Life Sciences, Vol. 43, pp. 2067-2075 Pergamon Press
Printed in the U.S.A.

EFFECT OF DIETARY ALPHA-LINOLENATE/LINOLEATE BALANCE ON MEAN SURVIVAL TIME, INCIDENCE OF STROKE AND BLOOD PRESSURE OF SPONTANEOUSLY HYPERTENSIVE RATS

Teruhiko Shimokawa, Atsuko Moriuchi, Takamitsu Hori, Masaki Saito, Yukio Naito, Hidehiro Kabasawa*, Yuji Nagae*, Michitaka Matsubara* and Harumi Okuyama§

From the Faculty of Pharmaceutical Sciences, and *the Faculty of Medicine, Nagoya City University, Mizuhoku, Nagoya 467, Japan

(Received in final form October 18, 1988)

Summary

Following the suckling period, stroke-prone spontaneously hypertensive rats (SHR-SP) were fed semi-purified diets supplemented either with safflower seed oil (rich in linoleic acid) or with perilla seed oil (rich in alpha-linolenic acid). The mean survival time of male SHR-SP fed the perilla diet was longer than that fed the safflower diet by 17% ($p<0.001$) while the difference was 15% in female SHR-SP ($p<0.05$). The mean survival times of female SHR-SP were more than 40% longer than those of male SHR-SP in both dietary groups. Postmortem examinations of brains revealed apoplexy-related symptoms as the major cause of the death in both dietary groups. The systolic blood pressure was lower by ca.10% (21 mmHg) in the perilla group than in both the safflower group and conventional diet group. The eicosapentaenoate (20:5 n-3)/ arachidonate (20:4 n-6) ratio of platelet phospholipids in spontaneously hypertensive rat (SHR), a measure of platelet aggregability, was much higher in the perilla group than in the safflower group. Thus, increasing the dietary alpha-linolenate/linoleate ratio resulted in an increased mean survival time of SHR-SP rats, possibly by lowering blood pressure and platelet aggregability.

The beneficial effects of dietary supplementation with fish oil were first suggested by epidemiological studies which showed that there was a lower incidence of thrombotic diseases in Eskimos as compared to Danes; this effect was suspected to be associated with a relatively larger intake by Eskimos of n-3 fatty acids as compared to n-6 fatty acids(1,2). Subsequent biochemical studies showed that eicosapentaenoate (20:5 n-3) competitively inhibits the incorporation of arachidonate (20:4 n-6) into phospholipids (3,4), thereby decreasing the availability of 20:4 n-6 for eicosanoid synthesis; in addition, 20:5 n-3 inhibits the conversion of 20:4 n-6 to eicosanoids (5,6). Thromboxane A_2, a potent proaggregatory agent is synthesized by platelets from 20:4

§ To whom correspondence should be addressed.

0024-3205/88 $3.00 + .00

n-6 (7), but 20:5 n-3 is not converted to thromboxane A_3 effectively (8,9). Furthermore, the proaggregatory activity of thromboxane A_3 is less than that of thromboxane A_2 (9). In contrast, both prostaglandin I_2 and prostaglandin I_3 are synthesized from 20:4 n-6 and 20:5 n-3, respectively, by endothelial cells, and both PG I derivatives have comparable antithrombotic activities (10). Thus by replacing n-6 fatty acids in phospholipids with n-3 fatty acids, platelet aggregability could be lowered thereby decreasing an important risk factor for thrombosis. These results provide at least a partial rationale for the claim that dietary supplementation with fish oil eicosapentaenoate has beneficial effects in humans.

Epidemiological studies have also revealed a higher incidence of apoplexy (stroke) in Eskimos than in Danes (2,11), and this has been postulated to result from a bleeding tendency resulting from too much n-3 fatty acid (11,12,13). In fact, the administration of 150 mg/kg/d of ethyl eicosapentaenoate is reported to increase the incidence of apoplexy and decrease the viability of SHR-SP (14) derived from SHR (15). If similar side effects could be expected at a comparable dose of 20:5 n-3 in humans, the applicability of fish oil supplement would be quite limited.

Here, we examined the effects of diets containing either safflower seed oil (rich in linoleic acid) or perilla seed oil (rich in α-linolenic acid) on the average survival time, incidence of apoplexy and blood pressure of SHR strains. A merit of using this combination of seed oils is that the proportions of saturated and monoenoic fatty acids are quite similar, the only major difference being in the proportions of linoleate and alpha-linolenate. Perilla oil has been used as a cooking oil in Asia while safflower oil had generaly been considered to be the best for health because of the highest linoleate content among the available vegetable oils.

