltd.
P.O. Box 6
Migdal HaEmeq 23106
Israel**

March 28, 2006

**NEW DIETARY INGREDIENT NOTIFICATION FOR
OMEGA-3 PHYTOSTEROL ESTERS**

Table of Contents

<u>1. NAME AND ADDRESS OF MANUFACTURER</u>	4
<u>2. NAME OF NEW DIETARY INGREDIENT (NDI)</u>	5
2.1 Statutory Basis for NDI Notification	5
<u>3. DESCRIPTION OF DIETARY SUPPLEMENT CONTAINING THE NDI</u>	7
3.1 Description of Dietary Supplement	7
3.2 Conditions of Recommended Use	7
3.3 Specifications of Product	8
3.4 Analyses of Pesticides, Heavy Metals and Other Impurities	8
3.5 Manufacturing Information	8
3.6 Substantial Equivalence of Phytosterol Content to Other GRAS Oils	9
3.7. Comparison of CardiaBeat Fatty Acid Content to Other GRAS Oils	10
<u>4. Evidence Regarding Safety of Dietary Ingredient and Components</u>	12
4.1 Absorption and Metabolic Transformation	12
4.2 Metabolism	13
4.3 Animal Studies	14
4.3.1 DHA and EPA Phytosterol Esters	14
4.3.2 CardiaBeat Studies	15
4.4 Clinical Studies	16
4.4.1 Phytosterols	16
4.4.2 EPA/DHA	17
4.4.3 CardiaBeat Clinical Trial	20

4.5 Regulatory Status	21
4.5.1 Phytosterols and Phytosterol Esters	21
4.5.2 Omega-3 Fatty Acids	23
4.6 Assessment of Safety	24
4.6.1 Phytosterols	24
4.6.2 EPA/DHA	25
4.6.3 CardiaBeat	25
<u>5.0 REFERENCES</u>	26

LIST OF APPENDICES

<u>APPENDIX A</u> Existence of Omega-3 LC-PUFA Phytosterol Esters in the Food Supply	34
<u>APPENDIX B</u> Specifications of Dietary Supplement Product and Batch Analyses	60
<u>APPENDIX C</u> Analyses for Pesticides, Heavy Metals and Other Impurities	66
<u>APPENDIX D</u> Efficacy Study of <i>CardiaBeat</i>[™] in Animal Model and Human Clinical Trial	79
<u>APPENDIX E</u> Reference Articles Attached	97
5.0 References Cover Sheet for NDI Notification Body	
6. 0 References Cover Sheet for COMPANY CONFIDENTIAL References for Appendix A; Existence of Omega-3 LC-PUFA Phytosterol Esters In the Food Supply	
Reference Cover Sheet for Efficacy Studies Appendix D	

Enzymotec Ltd.
New Dietary Ingredient Notification
March 28, 2006



**NEW DIETARY INGREDIENT NOTIFICATION FOR
OMEGA-3 PHYTOSTEROL ESTERS**

In accordance with the Dietary Supplement Health and Education Act of 1994 (DSHEA), 21 U.S.C. §350b (a) (2), and with final regulations published in the Federal Register (1997, 62:49886-49892, 21 C.F.R. § 190.6) "Requirement for Premarket Notification", the following information is submitted by Enzymotec Ltd. (Enzymotec) in support of a New Dietary Ingredient Notification for omega-3 phytosterol esters that will be formulated with DHA/EPA enriched fish oil as the **CardiaBeat™** (CardiaBeat) dietary supplement product. Enzymotec intends to market CardiaBeat as a dietary supplement in the United States. As per the statutes of the DSHEA, 21 U.S.C. § 350b (a) (2), Enzymotec will not introduce, market, distribute or sell omega-3 phytosterol esters or CardiaBeat until at least 75 days following official acknowledgement of the receipt of this notification by the U.S. Food and Drug Administration (FDA).

1. NAME AND ADDRESS OF MANUFACTURER

The name and complete address of the manufacturer of the dietary ingredient and the dietary supplement containing the dietary ingredient will be:

Enzymotec Ltd.
Hataasia 5 St.
P.O. Box 6
Migdal HaEmeq 23106
Israel

Contact: Iris Meiri-Bendek, M.Sc.
Regulatory Affairs
Enzymotec Ltd.
Hataasia 5 St.
P.O. Box 6
Migdal HaEmeq 23106
Israel
Tel: 972-4-6545112 (ex. 115)
Fax: 972-4-6443799
E-mail: irisb@enzymotec.com

2. NAME OF NEW DIETARY INGREDIENT (NDI)

The name of the new dietary ingredient (NDI) manufactured by Enzymotec is omega-3 phytosterol esters that will be marketed as a formulated dietary supplement under the product name CardiaBeat™ (CardiaBeat).

2.1 Statutory Basis for NDI Notification

Omega-3 phytosterol esters qualify as a new dietary ingredient under 21 U.S.C. 312(ff) (E) as "a dietary substance for use by man to supplement the diet by increasing the total dietary intake." The benefit of the combination of phytosterols esterified with long chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) is that the consumer can supplement his diet with two constituents with known health benefits, 1) phytosterols for cholesterol reduction and 2) DHA/EPA that have cardiovascular and other benefits. The omega-3 fatty acids and LC-PUFAs are in a class termed essential fatty acids. They are dietary precursors for eicosanoid formation, potent cellular regulatory substances and mediators of inflammation that include prostaglandins, thromboxanes, and leukotrienes.

Omega-3 phytosterol esters are in the human food supply in small amounts as a result of their ubiquitous presence in the seafood chain. Marine algae, microalgae and phytoplankton are sources of phytosterols that are the base of the seafood chain for vertebrates and invertebrates. Thus, when edible fish are consumed by man, these phytosterols and phytosterol esters with LC-PUFA content are present in the fish from ingestion of marine food chain phytosterols and subsequently esterified in the enterocytes by the fatty acid pools typical for marine fish species. This fatty acid pool in fish contains appreciable amounts of DHA, EPA and other LC-PUFAs. The esterification process associated with sterol absorption from the gut is described below.

The mechanisms by which phytosterols and phytosterol esters are handled and absorbed following ingestion are well understood and common to all vertebrate organisms. In the stomach and small intestine, the weak ester linkages of phytosterol esters are completely and rapidly hydrolyzed enzymatically by esterases to release free sterols from the ester bonded fatty acid constituents. This process is carried out by intestinal esterases, which have been reported to act at the same rate on both cholesterol and phytosterols, regardless of the chain length of the fatty acid (Mattson *et al.*, 1977). The free sterols are then absorbed into the enterocytes in the intestinal mucosa, where they are reesterified with fatty acids that are typical in composition of the total fatty acid pool for the animal species. These sterol esters are then absorbed into enterohepatic circulation to the liver for further storage and secondary metabolism. The pathways and important receptors involved in sterol absorption are

depicted graphically in the Figure 2.1 below. The fatty acids released are also absorbed via the enterocytes. Absorbed fatty acids and monoacylglycerols are derived from digestion by pancreatic lipase and other enzymes from di- and triacylglycerols, phospholipids and sterol esters (mainly cholesterol). In the enterocyte, the fatty acids and monoacylglycerides are reesterified to triglycerides, packed into chylomicrons with protein and cholesterol and exit the enterocyte by exocytosis, where they are taken up and transported in the lymphatic system for systemic absorption.

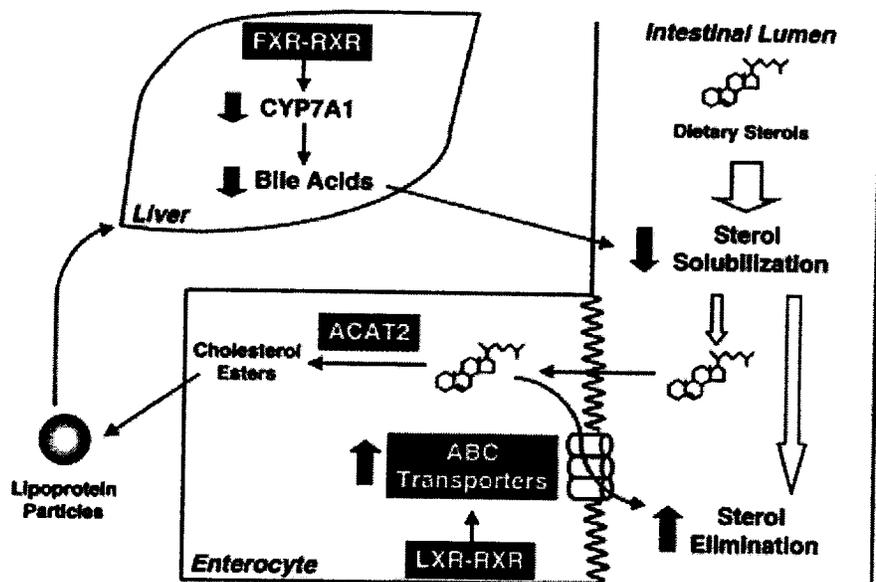


Figure 2.1 Molecular pathways involved in sterol absorption. The farnesoid X receptor–retinoid X receptor (FXR-RXR) heterodimer decreases bile acid production. The liver X receptor–retinoid X receptor (LXR-RXR) heterodimer increases ATP-binding cassette (ABC) transporter expression in enterocytes. Acyl CoA: cholesterol acyltransferase-2 (ACAT2) converts dietary free cholesterol into cholesterol esters. From Chen, H.C. 2001. Molecular Mechanisms of Sterol Absorption, *Journal of Nutrition*. 131:2603-2605.

