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Nutritional value of micro-encapsulated fish oils in rats

Der ernährungsphysiologische Wert von mikroverkapselten Fischölen in Ratten

Summary The nutritional value of a micro-encapsulated fish oil product has been investigated. Three groups of 10 male Wistar rats each were fed diets containing 20 % (w/w) of fat, and only the type and form of the fat added was different. In the test groups 5 % (w/w) of fish oil either as such or in a micro-encapsulated form was incorporated in the diets. The remaining fat was lard supplemented with corn oil to a dietary content of linoleic acid at 10 % (w/w). The control group received lard and corn oil only. A mixture similar to the dry matter in the micro-encapsulated product was also added to the diets not containing this product.

The uptake of marine (n-3) polyunsaturated fatty acids (PUFA) from both types of fish oil supplement was reflected in the fatty acid profiles of liver phosphatidyl cholines (PC), phosphatidyl ethanolamines (PE), triglycerides (TG) and cardiolipin (CL). A suppression of the elongation of linoleic acid leading to a higher concentration of this fatty acid in liver PC and PE was also observed.

The concentration of total lipids, triglycerides, cholesterol and phospholipids in liver was similar in all groups. Supplements of long chain (n-3) PUFA did not influence the concentration of plasma TG but lowered the level of plasma cholesterol.

No change in the oxidative status, measured as glutathione peroxidase activity and cytochrome P450 concentration in the liver, was found after feeding with fish oil either directly or in the micro-encapsulated form. Intake of (n-3) PUFA lowered the concentration of vitamin E in plasma while the content of vitamin E in the liver was unchanged.

Overall, fish oil and micro-encapsulated fish oil resulted in the same fatty acid pattern in the major lipid classes and the same concentrations of liver and plasma lipids. Furthermore, supplementation of fish oil or micro-encapsulated fish oil did not induce oxidative stress when the diets were supplemented with ambient concentrations of antioxidants. It is concluded that micro-encapsulated fish oil is suitable for increasing the intake of (n-3) PUFA by fortification of normal daily food ingredients.

Zusammenfassung Der ernährungsphysiologische Wert eines mikroverkapselten Fischölprodukts wurde untersucht. Drei Gruppen mit je zehn männlichen Wistar-Ratten erhielten isokalorisches Futter mit 20 Gew. % Fettanteil. In den beiden Testgruppen wurde 5 Gew. % Fischöl entweder in der freien oder in der mikroverkapselten Form der Nahrung beigemischt. Der restliche Fettanteil bestand aus Schmalz und Maisöl mit einem Anteil von 10 Gew. % Linolsäure. Die Kontrollgruppe erhielt nur Schmalz und Maisöl. Die fettfreie Matrix des mikroverkapselten Produkts wurde dem Futter der Kontrollgruppe beigemischt, so daß sie in jedem Futter vorhanden war.

Die Aufnahme von marinen, (n-3) mehrfach ungesättigten Fettsäuren (PUFA) aus den beiden unterschiedlichen Fischöldiäten spiegelt sich im Fettsäureprofil der Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Triglyceride (TG) und Cardiolipin (CL) der Leber wider. In diesen Gruppen wurde auch eine Hemmung der Elongation von Linolsäure beobachtet, was zu höheren Werten dieser Fettsäure in Leber-PC und -PE führte.

Die Konzentrationen an Gesamtlipiden, Triglyceriden, Cholesterin

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und Phospholipiden in der Leber waren in allen Gruppen gleich. Zusätze von langkettigen (n-3) PUFA beeinflußten die Konzentration der Plasma-TG nicht, erniedrigten jedoch den Plasma-Cholesterinspiegel.

Die Bestimmung der Glutathionperoxidase-Aktivität und Cytochrom-P450-Konzentration in der Leber zeigte keine Veränderung des oxidativen Zustands nach Fütterung von freiem bzw. mikroverkapseltem Fischöl. Die Aufnahme von (n-3) PUFA verringerte die Vitamin-E-Konzentration im Plasma, während der Gehalt an Vitamin E in der Leber unverändert blieb.

Zusammengefaßt kann gesagt werden, daß die Fütterung von Fischöl und mikroverkapseltem Fischöl zum gleichen Fettsäuremuster in verschiedenen Lipidklassen (TG, PC, PE, CL) und zu vergleichbaren Konzentrationen von Leber- und Plasmalipiden führt. Außerdem führen Zusätze von Fischöl oder mikroverkapseltem Fischöl zu keinem oxidativen Stress, wenn dem Futter Antioxidantien in ausreichender Menge zugesetzt wurden. Daraus kann man schließen, daß Zusätze von mikroverkapseltem Fischöl zu Lebensmitteln geeignet sind, die tägliche Aufnahme von (n-3) PUFA zu erhöhen.