Methods

Stroke-prone spontaneously hypertensive rats (SHR-SP) (72nd generation) were kindly provided by Prof. Kozo Okamoto, Kinki University School of Medicine (Minamikawachi, Osaka). They were fed a conventional laboratory chow containing 5% lipid (Nihon Clea Co. Ltd. Tokyo, CE-2, used as a normal diet) and mated at 12-17 weeks of age. The male and female pups at 3 weeks of age were weaned to either the safflower diet or the perilla diet. Spontaneously hypertensive rats (SHR) and normotensive Wistar/Kyoto rats (WKY) were obtained from Charles River of Japan.

The compositions of semi-purified diets (Nihon Clea Co.) were 24.6% milk casein, 47.0% corn starch, 2.0% alpha-starch, 8.0% cellulose, 5.0% sucrose, 2.0% vitamin mixture[1], 6.0% minerals[1], 0.4% DL-methionine[2] and 5% vegetable oil (safflower

---

1. The compositions of the vitamin mixture and mineral mixture were described elsewhere (16).
2. DL-Methionine was added to the basic diet since methionine is the limiting amino acid of casein.
3. Peroxide values were determined as described by Dahle and Holman (17).

Table I

Fatty Acid	Safflower Diet	Normal Diet	Perilla Diet
	% of total fatty acids		
14:0	0.5	0.5	0.6
16:0	8.6	15.5	8.1
16:1	N D	1.5	N D
18:0	2.2	2.5	1.9
18:1(n-9)	10.4	22.8	12.3
18:2(n-6)	78.0	48.8	12.8
18:3(n-3)	0.05	4.1	64.0
20:1(n-9)	N D	0.9	N D
20:3(n-9)	N D	N D	N D
20:4(n-6)	0.2	0.4	0.2
20:5(n-3)	N D	1.5	N D
22:6(n-3)	N D	1.1	N D

Fatty acids were designated by the carbon chain:the number of double bonds. The position of the first double bond numbered from the methyl terminus is designated as (n-9), (n-6) or (n-3). N D, not detected.

seed oil or perilla seed oil). The fatty acid compositions of the diets are shown in Table I. The diets with peroxide values below 30 meq/kg were used throughout the experiments. Rats were kept at 23±4°C. Since the SHR-SP strain is not "specific pathogen-free", but rather a carrier of pathogen(s) for pneumonia, pneumonia-like symptoms appeared toward the end of the survival time. Therefore, oxytetracycline hydrochloride (Wako Pure Chemical Ind., Osaka) was given to all rats at a concentration of 0.1% in water as recommended by the distributor.

Systolic blood pressure was measured by a plethysmographic tail method with an apparatus from Natsume Seisakusho Co.(Tokyo).

Platelets were isolated essentially as described by Billah et al (18). Lipids were extracted from the washed platelets according to the methods of Bligh and Dyer (19). After the extraction of lipids, the total platelet protein was collected from the water layer of lipid extraction by precipitation with trichloroacetic acid (20). For the separation of individual lipids, prewashed and activated Silica Gel plates (Merck 60) were used. Cholesterol and phospholipids were separated with petroleum ether/ diethyl ether/ acetic acid (80:30:1) as a solvent. Each phospholipid was separated by two-dimensional thin-layer chromatography with chloroform/ methanol/ 28% NH_4OH (65:35:6) and chloroform/ acetone/ acetic acid/ methanol/ water (50:20:15:10:5) as solvents for the first and second dimensions, respectively. Separated phospholipids were extracted from the Silica Gel, and their fatty acids were analyzed as methyl esters by gas-liquid chromatography (GLC) with a column of 10% EGSS-X on Chromosorb W (AW) at 190 °C (16). Cholesterol was quantitated by GLC of its trimethylsilyl ether using ergosterol as an internal standard. Total phospholipids and protein concentrations were determined by the methods of Eibl et al.(21) and Lowry et al.(22), respectively.

After death, the sliced brains were fixed in 10% formalin solution. The fixed brain slices were then stained by

hematoxylin-eosin stain method and examined by light microscopy. In addition to the cerebral softening, the encephalomalacia was judged by the hypertrophic astrocytes and by invasion of neutrophils and/or macrophages around or inside foci.