Enzymotec has prepared a report documenting the presence of phytosterol esters in various marine animals and microalgae including many species that are consumed by man. They have conducted analysis on an accepted phytosterol-rich dietary supplement and have demonstrated the presence of brassicasterol and stigmasterol esters of DHA. This report is attached as Appendix A. Due to the cost of conducting this proprietary research and the fact that this document bears only on the statutory basis for the NDI, not on safety or purity of the NDI or final product, Enzymotec is declaring this report to be Company Confidential.

Based on this report and the above discussion reviewing the mechanisms of digestion and absorption of sterols and fatty acids, and their reesterification in enterocytes prior to systemic absorption, it is evident that phytosterol esters with marine-derived LC-PUFAs including DHA

and EPA are present in the human diet in essentially all marine seafood. Therefore omega-3 phytosterol esters meet the statutory definition of a new dietary ingredient.

3. DESCRIPTION OF DIETARY SUPPLEMENT CONTAINING THE NDI

3.1 Description of Dietary Supplement

CardiaBeat, the formulated dietary supplement product, is a complex mixture of phytosterol esters transesterified with fatty acids derived from edible marine fish sources which have substantial amounts of long chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA) and eicosopentaenoic acid (EPA) as compared to current phytosterol esters on the market that utilize fatty acids derived from vegetable oil sources. Each 1 gram capsule of the CardiaBeat dietary supplement will typically contain a range of 75-85% of the omega-3 phytosterol esters component (the NDI). CardiaBeat also contains a significant amount of DHA and EPA fatty acid diglycerides and triglycerides derived from enriched fish oil. Each 1 gram capsule of the CardiaBeat dietary supplement will typically contain a range of 15-25% commercial fish oil.

A one gram capsule will typically include 750-850 mg of the omega-3 phytosterol esters component and 150-250 mg fish oil. Due to some variance in the degree of transesterification of phytosterols and DHA/EPA content of fish oil source used, the level of fish oil to be added will vary slightly. This fish oil addition is performed to achieve a final dietary supplement product containing a constant level of 325 mg DHA/EPA/gram capsule and 400 mg free phytosterol equivalents. The remaining 275 mg in the capsule will be comprised of other fatty acids found in fish oils and glycerol in the acylglycerol compounds.

The phytosterols used in the manufacture of the NDI are derived from vegetable oils like soybean oil. The DHA/EPA and other fatty acids used to transesterify the phytosterols are derived from edible marine fish sources. The fish oil added in final product formulation is also from edible fish oil sources. All of the ingredients used in the dietary supplement are of food-grade quality.

3.2 Conditions of Recommended Use

CardiaBeat is a yellow to brown liquid that is currently packaged in 1 g soft gel capsules. Enzymotec is also considering further applications of CardiaBeat, such as nutritional bars and liquid forms, which would provide the same daily dosages as the capsule formulation. Each 1 g capsule will contain 400 mg phytosterols (as free phytosterols) and 325 mg DHA/EPA. The

recommended use of CardiaBeat is the consumption of two capsules or 2.0 g/day, in the form of 1 capsule twice per day with meals. This recommended usage would provide an intake of not less than 700 -800 mg phytosterols (as free phytosterols) and 650 mg of DHA and EPA.

3.3 Specifications of Product

Specifications for CardiaBeat are presented in Appendix B (Table B-1). Analytical data from three manufacturing lots supporting the specifications are presented in Appendix B (Table B-2). Three omega-3 phytosterol esters batches (# 1900, #150 and #200) were produced as shown in Figure 3.1 below. Batches #1900 and #150 were further blended with fish oil to produce the final CardiaBeat product.

The phytosterol composition of CardiaBeat (see Table B-2) is in the same proportion as the starting phytosterol preparation. Analyses of heavy metal impurities and microbial presence demonstrate that the omega-3 phytosterol ester product and final blended CardiaBeat dietary supplement are of suitable purity for food and dietary supplement use.

3.4 Analyses of Pesticides, Heavy Metals and Other Impurities

Analyses of CardiaBeat blended final product and omega-3 phytosterol ester product, as well as supplied fish oil-based raw materials and phytosterol raw materials are provided in Appendix C, along with a specification table for chemical contaminants in the raw materials. Phytosterols were analysed for pesticides, PCBs, dioxins and furans. One lot of omega-3 phytosterol esters product (#1900) was analysed for PAH and one lot of CardiaBeat final product (# 1900 blended) was analysed for dioxins and furans, pesticides and PCBs. All analyses were carried out by Wellington Laboratories in Canada.

These analyses demonstrate that the raw materials used in the manufacture and formulation of omega-3 phytosterol esters, as well as the final products that are blended with fish oil, are free of notable pesticide, PCB, dioxin or furan impurities and are of suitable purity for dietary supplement use.

3.5 Manufacturing Information

The new dietary ingredient in CardiaBeat is manufactured *via* the esterification of vegetable or plant phytosterols with DHA/EPA derived from fish oil. The resulting omega-3 phytosterol esters component is then blended with DHA/EPA enriched fish oil containing fatty acid diglycerides and triglycerides to make the CardiaBeat product. The manufacturing process is described below.

- 1) Food grade vegetable oil phytosterols and DHA and EPA derived from food grade fish oil are used as the starting ingredients.
- 2) The vegetable oil phytosterols and DHA and EPA mixture are combined in the presence of a catalyst. A trans-esterification reaction takes place between the phytosterols, DHA, EPA and other fatty acids present in the mixture.
- 3) The reaction is neutralized by the addition of water or an aqueous citric acid solution. Following reaction and preliminary processing, the material is further processed by methods used in traditional vegetable oil processing.
- 4) The product is then bleached by the addition of a citric acid solution and trysil/bleaching earth. The trysil and bleaching earth are approved processing aids for use in vegetable oil processing. The resulting salts and the spent trysil/bleaching earth are removed *via* phase separation and filtration.
- 5) Further distillation is then employed to remove light impurities including those affecting the stability, taste and odor of the product. Small quantities of GRAS food grade antioxidants are added as preservatives in addition to GRAS approved flavoring agents. The resulting product is an omega-3 phytosterol ester solution.
- 6) Following an analysis of the total phytosterols and DHA/EPA content, DHA/EPA enriched fish oil is added and blended with the omega-3 phytosterol ester solution so as to achieve the CardiaBeat product's specifications for total phytosterols and DHA/EPA content.

3.6 Substantial Equivalence of Phytosterol Composition to Other GRAS Oils

In table 3.1 below, the composition of soybean derived phytosterols used by Enzymotec in its manufacture is compared against the EU limits and ADM phytosterol product for their major phytosterol constituents. As this table shows, the sterol composition of the Enzymotec phytosterol starting material is in conformance to EU specifications and substantially equivalent to the ADM and other typical phytosterol products that have received multiple regulatory approvals. The composition of the vegetable or plant phytosterols in the phytosterol ester component is similar to those employed in animal studies and clinical trials discussed below in the safety assessment. The substantial equivalence between the vegetable or plant phytosterols in the omega-3 phytosterol ester component of CardiaBeat and other phytosterol formulations allows for the use of prior published safety data and regulatory approvals in support of the safety and acceptability of the phytosterol composition of CardiaBeat.

Table 3.1. Comparison of Enzymotec Phytosterol Composition to EU Specifications and Typical Vegetable Oil-Sourced Product

Composition*	EU Sterol Composition Limits**	ADM vegetable oil-sourced phytosterols	CardiaBeat soybean oil-sourced phytosterols
β-sitosterol (%)	<80	40-58	40-60
β-sitostanol (%)	<35	0-5%	<5
Campesterol (%)	<40	20-30	20-30
Campestanol (%)	<15	-	<2
Stigmasterol (%)	<30	14-22	<30
Brassicasterol (%)	<3	0-6	<3
Other sterols/stanols (%)	<3		<3
Total sterols (%)	N/A Except for tall oil source	>90	>99

*With GC-FID or equivalent method

**Adopted from Opinion of the Scientific Committee on Food on *Applications for Approval of a Variety of Plant Sterol-Enriched Foods* (5 March 2003), the sterol specifications for plant derived phytosterols and phytosterol esters for food addition have been generalized for approval of new applications based

3.7. Comparison of CardiaBeat Fatty Acid Content to Other GRAS Oils

Table 3.2 presents the typical fatty acid content of 1) Enzymotec's omega -3 phytosterol esters, 2) a common vegetable oil (soy) used as source for vegetable oil fatty acids for phytosterol ester production, 3) canola oil commonly used as a food oil, and 4) two fish oils, menhaden and SPPFBO from anchovy and sardines. All of these substances have GRAS status for direct addition to a wide variety of foods. As this table shows, the new dietary ingredient, omega-3 phytosterol esters, has a much lesser content of saturated fatty acids than menhaden oil or vegetable oils. The most notable difference in fatty acid composition between the various oils and the new dietary ingredient is the enriched levels of total

polyunsaturated fatty acids at 75%, with DHA and EPA composing 57.1 and 5.5% respectively.