Key words Fish oil – micro-encapsulation – lipids – rats – fatty acids – oxidative status – vitamin E

Schlüsselwörter Fischöl – Mikroverkapselung – Lipide – Ratten – Fettsäuren – oxidativer Zustand – Vitamin E

Abbreviations *CL* = Cardiolipin *DHA* = docosahexaenoic acid *TG* = triglyceride · *PC* = phosphatidyl choline · *PE* = phosphatidyl ethanolamine · *PL* = phospholipid *PUFA* = polyunsaturated fatty acids

Introduction

The prophylactic effects of (n-3) polyunsaturated fatty acids (PUFA) against atherosclerotic and coronary heart diseases have increased the interest in elevating the intake of (n-3) PUFA (20). This can be obtained either by dietary supplementation of (n-3) PUFA, e.g. fish oil in gelatine capsules, or by increasing the level of fish consumed. It is, however, rather difficult to make people change their eating habits and the ingestion of the capsules may be unpleasant. Therefore, the idea of fortification of normal daily food ingredients has resulted in the development of a technique for micro-encapsulation of fish oils producing a free flowing powder without any flavor or smell of fish (Dry n-3, Danochemo A/S, Denmark).

The product consists of fish oil distributed in a food starch coated matrix of gelatine with ascorbate and tocopherol added as antioxidants. It is highly resistant to oxidation and thereby especially suitable for enrichment of e.g. bakery products, pasta, potato powder, infant formula and diet powders with long chain (n-3) fatty acids.

The main purpose of this study was to investigate the impact of dietary micro-encapsulated fish oils on the fatty acid pattern of liver PC, PE, TG and CL as compared to normal fish oil or no fish oil.

Polyunsaturated fish oil fatty acids are easily peroxidized and ingestion of polyunsaturated fat may therefore increase the risk of absorption of lipid oxidation products leading to oxidative stress *in vivo*. To examine whether micro-encapsulation diminishes this risk, induction of glutathione peroxidase activity and the concentration of cytochrome P450 present was measured. Glutathione peroxidase and cytochrome P450 participate in the cellular protection against lipid peroxidation *in vivo* and might be induced by a higher level of oxidative stress.

Enhanced ingestion of polyunsaturated fat is reflected in the fatty acid profile of the membranes leading to a higher susceptibility for *in vivo* oxidation. This effect can be neutralized by higher supplements of vitamin E (18). Therefore, the status of vitamin E was determined to ensure that enough vitamin E was administered.

To ensure detection of any potential negative effect of ingesting the micro-encapsulated product, relatively high doses of fish oil were added to the diets.

Materials and methods

Animals and diets

Thirty weanling male albino rats of Wistar SPF strain obtained from Møllegård Breeding Laboratories (Lille Skensved, Denmark) were divided into three groups of 10 animals and maintained on experimental diets for 8 weeks. The animals were caged in groups of 4 at 25 °C, a relative humidity of 45 % and with a 12/12 h dark-light cycle. Diets and water were supplemented *ad libitum*. The basal components of the diets were the same in all diets (Table 1). Only the type and form of fat in the diets was varied (Table 1). All groups received 10 % (w/w) of linoleic acid and the two fish oil groups received approximately the same fatty acids (Table 1). The feed was prepared in batches of 4 kg for each group. This was sufficient for about 14 days. The feed was stored at -80 °C until the day before use and then at -18 °C until immediately before use. As the feed was powdered it reached room temperature within a short while. The animals received fresh feed every day.