Table 3.2 Fatty Acid Composition of GRAS Fish Oils, Vegetable Oils and CardiaBeat Phytosterol Esters (as % of total fatty acids)

	Menhaden Oil *	SPPBO Oil**	Refined Soybean Oil *	Canola Oil *	CardiaBeat phytosterol esters
Fatty acids, total saturated	34.7	NR	16.0	7.5	1.8
4:0	9.1	6.5	0.0	0.0	0.4
16:0	17.3	15.5	11.2	4.2	0.5
18:0	4.3	2.9	4.2	1.9	0.4
20:0	0.0	NR	0.3	0.7	0.3
22:0	0.0	NR	0.3	0.4	0.2
24:0	0.0	NR	0.0	0.2	0.0
Fatty acids, total monounsaturated	30.4	NR	23.9	61.8	11.2
16:1 undifferentiated	11.9	8.3	0.0	0.2	0.3
18:1 undifferentiated	16.6	10.1	23.7	58.9	1.4
20:1	1.5	12.1	NR	1.8	2.2
22:1 undifferentiated	0.4	8.5	1.3	0.6	7.3
Fatty acids, total polyunsaturated	39.0	NR	60.2	31.1	74.9
18:2 undifferentiated	2.5	1.2	52.8	21.3	0.2

Table 3.2 Fatty Acid Composition of GRAS Fish Oils, Vegetable Oils and CardiaBeat Phytosterol Esters (as % of total fatty acids)

	Menhaden Oil *	SPPBO Oil**	Refined Soybean Oil *	Canola Oil *	CardiaBeat phytosterol esters
18:3 undifferentiated	1.7	0.7	7.4	9.8	0.1
18:4	3.1	NR	0.0	0.0	0.1
20:4 undifferentiated	1.3	NR	0.0	0.0	0.5
20:5 n-3 (EPA)	15.0	18.0	0.0	0.0	5.5
22:5 n-6	0.0	NR	0.0	0.0	3.4
22:5 n-3	5.6	NR	0.0	0.0	4.7
22:6 n-3 (DHA)	9.8	12.0	0.0	0.0	57.1
24:1	0.0	0.0	0.0	0.0	3.3

* Source - The USDA National Nutrient Database for Standard Reference

**Source GRAS Letter from FDA on Small Planktivorous Pelagic Fish (sardine and anchovy) Body Oil (SPPFBO) GRN 000102 September 3, 2002

NR=Not Reported

4. Evidence Regarding Safety of Dietary Ingredient and Components

4.1 Absorption and Metabolic Transformation

As discussed in section 2, following ingestion of phytosterol esters, the fatty acids are rapidly and completely cleaved from the phytosterol component by esterases in the gastrointestinal tract. Therefore, *in vivo*, any phytosterol ester, marine or vegetable oil based, will be presented to the intestinal lumen and absorbed as the free phytosterol and fatty acid constituents. In the enterocyte, the free phytosterols are reesterified with fatty acid triglycerides typical of the total pool for that species. Thus, there will be no systemic absorption or exposure to the originally ingested phytosterol esters and no systemic exposure to an enriched fraction of omega-3 fatty acid phytosterol esters would occur. As a practical

matter, the systemic exposure to phytosterol esters formed in the enterocyte for absorption would be essentially the same whether one was exposed to phytosterols with vegetable fatty acids or phytosterols with marine-derived fatty acids. The only difference is that with the marine-derived omega-3 phytosterol ester product, the consumer would absorb a much greater amount of beneficial omega-3 fatty acids than would be absorbed with the vegetable fatty acid phytosterol esters. Because of this initial gut metabolism, the safety of the new dietary ingredient has entirely the same toxicity profile as its two major components, phytosterols and omega-3 fatty acids. There is no difference in potential exposure or hazard from ingestion of 500 mg of omega-3 phytosterol esters to ingestion of an equivalent amount of, for example, 300 mg phytosterols and 200 mg of omega-3 fatty acids or fish oil equivalent.

4.2 Metabolism

The metabolism of phytosterol esters begins with the hydrolysis of the ester bond, separating the molecule into its component phytosterol and fatty acid (Mattson *et al.*, 1977). This process is carried out by intestinal esterases. Due to the fact that phytosterol esters will be rapidly and completely hydrolyzed in the gastrointestinal tract, the metabolism of the phytosterol component of CardiaBeat will be discussed separately from the metabolism of DHA and EPA, the major fatty acid components of CardiaBeat.

Phytosterols have been reported to be poorly absorbed from the gastrointestinal tract, and as a result low levels of phytosterols are present in the blood (Weststrate *et al.*, 1999). Of the phytosterols that are absorbed, the majority are quickly secreted back into the bile, where they are re-circulated back to the gastrointestinal tract (Salen *et al.*, 1970). In a study conducted by Sanders *et al.* (2000), the absorption and retention of various radiolabelled phytosterols was examined in rats. Of the administered dose, 13% of campesterol and 4% of β -sitosterol and stigmasterol was absorbed from the gastrointestinal tract. Phytosterols were reported to be widely distributed throughout the body, with a tendency to be present in most tissues at a very low level. After 96 hours, the greatest retention of the absorbed phytosterols was reported to occur in the adrenal gland, intestinal epithelium and the ovaries. Sanders *et al.* (2000) reported that the predominant route of excretion was via the feces, in which 75 to 96% of the administered dose was recovered within 24 hours of dosing.

While human absorption of phytosterols has been reported to be similar to that of laboratory animals, an exception exists for individuals with a condition known as phytosterolemia, also referred to as sitosterolemia (Miettinen *et al.*, 1990; Heinemann *et al.*, 1993; Ratnayake and Vanasour, 2004). Phytosterolemic individuals have blood phytosterol levels that are 50 to 60 times greater than those of normal individuals, resulting from an increase in phytosterol absorption, reduced cholesterol synthesis, and impaired biliary secretion of phytosterols (Ikeda *et al.*, 2001; Ratnayake and Vanasour, 2004; Yu *et al.*, 2004). Individuals with

phytosterolemia are at a greater risk of developing xanthomas (cholesterol deposits in the skin and tendons) in the first decade of life and premature coronary heart disease (CHD) (Stalenhoef *et al.*, 2001).

The metabolism of EPA and DHA is very different from that of phytosterols, as approximately 60% of an administered dose of EPA or DHA is absorbed from the gastrointestinal tract, with absorption improving to almost 90% when consumed with other fats (Nordøy *et al.*, 1991; Banno *et al.*, 2001). Once EPA and DHA are absorbed from the gastrointestinal tract, they are incorporated into plasma phospholipids primarily in the liver and circulate in the blood stream (Hansen *et al.*, 1993). From plasma phospholipids, DHA and EPA can distribute to cellular membranes in a variety of tissues in the body including brain, eye, liver, kidney, red blood cells and adipose tissue (Vidgren *et al.*, 1997; Fenton *et al.*, 2001; Yasui *et al.*, 2001; Berson *et al.*, 2004). Further metabolism of DHA and EPA occurs through their removal from the membrane phospholipids, for use as essential fatty acid precursors in the synthesis of eicosanoids, which are endogenous compounds involved in blood clotting and immune responses.

4.3 Animal Studies

FDA has reviewed the basis for the safety of phytosterols and omega-3 fatty acids as described in section 4.5. Key animal studies pertinent to the safety evaluation of CardiaBeat and its components are discussed below. The phytosterol composition of phytosterol oils employed in the animal studies discussed below is similar to that of CardiaBeat as the oils are all derived from vegetable sources such as soybean oil. For this reason, these studies are considered relevant to the safety evaluation of CardiaBeat.

4.3.1 DHA and EPA phytosterol esters

Two studies were identified that examined the safety of phytosterols esterified to fish oil fatty acids, producing a product similar to the CardiaBeat omega-3 phytosterol ester. In the first study JCR:LA-cp rats, who exhibit the symptoms of obesity and insulin resistance syndrome, were administered diets containing phytosterol esters produced from canola oil and marine oil (Russell *et al.*, 2002). The rats consumed diets providing 86, 500, or 2,600 mg/kg body weight/day of phytosterol esters containing DHA and EPA for a period of 4 weeks. The phytosterol fraction was composed of 85% stigmasterol. No differences were observed in the food consumption or body weights of the rats consuming phytosterol esters and the control animals. Consumption of DHA and EPA phytosterol esters was reported to have a beneficial effect on serum triglycerol levels and on the vasculature.

In the second study, phytosterol esters containing DHA and EPA were administered through the diet to guinea pigs (Ewart *et al.*, 2002). These phytosterol esters were produced from

corn oil phytosterols and marine oil, in the presence of sodium methoxide, to produce phytosterol esters in which the fatty acid components were primarily DHA and EPA. The phytosterol component again was 85% stigmasterol. A diet containing 0.8 g of cholesterol/kg was supplemented with 25 g/kg of the phytosterol esters and 5 g/kg of corn oil, while 30 g/kg of corn oil was added to the control diet. Over the course of the 28 day study, the guinea pigs consumed an average of 28 to 36 g of food per day, providing 700 to 900 mg of phytosterol esters/day, equivalent to approximately 933 to 1,200 mg phytosterol esters/kg/day, 597 to 768 mg of phytosterols/kg/day, and 202 to 260 mg DHA and EPA/kg/day. No adverse effects or signs of toxicity were reported to result from the guinea pigs phytosterol ester consumption. Additionally, the authors reported a beneficial effect on blood levels of triacylglycerol, total cholesterol, and non-high-density cholesterol in the guinea pigs consuming phytosterol esters containing DHA and EPA.

4.3.2 CardiaBeat Studies

Enzymotec has conducted one study in which apolipoprotein E deficient (apoE) mice were administered an earlier formulation of the CardiaBeat product (Appendix D). This formulation was produced by enzymatic transesterification using a single step of transesterification of soy-derived phytosterols with fish oil glycerides (the reaction is taken place inside the fish oil).

This process used the naturally present omega-3 fatty acids in fish oil as the source of the esterified fatty acids. This earlier CardiaBeat formulation contained approximately 25% phytosterol esters, whereas the current method of esterifying the phytosterols to concentrated DHA and EPA fatty acids and diluting the product with fish oil acylglycerols contains approximately 85% phytosterol esters with enriched EPA and DHA content. In other words, the earlier formulation can be described as a blend of approximately 25% phytosterol esters and 75% fish oil while the current formulation is a blend of approximately 85% phytosterol esters and 15% fish oil.

In the animal trial conducted by Enzymotec, apoE deficient mice were chosen as they represent an animal model for atherosclerosis. Apolipoprotein E is involved in the transport of lipoproteins, and in apoE mice the impairment of these functions lead to a premature rise in LDL-cholesterol levels and the development of atherosclerotic lesions (Bobková *et al.*, 2004). In this study, 8-week-old apoE mice were administered gavage doses of fish oil-based omega-3 phytosterols in a fish oil matrix, canola oil or placebo, with 5 mice used in each dosing group, for a period of 10 weeks. The administered doses of treatment oils were equivalent to approximately 13.9 mg/day or 463 mg/kg/day. The administered formulation contained 2.5 mg/day of phytosterols, representing an exposure of 83 mg/kg/day in mice. It also contained approximately 7.5 mg DHA/EPA/day, equivalent to 250 mg/kg/day,

At the conclusion of the treatment period, the plasma lipid profile of the mice and parameters pertaining to lipid peroxidation were examined. No fatalities or incidence of toxicity were reported in any of the three treatment groups. The consumption of CardiaBeat was observed to induce a distinct tendency to lower serum cholesterol levels (19% reduction with respect placebo; $P < 0.08$), however this effect was not significantly different from that observed in the mice administered canola oil. The mice receiving CardiaBeat were reported to exhibit a significant decrease in the oxidative stress level of peritoneal macrophages as compared to both the canola oil and control mice.

4.4 Clinical Studies

4.4.1 Phytosterols

Although the majority of the clinical trials were not conducted specifically for the assessment of the safety of phytosterol consumption, they measured endpoints that can be used to lend support to the safety of phytosterols. The phytosterols employed in these studies were generally derived from vegetable oil sources and were similar in composition to the phytosterols used in the CardiaBeat product ingredient. The clinical trials reviewed have utilized administration of phytosterol doses of up to 8.6 g/day and for periods as long as 52 weeks. None of the clinical trials reviewed reported any adverse effects resulting from the consumption of phytosterols by healthy, normolipidemic, hyperlipidemic, normocholesterolemic, and hypercholesterolemic male and female adults, individuals heterozygous for phytosterolemia and adults with type 2 diabetes.

The primary concern regarding phytosterol supplementation is the effect it may have on the absorption and circulating levels of lipid soluble vitamins and carotenoids. The majority of the clinical trials reviewed examined the effect of phytosterol consumption on lipid soluble vitamins and carotenoids. No trials were identified in which phytosterol supplementation resulted in a significant decrease in the circulating levels of lipid soluble vitamins following consumption of 0.83 to 3.6 g of phytosterols for periods of 3.5 to 52 weeks (Hendriks *et al.*, 1999; Hallikainen *et al.*, 2000; Christiansen *et al.*, 2001; Judd *et al.*, 2002; Ntanios *et al.*, 2002; Hendriks *et al.*, 2003; Seki *et al.*, 2003). Additionally, in a study conducted by Noakes *et al.* (2002), individuals who consumed one or more servings of carrots, sweet potatoes, pumpkins, tomatoes, apricots, spinach or broccoli per day while receiving 2.3 g of phytosterols/day were reported to exhibit no significant change in plasma carotenoid concentrations.

Individuals with phytosterolemia are known to absorb and store larger amounts of phytosterols than normal individuals, leading to an increased risk of premature CHD. Of the clinical trials reviewed, 2 examined the effect of phytosterol supplementation on individuals

heterozygous for phytosterolemia (Stalenhoef *et al.*, 2001; Kwiterovich *et al.*, 2003). As phytosterolemia is an autosomal recessive disorder, heterozygotes do not show symptoms of phytosterolemia; however, the impact of large doses of phytosterols on them was previously unclear. In the first trial, consumption of 2.2 g of phytosterol/day was reported to significantly increase plasma campesterol and sitosterol levels; however, the increases were not significantly different from those seen in control populations that were not phytosterolemia heterozygotes (Kwiterovich *et al.*, 2003). In the second trial, the consumption of approximately 3.0 g phytosterols/day was also reported to increase campesterol and sitosterol levels and again this increase was not significantly different from that seen in normal individuals (Stalenhoef *et al.*, 2001). The authors of both studies concluded that individuals heterozygous for phytosterolemia do not accumulate phytosterols and therefore do not experience the problems of phytosterolemic individuals.

All of the clinical trials reviewed indicated that consumption of phytosterols produced beneficial effects on plasma cholesterol levels. These beneficial effects were significant in individuals consuming as little as 450 mg of phytosterols/day. Phytosterol consumption was reported to be well tolerated in all the clinical trials reviewed and no side effects were reported to result from the consumption of as much as 8.6 g phytosterols/day. In a meta-analysis conducted by Katan *et al.* (2003), forty one clinical trials examining phytosterols and phytosterol esters were reviewed. The authors concluded that sufficient evidence was present to promote the use of phytosterols for the purpose of lowering LDL cholesterol levels in persons at risk for coronary heart disease (Katan *et al.*, 2003).

4.4.2 EPA/DHA

Searches of the scientific literature were conducted to determine if there were any new publications relating to the safety of EPA and DHA since FDA's final regulation on the GRAS affirmation of menhaden oil. No new safety issues were identified. A few pertinent current reviews were identified where the utility of fish oils were reviewed and their clinical usefulness and significance discussed. These are briefly discussed below along with conclusions reached from review of numerous clinical studies on EPA, DHA and omega-3 intake from dietary supplementation.

FDA addressed the safety of DHA and EPA supplementation in the 1997 final rule on the approved use of menhaden oil as a direct food ingredient (FDA, 1997) and regarding the use of ω -3 fatty acids as a dietary supplement (FDA, 2000a). In both of these decisions, the FDA set a maximum intake of 3 g DHA and EPA/day based on scientific literature. In setting the upper limit for daily DHA and EPA intake, the FDA identified three areas of concern regarding DHA and EPA supplementation related adverse effects (FDA, 1997). Increased bleeding times were identified as an area of concern as a result of observational studies in populations

traditionally consuming fish-based diets containing high quantities of DHA and EPA (Bang and Dyerberg, 1980). Increases in low-density lipoprotein (LDL)-cholesterol levels were also identified as an area of concern by the FDA as they are known to be a risk factor in the development of CHD (Balestrieri *et al.*, 1996). The third area of concern identified by the FDA was the effects on DHA and EPA supplementation on the control of fasting glucose levels in individuals with non-insulin dependent diabetes mellitus (NIDDM) (FDA, 1997, 2000).

Due to the totality of evidence at the time of ruling concerning DHA and EPA intake, the FDA reported that increased bleeding time was a relevant determinant in limiting the consumption of DHA and EPA to 3 g/person/day; however, the FDA also acknowledged the equivocal nature of the results reported in the literature regarding increased bleeding time and dietary intake of DHA and EPA, and that the clinical significance of an increased bleeding time resulting from the consumption of DHA and EPA is lacking.

More than forty clinical trials have examined the effect of DHA and EPA consumption on bleeding time and parameters related to blood clotting and fibrinolysis. Eleven of these trials reported that bleeding times were either unchanged from baseline or within normal ranges following consumption of DHA and EPA doses ranging from 3.3 g/day for 3 weeks, to 10 g/day for 6 weeks. Clinical trials examining parameters of blood clotting revealed that the incorporation of DHA and EPA into phospholipids membranes affected clotting mediators and responses (Leaf *et al.*, 1994; Prisco *et al.*, 1994; Turini *et al.*, 1994; Mori *et al.*, 1997). The physiological response to these changes was varied, as some studies reported decreases in platelet aggregation and clotting factor levels, while others reported that these parameters did not change significantly (Nilsen *et al.*, 1993; Scheurlen *et al.*, 1993; Eritsland *et al.*, 1995, 1996; Luostarinen *et al.*, 1995; Prisco *et al.*, 1995; Freese and Mutanen, 1997; Mori *et al.*, 1997; Hansen *et al.*, 2000).

As part of a review of marine n-3 PUFAs and coronary heart disease, Schmidt *et al.*, (2005) recently reviewed the safety of omega-3 fatty acids and reported that while there has been concern about an increased risk of bleeding, especially after consumption of large doses of fish oil concentrates, there is little clinical evidence in support of this even in patients treated with aspirin or anticoagulants. They noted that it has been claimed that n-3 PUFA may deteriorate glycemic control in patients with diabetes mellitus, but recent data have discarded this hypothesis. In addition, while immune responses can be modulated by n-3 PUFA, there is no evidence that intake of n-3 PUFA is associated with an increased risk of serious infections or cancer (Larsson *et al.*, 2004).