Table 1 Composition of the diets

Dietary fat	Diet group		
% (w/w)	Control	Fish oil	Micro-encaps. fish oil
Lard	19.0	13.5	13.5
Corn oil	1.0	1.5	1.5
Fish oil	0.0	5.0	5.0
Total	20.0	20.0	20.0
FA % (w/w)	Control	Fish oil	Micro-encaps. fish oil
14:0	1.7	3.3	3.3
16:0	28.1	25.3	23.9
18:0	18.4	13.7	13.6
20:0	0.2	0.2	0.2
24:0	0.0	0.2	0.3
14:1	0.0	0.2	0.2
16:1	2.7	4.3	4.4
18:1	35.7	29.6	29.6
20:1	0.7	1.0	1.1
24:1	0.0	0.2	0.2
18:2 (n-6)	10.2	9.8	9.6
20:2 (n-6)	0.2	0.2	0.2
20:4 (n-6)	0.2	0.4	0.4
18:3 (n-3)	0.6	0.7	0.8
20:5 (n-3)	0.0	4.0	4.7
22:5 (n-3)	0.0	0.5	0.5
22:6 (n-3)	0.0	2.6	3.0
Others	1.3	3.8	4.0
Total	100.0	100.0	100.0
Σ Saturated	48.4	42.7	41.3
Σ Monoenes	39.1	35.3	35.3
Σ (n-6)	10.6	10.4	10.2
Σ (n-3)	0.6	7.8	9.0

All the diets contained 20 % (w/w) fat, 34 % (w/w) starch, 15 % dry matter in micro-encapsulated product, 15 % (w/w) casein, 6 % (w/w) sucrose, 5 % (w/w) salts and trace elements (1), 4 % (w/w) cellulose fibers, 0.5 % (w/w) choline chloride and 0.5 % (w/w) vitamins (1). The dry matter of the micro-encapsulated product consists of a food starch coated matrix of gelatine and sucrose with ascorbate and tocopherol as antioxidants.

Isolation of blood and liver

The rats were sacrificed in random order over a period of 4 days. The rats were fasted for 18 h and anaesthetized by abdominal injection of 0.2 ml Mebumal (Nycomed DAK, Copenhagen, Denmark) (50 g/l) pr. 100 g body mass. After opening the chest, blood was collected from the heart using the EDTA Venoject system (Terumo, Leuven, Belgium). The liver was isolated and rinsed on the outside in cold 0.9 % (w/w) saline. Enzyme activity assays were carried out on the day of the killing. The rest of the liver was frozen in liquid N_2 and stored at -40 °C until the time of analysis.

Plasma

The blood was centrifuged at 1000 g_{av} for 10 min. Plasma was transferred to another test tube and stored at -20 °C until the time of analysis.

Preparation of liver fractions

2 g liver was homogenized in 8 ml solution (220 mM D-mannitol, 70 mM sucrose and 2 mM Hepes, pH 7.4) using a Potter-Elvehjem glass/Teflon homogenizer. The homogenate was centrifuged at 660 g_{av} and 4 °C for 15 min. The supernatant (S660) was centrifuged at 10 000 g_{av} and 4 °C for 15 min. 1 ml of the new supernatant (S10,000) was used for measuring glutathione peroxidase activity. The remaining supernatant was centrifuged at 105 000 g_{av} and 4 °C for 60 min in order to isolate the microsomes. The pellet (P105,000) was used for measuring the concentration of cytochrome P450).

Glutathione peroxidase

The activity in S10,000 from the liver was measured as oxidation of NADPH and monitored spectrophotometrically (10).

Cytochrome P450

The concentration in P105,000 from the liver was measured as the difference spectrum of cytochrome P450-CO and P450 (25).

Protein

The concentration was measured in S10,000 and P105,000 from the liver as described by Lowry (23).

Vitamin E

Extraction from plasma and liver with hexane was carried out after solubilizing the membranes with SDS (8). The concentration was measured by HPLC according to Leenheer et al. (21).

Extraction of total lipids

Lipids were extracted from the liver according to Folch (11).

Fatty acids

The composition of liver fatty acids in TG, PC, PE and CL was measured after isolation on TLC. The solvent system was hexane/ethyl ether/acetic acid, 80:20:1 (v/v/v)

for isolation of TG and methanol/acetic acid/chloroform/hexane, 20:10:40:30 (v/v/v/v) containing 18 g/l boric acid for isolation of the phospholipids. The lipids were converted to methyl esters by transmethylation with BF_3 in methanol (16), and analyzed by GC using a Hewlett-Packard 5830A chromatograph with a packed silica column (SP-2330, 2.75 m x 2 mm, Supelco, Inc.) using on-column injection. Initial oven temperature was 170 °C for 1 min, followed by a temperature gradient of 1 °C/min until 230 °C. The carrier gas was helium at an initial flow rate of 30 ml/min.

Total lipids

The total concentration of lipid in the extracts was determined gravimetrically.