In the clinical trials pertaining to the second area of concern identified by the FDA, an increase in LDL-cholesterol, the results are much more unified. Again more than 40 clinical

trials were identified in which the effect on DHA and EPA supplementation on plasma lipids and triglycerols, and specifically LDL-cholesterol were examined. Of these trials, the majority report that consumption of DHA and EPA did not significantly change, or resulted in a decrease in LDL-cholesterol levels. It should also be noted that in all of the studies in which an increase in LDL-cholesterol was observed, the subjects were those with established health conditions such as NIDDM, hypertriglyceridemia, IDDM, hypercholesterolemia, or hypertension (Connor *et al.*, 1993; Harris *et al.*, 1997; Gray *et al.*, 1996; Otto *et al.*, 1996; Rossing *et al.*, 1996; Swahn *et al.*, 1998; Mori *et al.*, 2000). Additionally, the physiological relevance of these results is uncertain as other CHD risk factors were unaffected by DHA and EPA consumption. Furthermore, of the clinical trials where no increase in LDL-cholesterol was reported, both healthy patients and patients with pre-existing conditions including CHD were not affected by doses as high as 10.1 g DHA and EPA/day, lending support to the safety of DHA and EPA doses >3 g/day.

The third area of concern regarding DHA and EPA consumption identified by the FDA was the control of fasting glucose levels in individuals with NIDDM. Concerning the effect of fish oil administration on the glycemic control in diabetic patients with type 2 and type 1 diabetes mellitus, conflicting results have been obtained. Some early studies on type 2 diabetes mellitus showed that fish oil may worsen glycemic control; however, the adverse effect of fish oil has almost invariably been established with large doses of omega-3 fatty acids (5.5–8 g/day) in studies lacking an appropriate control group and with a small number of subjects. Other authors found no changes or even improved insulin sensitivity in patients with type 2 diabetes mellitus. Approximately twenty clinical trials were identified in which the effects of DHA and EPA on variables related to glycemic control were examined. Of these trials, the vast majority reported that doses of >3 g DHA and EPA/day did not alter blood glucose or glycosylated hemoglobin concentrations. Furthermore, the majority of the trials also indicated that DHA and EPA had no effect on plasma insulin levels either.

In diabetics, it has been reported that fish oil intake delays the development of glucose intolerance (Feskens *et al.*, 1991). Interestingly, a recent work by Stene and Joner (2004), in a larger nationwide case-control study in Norway, found a significant association between the use of cod liver oil during the first year of life and a lower risk of type 1 diabetes mellitus, perhaps through the anti-inflammatory effects of long-chain n-3 fatty acids.

A meta analysis (Friedberg *et al.*, 1998) of 26 different trials on the effect of fish oil administration on both glycemic control and lipid parameters in type 2 and type 1 diabetic subjects showed (i) a decrease in plasma triglyceride concentration and slight but significant increase in LDL-C, both findings being more prominent in type 2 diabetes mellitus; no changes in total cholesterol and HDL-C were observed, (ii) no significant changes in HbA_{1c} occurred in diabetic patients, (iii) fasting blood glucose levels increased with borderline

significance in type 2 diabetic subjects and were significantly lower in type 1 diabetic subjects; (iv) besides, significantly, dose-response effects of EPA (g/day) on HbA_{1c} and triglycerides and DHA (g/day) on fasting blood glucose levels, HbA_{1c} and triglycerides, were demonstrated only in type 2 diabetic subjects.

The diabetes and nutrition study group of the Spanish Diabetes Association, in a 7-year prospective, population-based, multicenter study showed that normoalbuminuria and nephropathy regression in well-controlled diabetic patients with long-term diabetes (type 1 and 2) are associated with enhanced PUFAs and less saturated fatty acid consumption (Spanish Diabetes Association, 2004).

While the primary focus of the majority of the clinical trials examined were related to the three concerns identified by the FDA, several additional parameters on the immune system, and liver and kidney function were examined in the clinical trials. The results of these trials indicated that DHA and EPA had no adverse effects on any of these parameters.

Overall, clinical trials have examined the effects of DHA and EPA supplementation for as long as 4 years and provided doses as high as 10 g DHA and EPA/day. These trials demonstrate that doses of DHA and EPA significantly larger than the amount provided by the recommended use of CardiaBeat do not have an adverse effect on homeostasis, LDL-cholesterol levels, glycemic control, immune responses, or the function of the kidney or the liver. Specifically, the reported significant effects of DHA and EPA on various parameters of homeostasis are inconsistent, no longer supported by the weight of evidence, and clinical findings indicative of an adverse health effect are generally absent.

4.4.3 CardiaBeat Clinical Trial

A clinical trial conducted by Enzymotec examined the efficacy of CardiaBeat consumption (Appendix D). The trial was of crossover design and was completed by 21 volunteers who consumed olive-oil based diets supplemented with olive oil, DHA and EPA, commercially available sunflower oil phytosterol esters, or CardiaBeat for periods of 4 weeks. This study used an earlier formulation of the CardiaBeat product as described above (see section 4.3.2.).

Consumption of the diets was preceded by, and separated by, a 4-week period in which the volunteers consumed their typical diets. Baseline control diets provided either up to 200 mg/day phytosterols in the olive-oil base diet or 21.4 g of commercial product of a low-fat sunflower oil-phytosterol ester margarine containing 1.7 g/day phytosterols. In the fish oil-derived CardiaBeat treated group, DHA and EPA intakes were 5.1 g/day and 1.7 g/day soy phytosterols in a total of 9.2 g/day product. Likewise, in the group given fish oil only, DHA and

EPA intakes were 5.1 g/day in 7.6 g/day fish oil product. At the end of all dietary phases, hematological parameters, total, HDL- and LDL-cholesterol levels, as well as fasting and postprandial triglyceride concentrations, were examined.

No adverse effects were reported to result from the consumption of diets supplemented with DHA and EPA or CardiaBeat. In fact, the consumption of CardiaBeat was well tolerated by the volunteers, with only a few minor complaints of fishy aftertaste, GI upset and burping from personal interviews, as was reported for the fish oil product. Additionally, all of the hematological parameters examined remained within reference levels following consumption of both the DHA and EPA and CardiaBeat supplemented diets. The consumption of CardiaBeat was reported to have the same beneficial effect on total and LDL-cholesterol as obtained for the commercial sunflower oil based phytosterol esters and similar beneficial efficacy on reducing fasting and postprandial triglycerides levels as obtained for the fish oil administration.

4.5 Regulatory Status

4.5.1 Phytosterols and Phytosterol Esters

There have been many published studies evaluating the safety of phytosterol and phytosterol esters and these have all been evaluated by FDA in arriving at the determinations regarding GRAS status and qualified health claims. The phytosterols and phytosterol esters have been the topic of multiple GRAS Notices submitted to the Food and Drug Administration (FDA) for use as ingredients in a variety of foods that are used as a part of a low fat diet to reduce serum cholesterol. FDA has responded to each of these submissions with a statement that they had no questions regarding the conclusions that the phytosterols and phytosterol esters are GRAS for the intended applications. The phytosterol product used to manufacture the NDI, as demonstrated in the substantial equivalence discussion and table in section 3, is essentially equivalent to previously notified phytosterols in GRAS notices. Phytosterols and phytosterol esters are already incorporated in products that are currently available in the marketplace.

There were several other GRAS notice submissions to the agency that followed in subsequent years. In each case, FDA responded that they had no questions on the proposed use and did not object to the GRAS notice. These include the following: GRN 000039: Responses dated April 24 and November 2, 2000 from FDA to Novartis Consumer Health, Inc; GRN 000048: Response on November 27, 2000 from FDA to Cargill Incorporated; GRN 000053: Response from FDA dated December 20, 2000 to Proctor and Gamble; GRN 000061: Response from FDA dated April 18, 2001 to a submission from

Archer Daniels Midland Company; GRN. 000112: Response from FDA dated February 4, 2003 to Teriaka Ltd that are available at <http://www.cfsan.fda.gov/~rdb/opa-gras.html>.

FDA has previously recognized their use in foods in a final rule published in the Federal Register (65 FR 54685, 2000) on September 8, 2000 in response to petition filed by Lipton (plant sterols petitioner) and a petition filed by McNeil Consumer Health Care for a health claim associating plant sterol/stanol esters and reduced risk of coronary heart disease. Based on the totality of publicly available evidence, the agency concluded that plant sterol/stanol esters may reduce the risk of coronary heart disease. The applications in these petitions were for spreads and dressings for salads containing at least 1.6 grams of plant sterol esters per reference amount customarily consumed to bear a health claim about reduced risk of CHD. McNeil's petition requested a similar application primarily as a spread at a level of 1.7 grams of plant stanol esters per serving of spread per reference amount customarily consumed to bear a health claim about reduced risk of CHD.

FDA later issued a letter dated February 14, 2003 to Cargill regarding enforcement discretion with respect to expanded use of an interim health claim rule about plant sterol/stanol esters and reduced risk of coronary heart disease. Cargill had cited new scientific evidence and comments submitted to FDA in the plant sterol/stanol esters health claim rulemaking, and requested that FDA exercise enforcement discretion until such time as a final rule is issued that extends the authorized health claim to all the forms and sources of phytosterols, and product forms, that may effectively reduce blood cholesterol levels. Cargill further stated that the scientific literature now supports expanding the health claim to free forms of plant sterols and stanols; and to a wider range of products, including low-fat products. Cargill further stated that current science shows that the lowest effective daily intake of free phytosterols is 800 mg/day with the minimum addition of 400 mg free phytosterols per serving, and that the phytosterol substance should consist of at least 80 percent sitosterol, campesterol, stigmasterol, sitostanol, and campestanol (combined weight).

Based on preliminary review of the comments and additional scientific evidence, FDA's response was that it intends to consider the exercise of enforcement discretion, pending publication of the final rule, with respect to certain requirements of the health claim. The agency will consider exercising enforcement discretion with regard to the use of a claim about reduced risk of CHD in the labeling of a phytosterol-containing food, including foods other than those specified in §101.83(c)(2)(iii)(A), if: (1) the food contains at least 400 mg per reference amount customarily consumed (RACC) of phytosterols; (2) mixtures of phytosterol substances (i.e., mixtures of sterols and stanols) contain at least 80 percent beta-sitosterol, campesterol, stigmasterol, sitostanol, and campestanol (combined weight); (3) the food meets the requirements of §101.83(c)(2)(iii)(B)-(D); (4) products containing phytosterols, including mixtures of sterols and stanols or free forms, use a collective term in lieu of the

terms required by §101.83(c)(2)(i)(D) in the health claim to describe the substance (e.g., "plant sterols" or "phytosterol"); (5) the claim specifies that the daily dietary intake of phytosterols that may reduce the risk of CHD is 800 milligrams (mg) or more per day, expressed as the weight of free phytosterol; (6) vegetable oils for home use that exceed the total fat disqualifying level bear the health claim along with a disclosure statement that complies with §101.13(h); and (7) the use of the claim otherwise complies with §101.83. FDA is developing a final rule on this health claim and intends to publish it as expeditiously as possible.

In summary, based on the data and information that FDA has considered, which includes data and information that FDA relied upon in reaching its conclusions about the safety of phytosterols and phytosterol esters for multiple subsequent GRAS notifications to which FDA did not object, as well as the health claim for CHD, FDA has concluded that the use of phytosterols and phytosterol esters as direct food additives and dietary supplements is safe.

4.5.2 Omega-3 Fatty Acids

There have been many published studies evaluating the safety of omega-3 fatty acids and these have all been evaluated by FDA in arriving at the first and broadest determination that menhaden oil and its component fatty acids including EPA and DHA are GRAS as food ingredients subject to the limitations in 21 CFR §184.1472 and the final rule affirming as Generally Recognized as Safe (GRAS) menhaden oil published on March 23, 2005, 70 FR Number 55 Part 184 at page 14530. FDA also permitted the use of a Qualified Health Claim on dietary supplements containing EPA and DHA on October 31, 2000 as well as for conventional foods on September 8, 2004. In the October 31, 2000 letter, FDA concluded that the use of EPA and DHA omega-3 fatty acids as dietary supplements is safe and lawful under 21 CFR § 101.14, provided that daily intakes of EPA and DHA omega-3 fatty acids do not exceed 3 g/p/d from conventional food and dietary supplement sources. Further, FDA concluded that in order to help ensure that a consumer does not exceed an intake of 3 g/p/d of EPA and DHA omega-3 fatty acids from consumption of a dietary supplement with the qualified claim, an EPA and DHA omega-3 fatty acid dietary supplement bearing a qualified claim should not recommend or suggest in its labeling, or under ordinary conditions of use, a daily intake exceeding 2 grams EPA and DHA.

In addition, FDA has not objected to multiple GRAS notifications for additional sources of EPA and DHA as food ingredients in fish oils other than menhaden oil as follows: GRN 000102: Response dated September 3, 2002 to Jedwards International on a small planktivorous pelagic fish body oil (SPPFBO); GRN 000105: Response dated October 15, 2002 from FDA to Unilever United States on a fish oil concentrate; GRN 000109: Response dated December 4, 2002 to Clover Corporation on tuna oil; GRN 000137 Response dated

February 12, 2004 to Martek Biosciences on an algal oil (*Schizochytrium* sp) and GRN 000138: Response dated April 20, 2004 to Ocean Nutrition Canada, Ltd on a fish oil predominantly derived from anchovy. These GRAS notices proposed maximum use levels consistent with those specified in the tentative final rule affirming, as GRAS, menhaden oil as a direct human food ingredient with specific limitations of use.

Based on the data and information that FDA has considered, which includes data and information that FDA relied upon in reaching its conclusions about the safety of EPA and DHA omega-3 fatty acids in its GRAS affirmation of menhaden oil and multiple subsequent GRAS notifications to which FDA did not object, FDA has concluded that the use of EPA and DHA omega-3 fatty acids as direct food additives and dietary supplements is safe and lawful under 21 CFR §101.14 provided that daily intakes of EPA and DHA omega-3 fatty acids from conventional food and dietary supplement sources do not exceed 3.0 g/p/d.

4.6 Assessment of Safety

4.6.1 Phytosterols

The recommended use of two capsules of CardiaBeat will provide not less than 800 mg of phytosterols. Both *in vitro* and *in vivo* testing has indicated that phytosterols are not mutagenic nor genotoxic. Acute, subchronic, and chronic animal studies have indicated that phytosterols possess little oral toxicity to normal animals. Clinical trials that have examined the effects of the consumption of phytosterol doses as high as 10 g/day have reported that phytosterols are well tolerated and produce no adverse effects. The exposure to phytosterols resulting from the recommended use of CardiaBeat is not expected to result in any adverse effects.

As noted in the ADM GRAS notification (GRN 61) and the Lipton GRAS notification (FMF 625), the acceptable daily intake (ADI) for phytosterols based on animal studies at the highest doses tested is about 130 mg/kg/day. This equates to a daily intake of 9.1 g/day for a 70 kg adult. The ADM GRAS notice concluded that a higher ADI was appropriate, of 10.6 g/day, and FDA did not object to this conclusion. The recommended intake of phytosterols in CardiaBeat is 800 mg/day, a level twelve fold below the likely ADI from animal studies. Moreover, even this ADI is likely to be conservative. The data support a conclusion that dietary phytosterols have a very low order of toxicity and are safe at significant levels of consumption. For example, Eli Lilly conducted clinical trials and marketed Cytellin, a drug for hypercholesterolemia through 1985, which was voluntarily withdrawn due to the advent of the statins and its poor palatability. The drug contained mixed phytosterols, but was mainly composed of β -sistosterol. As noted in the review by Pollak and Kritchevsky (1981), subjects received doses as high as 24 g/day for three years without any apparent adverse effects. In a

trial with 13 patients with coronary atherosclerosis, doses as high as 52 g/day were given with no reported adverse clinical effects.

In summary, the consumption of phytosterols according to the recommended daily intake of CardiaBeat is not considered to pose any safety concerns. CardiaBeat is expected to provide phytosterol esters at levels that will have a beneficial effect on the risk of developing CHD.

4.6.2 EPA/DHA

The amount of DHA and EPA provided by the recommended daily consumption of CardiaBeat is approximately 650 mg, a level nearly five-fold below the 3.0 g/day limit established by the FDA (1997, 2004). Animal studies and human trials have demonstrated that DHA and EPA possess low oral toxicity. Clinical trials conducted with DHA and EPA doses at least 4.5 times larger than the amount present in CardiaBeat have indicated that DHA and EPA consumption produced no adverse effects on bleeding times, LDL-cholesterol levels, and fasting glucose levels in individuals with NIDDM. The amount of DHA and EPA provided by the recommended daily intake of CardiaBeat is not expected pose any safety concerns.

4.6.3 CardiaBeat

Enzymotec has conducted both animal studies and clinical efficacy trials with CardiaBeat, and no adverse effects were reported in either of the studies. Additionally, the safety of CardiaBeat and the new dietary ingredient, omega-3 phytosterol esters, are supported by extensive safety data relating to its primary components, plant phytosterols and the omega-3 fatty acids DHA and EPA. Therefore, the recommended daily consumption of CardiaBeat with its formulated ingredients is not considered to pose any safety concerns.

5.0 REFERENCES

- Balestrieri, G.P.; Maffi, V.; Sleiman, I.; Spandrio, S.; Di Stefano, O.; Salvi, A.; Scalvinin, T. 1996. Fish oil supplementation in patients with heterozygous familial hypercholesterolemia. *Recenti Prog Med* 87:102-105.
- Bang, H.O.; Dyerberg, J. 1980. The bleeding tendency in Greenland Eskimos. *Danish Med Bull* 27(4):202-205.
- Banno, F.; Doisaki, S.; Shimizu, N.; Fujimoto, K. 2002. Lymphatic absorption of docosahexaenoic acid given as monoglyceride, diglyceride, triglyceride, and ethyl ester in rats. *J Nutr Sci Vitaminol* 48(1):30-35.
- Bobková, D.; Honsová, E.; Kovář, J.; Pledne, R. 2004. Effect of diets on lipoprotein concentrations in heterozygous apolipoprotein E-deficient mice. *Physiol Res* 53(6):635-643.
- Berson, E.L.; Rosner, B.; Sandberg, M.A.; Weigel-Difranco, C.; Moser, A.; Brockhurst, R.J.; Hayes, K.C.; Johnson, C.A.; Anderson, E.J.; Gaudio, A.R.; Willett, W.C.; Schaefer, E.J. 2004. Clinical trial of docosahexaenoic acid in patients with retinitis pigmentosa receiving vitamin A treatment. *Arch Ophthalmol* 122(9):1297-1305.
- Christiansen, L.I.; Lähteenmaki, P.L.; Mannelin, M.R.; Seppänen-Laakso, T.E.; Hiltunen, R.V.; Yliruusi, J.K. 2001. Cholesterol-lowering effect of spreads enriched with microcrystalline plant sterols in hypercholesterolemic subjects. *Eur J Nutr* 40(2):66-73.
- Chen, H.C. 2001. Molecular mechanisms of sterol absorption, *Journal of Nutrition*, 131:2603-2605.
- Connor, W.E.; Prince, M.J.; Ullmann, D.; Riddle, M.; Hatcher, L.; Smith, F. E.; Wilson, D. 1993. The hypotriglyceridemic effect of fish oil in adult-onset diabetes with adverse glucose control. *Ann N Y Acad Sci* 683:337-340.
- EC Decision 2004/845/EC dated November 12, 2004 to Forbes Medi-Tech on phytosterols and phytosterol esters for novel food applications at http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2004/l_366/l_36620041211en00140016.pdf
- Eritsland, J.; Arnesen, H.; Seljeflot, I.; Kierulf, P. 1995. Long-term effects of n-3 polunsaturated fatty acids on haemostatic variables and bleeding episodes in patients with coronary artery disease. *Blood Coagul Fibrinolysis* 6(1):17-22.