Triglycerides (TG)

Total concentration of triglycerides in the liver lipid extracts was determined by comparing the concentration of fatty acid methyl esters from the triglycerides with an internal standard (pentadecanoic acid). The concentration of plasma TG was determined by an enzymatic colorimetric method (Boehringer Mannheim GmbH, no. 701 912).

Phospholipids (PL)

The concentration of phosphorus in the lipid extracts was measured spectrophotometrically. The concentration of total PL was calculated from phosphorus values by a conversion factor based on PC with one palmitic acid and one arachidonic acid (29).

Cholesterol

The concentration of cholesterol in the extracts was determined spectrophotometrically upon reaction with 2,5-dimethyl benzenesulphonic acid (33). The concentration in plasma was determined by an enzymatic colorimetric method (Boehringer Mannheim GmbH, no. 237 574).

Statistics

The results were evaluated by one way ANOVA and Duncan's multiple range test. The results are presented as mean ± standard deviation.

Results and discussion

The rats were weighed weekly and the food intake was recorded for 1 week. No signs of abnormalities or deficiencies were observed. There were no differences in body weight, liver weight or food intake between any of the groups ($p < 0.05$).

The fatty acid composition of the two main classes of PL in the membranes of the liver, PC and PE, is shown in Figs. 1 and 2. Both groups receiving fish oil, independent of the form of the supplement, had a higher content of (n-3) fatty acids in liver PC and PE at the expense of the (n-6) PUFA, arachidonic acid. Although the concentration of saturated fatty acids was higher in the control diet as compared to the other diets the ratio of saturated fatty acids to unsaturated fatty acids was the same in all groups. This indicates the necessity of maintaining a certain fluidity in the membranes. The constancy of this parameter agrees with results previously reported (12, 18, 19).

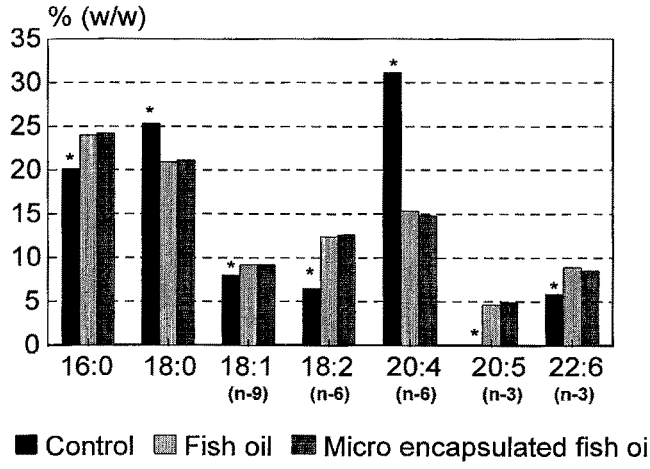

Fig. 1 Effect of dietary supplementation of fish oil or micro-encapsulated fish oil on the fatty acid composition of liver PC. An asterisk indicates that a group differs significantly from the other groups ($p < 0.05$).

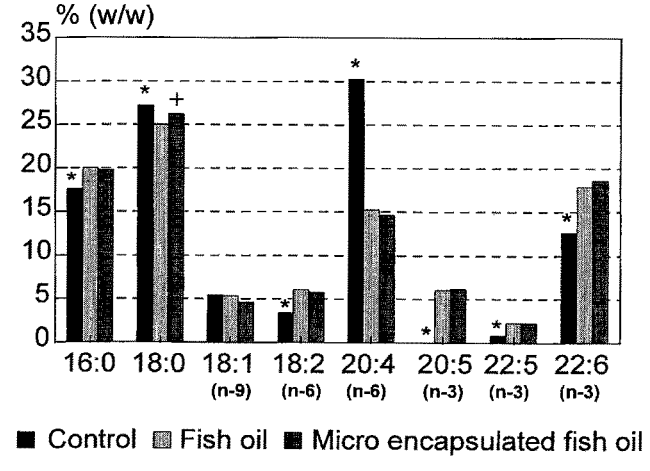

Fig. 2 Effect of dietary supplementation of fish oil or micro-encapsulated fish oil on the fatty acid composition of liver PE. An asterisk indicates that a group differs significantly from the other groups ($p < 0.05$).