Enzymotec Ltd.
New Dietary Ingredient Notification
March 28, 2006

Eritsland, J.; Arnesen, H.; Grønseth, K.; Fjeld, N.B.; Abdelnoor, M. 1996. Effect of dietary supplementation with n-3 fatty acids on coronary artery bypass graft patency. *Am J Cardiol* 77(1):31-36.

Ewart, H.S.; Cole, L.K.; Kralovec, J.; Layton, H.; Curtis, J.M.; Wright, J.L.; Murphy, M.G. 2002. Fish oil containing phytosterol esters alters blood lipid profiles and left ventricle generation of thromboxane A2 in adult guinea pigs. *J Nutr* 132(6):1149-1152.

FDA. 1997. Final rule Menhaden oil - Substances affirmed as generally recognized as safe. 62 FR 30751; June 5, 1997).

FDA Letter 2000. Dated October 31, 2000 Regarding Dietary Supplement Health Claim for Omega-3 Fatty Acids and Coronary Heart Disease (Docket No. 91N-0103) to Jonathan W. Emord, Emord & Associates, P.C. at <http://vm.cfsan.fda.gov/~dms/ds-ltr11.html>.

FDA Letter 2003. Dated February 14, 2003 Regarding Enforcement Discretion with Respect to Expanded Use of an Interim Health Claim Rule about Plant Sterol/Stanol Esters and Reduced Risk of Coronary Heart Disease to Cargill Health and Food Technologies at <http://www.cfsan.fda.gov/~dms/ds-ltr30.html>

FDA Letter 2004. Dated September 8, 2004 Letter Responding to Health Claim Petition Dated November 3, 2003 (Wellness): Omega-3 Fatty Acids and Reduced Risk of Coronary Heart Disease to Jonathan W. Emord, Emord & Associates, P.C. at <http://www.cfsan.fda.gov/~dms/ds-ltr38.html>.

Federal Register 2000. Interim Final Rule on Plant Sterol/Stanol Esters and Coronary Heart Disease Health Claim 65 FR 54685, September 8, 2000 at <http://www.cfsan.fda.gov/~lrd/fr000908.html>

Federal Register 2005. Final Rule on Menhaden Oil 70 FR 14530, March 23, 2005: Substances Affirmed as Generally Recognized as Safe at <http://www.cfsan.fda.gov/~lrd/fr050323.html>

Fenton, W.S.; Dickerson, F.; Boronow, J.; Hibbeln, J.R.; Knable, M. 2001. A placebo-controlled trial of omega-3 fatty acid (ethyl eicosapentaenoic acid) supplementation for residual symptoms and cognitive impairment in schizophrenia. *Am J Psychiatry* 158(12):2071-2074.

Feskens, E, Bowles, CH, Kromhout, D 1991. Inverse association between fish intake and risk of glucose intolerance in normoglycemic men and women, *Diabetes Care* 14: 935-941.

Enzymotec Ltd.
New Dietary Ingredient Notification
March 28, 2006

Freese, R.; Mutanen, M. 1997. α -Linolenic acid and marine long-chain n-3 fatty acids differ only slightly in their effects on hemostatic factors in healthy subjects. *Am J Clin Nutr* 66(3):591-598.

Friedberg, CE, Janssen, MJEM, Heine, RJ, Grobbee, DE. 1998. Fish oil and glycemic control in diabetes. A meta-analysis, *Diabetes Care* 21:494-500.

GRAS Notice No. GRN 000039: (April 24 and November 2, 2000) *FDA/CFSAN/OPA*: Agency Response Letter to Novartis Consumer Health, Inc on tall oil phytosterols at <http://www.cfsan.fda.gov/~rdb/opa-g039.html>

GRAS Notice No. GRN 000048: (November 27, 2000) *FDA/CFSAN/OPA*: Agency Response Letter to Cargill Incorporated on vegetable oil phytosterol esters at <http://www.cfsan.fda.gov/~rdb/opa-g048.html>

GRAS Notice No. GRN 000053: (December 20, 2000): *FDA/CFSAN/OPA*: Agency Response Letter to Proctor and Gamble on phytosterol esters at <http://www.cfsan.fda.gov/~rdb/opa-g053.html>

GRAS Notice No. GRN 000061: Response from FDA dated April 18, 2001): *FDA/CFSAN/OPA*: Agency Response Letter to Archer Daniels Midland Company on plant sterols and plant sterol esters at: <http://www.cfsan.fda.gov/~rdb/opa-g061.html>

GRAS Notice No. GRN 000102 (September 3, 2002): *FDA/CFSAN/OPA*: Agency Response Letter to Jedwards International on small planktivorous pelagic fish body oil. <http://www.cfsan.fda.gov/~rdb/opa-g102.html>

GRAS Notice No. GRN 000105 (October 15, 2002): *FDA/CFSAN/OPA*: Agency Response Letter to Unilever United States, Inc. on Marinol omega-3 concentrate. <http://www.cfsan.fda.gov/~rdb/opa-g105.html>

GRAS Notice No. GRN 000109 (December 4, 2002): *FDA/CFSAN/OPA*: Agency Response Letter to Clover Corporation on tuna oil. <http://www.cfsan.fda.gov/~rdb/opa-g109.html>

GRAS Notice No. GRN. 000112: (February 4, 2003): *FDA/CFSAN/OPA*: Agency Response Letter to Teriaka Ltd on phytosterols at <http://www.cfsan.fda.gov/~rdb/opa-gras.html>

GRAS Notice No. GRN 000137 (February 12, 2004): *FDA/CFSAN/OPA*: Agency Response Letter to Martek Biosciences Corporation on algal oil. <http://www.cfsan.fda.gov/~rdb/opa-g137.html>

GRAS Notice No. GRN 000138 (April 20, 2004): *FDA/CFSAN/OPA*: Agency Response Letter to Ocean Nutrition Canada, Ltd on fish oil from anchovy. <http://www.cfsan.fda.gov/~rdb/opa-g138.html>

GRAS Notice No. GRN 000146 (August 17, 2004): *FDA/CFSAN/OPA*: Agency Response Letter to Jedwards International on salmon oil. <http://www.cfsan.fda.gov/~rdb/opa-g146.html>

Gray, D.R.; Gozzip, C.G.; Eastham, J.H.; Kashyap, M.L. 1996. Fish oil as an adjuvant in the treatment of hypertension. *Pharmacotherapy* 16(2):295-300.

Hallikainen, M.A.; Sarkkinen, E.S.; Gylling, H.; Erkkila, A.T.; Uusitupa, M.I. 2000. Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. *Eur J Clin Nutr* 54(9):715-725.

Hansen, J.-B.; Olsen, J.O.; Wilsgard, L.; Lyngmo, V.; Svensson, B. 1993. Comparative effects of prolonged intake of highly purified fish oils as ethyl ester or triglyceride on lipids, haemostasis and platelet function in normolipaemic men. *Eur J Clin Nutr* 47(7):497-507.

Hansen, J.-B.; Grimsgaard, S.; Nordøy, A.; Bønaa, K.H. 2000. Dietary supplementation with highly purified eicosapentaenoic acid and docosahexaenoic acid does not influence PAI-1 activity. *Thromb Res* 98(2):123-132.

Harris, W.S.; Ginsberg, H.N.; Arunakul, N.; Shachter, N.S.; Windsor, S.L.; Adams, M.; Berglund, L.; Osmundsen, K. 1997. Safety and efficacy of Omacor in severe hypertriglyceridemia. *J Cardiovasc Risk* 4:385-391.

Heinemann, T.; Axtmann, G.; von Bergmann, K. 1993. Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur J Clin Invest* 23:827-831.

Hendriks, H.F.; Weststrate, J.A.; van Vliet, T.; Meijer, G.W. 1999. Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur J Clin Nutr* 53(4):319-327.

Hendriks, H.F.; Brink, E.J.; Meijer, G.W.; Princen, H.M.; Ntanios, F.Y. 2003. Safety of long-term consumption of plant sterol esters-enriched spread. *Eur J Clin Nutr* 57(5):681-692.

Ikeda, I.; Nakagiri, H.; Sugano, M.; Ohara, S.; Hamada, T.; Nonaka, M.; Imaizumi, K. 2001. Mechanisms of phytosterolemia in stroke-prone spontaneously hypertensive and WKY rats. *Metabolism* 50(11):1361-1368.

Judd, J.T.; Baer, D.J.; Chen, S.C.; Clevidence, B.A.; Muesing, R.A.; Kramer, M.; Meijer, G.W. 2002. Plant sterol esters lower plasma lipids and most carotenoids in mildly hypercholesterolemic adults. *Lipids* 37(1):33-42.