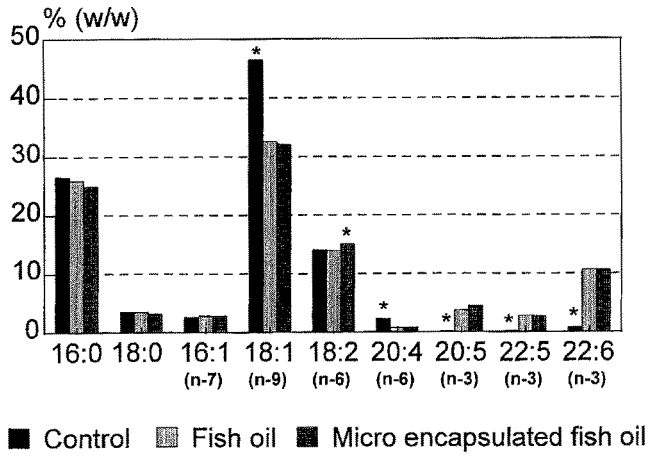

Fig. 3 Effect of dietary supplementation of fish oil or micro-encapsulated fish oil on the fatty acid composition of liver TG. An asterisk indicates that a group differs significantly from the other groups ($p < 0.05$).

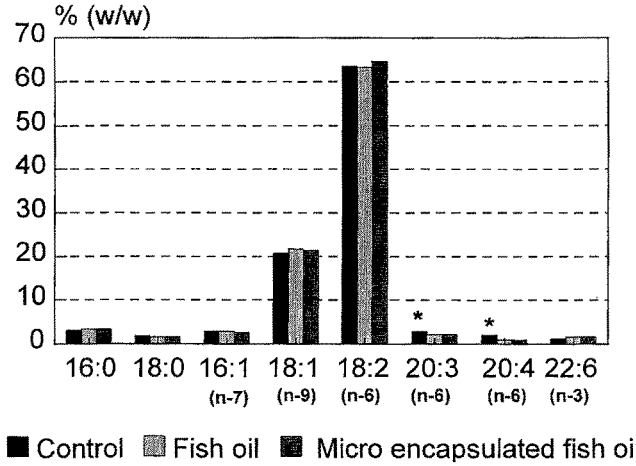

Fig. 4 Effect of dietary supplementation of fish oil or micro-encapsulated fish oil on the fatty acid composition of liver CL. An asterisk indicates that a group differs significantly from the other groups ($p < 0.05$).

The higher level of (n-3) fatty acids in the fish oil diets inhibits the transformation of linoleic acid leading to an increased level of linoleic acid in PC and PE. Similar results has been reported elsewhere (15).

The higher concentration of stearic acid in liver PC and PE from the control group may be a result of a higher content of this fatty acid in the diet. However, it might also be a reflection of a prefered distribution of molecular species maintaining a certain membrane fluidity.

The composition of the fatty acids in liver TG is shown in Fig. 3. The control group compensates for the lack of dietary (n-3) fatty acids by incorporating oleic acid in the liver TG since this fatty acid is not needed as a major fatty acid in the membranes (26). High contents of oleic acid are present in the diets as a convenient energy source. The saturated fatty acid pattern of the liver TG is the same in all groups. It is surprising that as much as 10 % of liver TG is docosahexanoic acid (DHA). However, this indicates that the concentration of DHA in PC and PE is sufficient. It may therefore be concluded that the preferential levels of DHA in PC and PE are about 8 % and 18 %, respectively.

Liver cardiolipins (CL) contained about 64 % (w/w) linoleic acid, 18:2 (n-6), and about 21 % (w/w) oleic acid, 18:1, regardless of if the diet contained fish oil or not. This is illustrated in Fig. 4. The influence of the marine (n-3) fatty acids is neglected in this phospholipid class as long as the dietary linoleic acid is ambient. These results are consistent with previous findings (17). If *in vivo* oxidation had taken place and the level of unsaturated fatty acids in the diets had been insufficient for substitution, this might influence the CL fatty acid profile since this phospholipid contains mostly unsaturated fatty acids. Apparently this was not the case.

The described changes in fatty acid profiles as a consequence of ingesting fish oil are the same whether the oil is in the micro-encapsulated form or simply mixed in the food. Accordingly, the absorption of the two forms of fish oil seems equally efficient.

Results for total lipid and the content of various lipid classes in the liver and plasma are given in Table 2. There is a tendency to higher values for total liver lipids in fish oil supplemented groups but the tendency is not significant. Likewise, no significant changes were observed in the level of liver TG, PL or cholesterol due to fish oil supplementation in any form. However, two animals in the group receiving micro-encapsulated fish oil had abnormal high concentrations of triglyceride in the liver resulting in high concentrations of total lipid in the liver as well as high liver weights. This is the reason why the average and standard deviation of these parameters are higher for the group receiving micro-encapsulated fish oil as compared to the other groups.