Katan, M.B.; Grundy, S.M.; Jones, P.; Law, M.; Miettinen, T.; Paoletti, R. 2003. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clin Proc* 78(8):965-978.

Kwiterovich, P.O. (Jr.); Chen, S.C.; Virgil, D.G.; Schweitzer, A.; Arnold, D.R.; Kratz, L.E. 2003. Response of obligate heterozygotes for phytosterolemia to a low-fat diet and to a plant sterol ester dietary challenge. *J Lipid Res* 44(6):1143-1155.

Larsson, SC, Kumlin, M, Ingelman-Sundberg, M, Wolk, A. 2004. Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am. J. Clin. Nutr.* 79 :935-945.

Leaf, A.; Jorgensen, M.B.; Jacobs, A.K.; Cote, G.; Schoenfeld, D.A.; Scheer, J.; Weiner, B.H.; Slack, J.D.; Kellett, M.A.; Raizner, A.E.; Weber, P.C.; Mahrer, P.R.; Rossouw, J.E. 1994. Do fish oils prevent restenosis after coronary angioplasty? *Circulation* 90(5):2248-2257.

Luostarinen, R.; Wallin, R.; Wibell, L.; Saldeen, T. 1995. Vitamin E supplementation counteracts the fish oil-induced increase of blood glucose in humans. *Nutr Res* 15(7):953-968.

Mattson, F.H.; Volpenhein, R.A.; Erickson, B.A. 1977. Effect of plant sterol esters on the absorption of dietary cholesterol. *J Nutr* 107(7):1139-1146.

Miettinen, T.A., Tilvis, R.S.; Kesäniemi, Y.A. 1990. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am J Epidemiol* 131(1):20-31.

Mori, T.A.; Beilin, L.J.; Burke, V.; Morris, J.; Ritchie, J. 1997. Interactions between dietary fat, fish and fish oils and their effects on platelet function in men at risk of cardiovascular disease. *Arterioscler Thromb Vasc Biol* 17(2):279-286.

Mori, T.A.; Burke, V.; Puddey, I.B.; Watts, G.F.; O'Neal, D.N.; Best, J. D.; Beilin, L.J. 2000. Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. *Am J Clin Nutr* 71(5):1085-1094.

- Nilsen, D.W.T.; Almdahl, S.M.; Svensson, B.; Vaage, J.; Rasmussen, K.; Østerud, B. 1993. Lipopolysaccharide induced monocyte thromboplastin synthesis and coagulation responses in patients undergoing coronary bypass surgery after preoperative supplementation with n-3 fatty acids. *Thromb Haemost* 70(6):900-902.
- Nordøy, A.; Barstad, L.; Hatcher, L.; Connor, W.E. 1991. Absorption of the n-3 eicosapentaenoic and docosahexaenoic acids as ethyl esters and triglycerides by humans. *Am J Clin Nutr* 53(5):1185-1190.
- Ntanios, F.Y.; Homma, Y.; Ushiro, S. 2002. A spread enriched with plant sterol-esters lowers blood cholesterol and lipoproteins without affecting vitamins A and E in normal and hypercholesterolemic Japanese men and women. *J Nutr* 132(12):3650-3655.
- Otto, C.; Ritter, M.M.; Soennichsen, A.C.; Schwandt, P.; Richter, W.O. 1996. Effects of n-3 fatty acids and fenofibrate on lipid and hemorrheological parameters in familial dysbetalipoproteinemia and familial hypertriglyceridemia. *Metabolism Clin Exp* 45(1):1305-1311.
- Pollak, O and Kritchevsky, D. 1981. *Sitosterol. Monographs on atherosclerosis*. Basel; S. Karger
- Prisco, D.; Paniccia, R.; Filippini, M.; Francalanci, I.; Bandinelli, B.; Comeglio, P.; Rostagno, C.; Abbate, R.; Neri Serneri, G.G. 1994. No changes in PAI-1 levels after four-month n-3 PUFA ethyl ester supplementation in healthy subjects. *Thromb Res* 76(3):237-244.
- Prisco, D.; Filippini, M.; Francalanci, I.; Paniccia, R.; Gensini, G.F.; Neri Serneri, G.G. 1995. Effect of n-3 fatty acid ethyl ester supplementation on fatty acid composition of the single platelet phospholipids and on platelet functions. *Metabolism Clin Exp* 44(5):562-569.
- Ratnayake, W.M.N.; Vavasour, E. 2004. Potential health effects associated with large intakes of plant sterols. In: Dutta, P. (Ed.). *Phytosterols as Functional Food Components and Nutraceuticals*. Marcell Dekker; New York, Vol. 1, pp. 365-395.
- Rossing, P.; Hansen, B.V.; Nielsen, F.S.; Myrup, B.; Hølmer, G.; Parving, H.H. 1996. Fish oil in diabetic nephropathy. *Diabetes Care* 19(11):1214-1219.
- Russell, J.C.; Ewart, H.S.; Kelly, S.E.; Kralovec, J.; Wright, J.L.; Dolphin, P.J. 2002. Improvement of vascular dysfunction and blood lipids of insulin-resistant rats by a marine oil-based phytosterol compound. *Lipids* 37(2):147-152 & Erratum 37(9):929.
- Salen, G.; Ahrens, E.H. (Jr.); Grundy, S.M. 1970. Metabolism of β -sitosterol in man. *J Clin Invest* 49:952-967.

Sanders, D.J.; Minter, H.J.; Howes, D.; Hepburn, P.A. 2000. The safety evaluation of phytosterol esters. Part 6. The comparative absorption and tissue distribution of phytosterols in the rat. *Food Chem Toxicol* 38(6):485-491.

SCF 2003. Opinion of the Scientific Committee on Food on *Applications for Approval of a Variety of Plant Sterol-Enriched Foods* at http://europa.eu.int/comm/food/fs/sc/scf/out174_en.pdf

Scheurlen, M.; Kirchner, M.; Clemens, M.R.; Jaschonek, K. 1993. Fish oil preparations rich in docosahexaenoic acid modify platelet responsiveness to prostaglandin-endoperoxide/thromboxane A₂ receptor agonists. *Biochem Pharmacol* 46(2):245-249.

Schmidt, EB, Arnesen, H, Christensen, JH, Rasmussen, LH, De Caterina, R, Kristensen, SD. 2005. Marine n-3 polyunsaturated fatty acids and coronary heart disease. Part I: Background, epidemiology, animal data, effects on risk factors and safety. *Thromb Res*.115: 163-170.

Seki, S.; Hidaka, I.; Kojima, K.; Yoshino, H.; Aoyama, T.; Okazaki, M.; Kondo, K. 2003. Effects of phytosterol ester-enriched vegetable oil on plasma lipoproteins in healthy men. *Asia Pac J Clin Nutr* 12(3):282-291.

Spanish Diabetes Association;The Diabetes and Nutrition Study Group (GSEDNU). 2004. Polyunsaturated fatty acid consumption may play a role in the onset and regression of microalbuminuria in well-controlled Type 1 and Type 2 diabetic peopleA 7-year prospective, population-based, observational multicenter study, *Diabetes Care* 27:1454–1457.

Stalenhoef, A.F.; Hectors, M.; Demacker, P.N. 2001. Effect of plant sterol-enriched margarine on plasma lipids and sterols in subjects heterozygous for phytosterolaemia. *J Intern Med* 249(2):163-166.

Stene, LC, Joner, G. (2003). Norwegian Childhood Diabetes Study Group, Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type 1 diabetes: a large, population-based, case-control study, *Am J Clin Nutr* 78:1128–1134.

Swahn, E.; von Schenck, H.; Olsson, A.G. 1998. Omega-3 ethyl ester concentrate decreases total apolipoprotein CIII and increases antithrombin III in postmyocardial infarction patients. *Clin Drug Invest* 15:473-482.

Turini, M.E.; Powell, W.S.; Behr, S.R.; Holub, B.J. 1994. Effects of a fish-oil and vegetable-oil formula on aggregation and ethanolamine-containing lysophospholipid generation in activated human platelets and on leukotriene production in stimulated neutrophils. *Am J Clin Nutr* 60:717-724.

Vidgren, H.M.; Ågren, J.J.; Schwab, U.; Rissanen, T.; Hänninen, O.; Uusitupa, M.I. 1997. Incorporation of n-3 fatty acids into plasma lipid fractions, and erythrocyte membranes and platelets during dietary supplementation with fish, fish oil, and docosahexaenoic acid-rich oil among healthy young men. *Lipids* 32(7):697-705.

Weststrate, J.A.; Ayesch, R.; Bauer-Plank, C.; Drewitt, P.N. 1999. Safety evaluation of phytosterol esters. Part 4. Faecal concentrations of bile acids and neutral sterols in healthy normolipidemic volunteers consuming a controlled diet either with or without a phytosterol ester-enriched margarine. *Food Chem Toxicol* 37(11):1063-1071.

Yasui, T.; Tanaka, H.; Fujita, K.; Iguchi, M.; Kohri, K. 2001. Effects of eicosapentaenoic acid on urinary calcium excretion in calcium stone formers. *Eur Urol* 39(5):580-585.

Yu, L.; von Bergmann, K.; Lutjohann, D.; Hobbs, H.H.; Cohen, J.C. 2004. Selective sterol accumulation in ABCG5/ABCG8-deficient mice. *J Lipid Res* 45(2):301-307.