In the plasma, the values for TG were also equal for all three dietary groups. A TG-lowering effect in plasma induced by dietary (n-3) PUFA has been described by Lenz (22). In this study, rats were fed diets containing 20 % (w/w) fish oil. Huang et al. (15) detected a similar effect after administration of oil containing 16 % (w/w) (n-3) fatty acids while dietary fat containing 9 % (w/w) (n-3) fatty acids had no effect. This might explain why no TG-lowering effect was observed following administration of fat containing 8 % (w/w) (n-3) fatty acids.

A decline in plasma TG has also been observed in humans after ingestion of fairly high doses of fish oil (8–50 g pr day) (30, 7, 14, 31, 24, 27).

The lowered concentration of cholesterol in plasma of the fish oil groups compared to the control group is consistent with results previously found in rats (28, 22,

Table 2 Liver and plasma lipids in rats

Diet group			Control	Fish oil	Micro-encaps. fish oil
Liver	Total lipid	(mg/g tissue)	47 ± 4	52 ± 5	56 ± 13
	TG	(mg/g tissue)	10 ± 4	11 ± 2	16 ± 10
		(mg/g lipid)	200 ± 70	220 ± 80	280 ± 100
	PL	(mg/g tissue)	29 ± 3	31 ± 2	31 ± 5
		(mg/g lipid)	610 ± 70	610 ± 60	560 ± 90
	Cholesterol	(mg/g tissue)	2.9 ± 0.5	3.0 ± 0.7	3.0 ± 1.0
		(mg/g lipid)	61 ± 6	57 ± 9	61 ± 6
Plasma	TG	(mmol/l)	1.1 ± 0.3	1.0 ± 0.2	1.0 ± 0.3
	Cholesterol	(mmol/l)	1.9 ± 0.2*	1.6 ± 0.3	1.5 ± 0.3

Results are shown as average ± standard deviation (n = 10)
* Values are significantly different from other groups ($p < 0.05$)

Table 3 Liver glutathione peroxidase activity, liver cytochrome P450 concentration and liver and plasma vitamin E concentration in rats

Diet group	Control	Fish oil	Micro-encaps. fish oil
Glutathione peroxidase			
Liver (U/mg protein)	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.2
Cytochrome P450			
Liver (nmol/mg protein)	0.8 ± 0.3	0.7 ± 0.3	0.8 ± 0.3
Vitamin E			
Liver (μg/g tissue)	26 ± 3	20 ± 3	24 ± 9
Plasma (μg/ml)	7 ± 1*	6 ± 1	5 ± 2

Results are shown as average ± standard deviation
n = 10 except for vitamin E in liver where n = 4
* Values are significantly different from other groups ($p < 0.05$)

32, 3) and in humans (9, 4, 5). In other experiments, however, changes in the concentration of cholesterol in plasma as a result of administration of (n-3) PUFA to humans have not been found (2, 6, 30). This might be due to differences in control diets or the level of (n-3) fatty acids ingested.

To examine whether a fish oil supplement leading to deposition of highly unsaturated fatty acids in the membranes influences the oxidative status, the activity of glutathione peroxidase and the concentration of cytochrome P450 was measured (Table 3). Glutathione peroxidase and cytochrome P450 participate in the cellular protection against lipid peroxidation *in vivo* (13). No significant changes were observed after feeding the fish oil supplements, neither as pure fish oil or as the encapsulated product. Therefore, ingestion of moderate levels long chain (n-3) fatty acids is not likely to increase the level of oxidative stress. The level of vitamin E in plasma is only slightly diminished in the diet groups receiving fish oil. Accordingly, the concentration of vitamin E in the diets has been sufficient for compensating for the increased risk of *in vivo* oxidation due to the higher level of unsaturation of the membrane fatty acids or the ingestion of lipid oxidation products. The finding that the content of vitamin E in the liver is unaffected by the diet supports this conclusion.

The present results indicate that micro-encapsulated (n-3) PUFAs are well absorbed and incorporated into the biological membranes of the organism without inducing oxidative stress though the concentration of (n-3) PUFAs in the diets was high. It is, therefore, concluded that dry n-3 is suitable for increasing the human intake of (n-3) PUFA, e.g., by fortification of normal daily food ingredients.

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