In this paper, results were expressed as mean ± Standard Deviation. Statistical analyses were performed by Student's t-test.

Results

The safflower diet and the perilla diet were fed to various strains of rats (SHR-SP, SHR, WKY, Wistar, Donryu) for up to 2 generations in our laboratory. So far, no significant differece was observed in the appearance, growth rate, weight gain or litter size between the two dietary groups. The average survival time of male SHR-SP fed the safflower diet was 50.9±4.0 weeks while that of the perilla group was 59.5±3.4 weeks (Fig.1).

Similarly, the average survival time of female SHR-SP fed the safflower diet and perilla diet were 75.7±11.0 weeks and 86.7±3.4 weeks, respectively. The differences between the two dietary groups were statistically significant (male; $p<0.001$, female; $p<0.05$). The average survival time of females were longer than those of males by 49% in the safflower group and 46% in the perilla group.

In a separate experiment, the average survival time of male SHR-SP fed a conventional diet (normal diet) was found to be 52.7 ± 8.7 weeks (n=4). The number of rats in the normal diet group

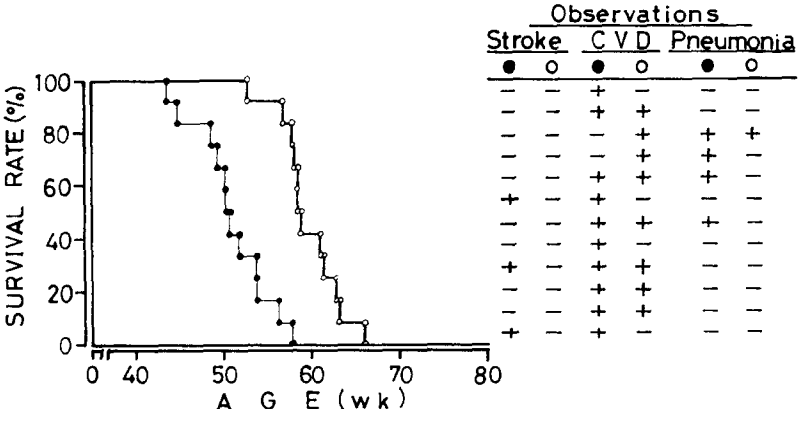

FIG. 1

Male SHR-SP rats (n=12) were fed the test diets from 3 weeks of age (weaning). Apparent symptoms of strokes and pneumonia are indicated as stroke (+ or -) and pneumonia (+ or -). Symptoms of cerebrovascular disease (CVD) such as hemorrhage and encephalomalacia were judged for the fixed brain slices prepared after the death and are shown as CVD (+ or -). (●); Safflower diet group, (○); Perilla diet group, See text for details.

was too small to show statistical differences among the three dietary groups.

While alive, 3 male rats in the safflower group showed typical symptoms of stroke; paralysis of limbs and incontinence of urine, but appreared to recover relatively easily from the stroke. No such typical symptoms were observed in the perilla group. The pneumonia-like symptoms (sneezing and wheezing) were observed in 4 rats among 12 rats fed the safflower diet (33%) and in 1 rat among 12 rats fed the perilla diet (8%). After death, brains were examined by light microscopy. Symptoms of cerebrovascular disease (CVD) such as intracerebral hemorrhage, subarachnoid hemorrhage and/or encephalomalacia caused by cerebral embolism were observed in most of rats, regardless of diet. The anemic encephalomalacia recognized was 67% (n=8) in the safflower group and 58% (n=7) in the perilla group, while the incidence of intracerebral hemorrhage was 33% (n=4) in the safflower group and 8% (n=1) in the perilla group. Hemorrhage into pia-arachnoid space was seen in 75% (n=9) of the safflower group and 67% (n=8) of the perilla group. The maximal area of encephalomalacia was measured for each rat, but no significant difference was observed in the average maximal area between the two dietary groups. Thus, the symptoms of cerebrovascular diseases tend to be more severe in the safflower group than in the perilla group, but the number of observations was too small to establish a statistical significance.

As shown in Table II, the systolic blood pressure of the perilla group was significantly ($p<0.01$) lower by about 10% than those of the safflower group at 10 and 18 weeks of age. The blood pressure of the normal diet group at 18 weeks of age was about the same as that of the safflower group although they were not litter mates.

Similarly, the blood pressure of SHR and normotensive control WKY strains were measured (Fig.2). Diets did not affect the blood pressure of SHR for up to 7 weeks of age. However, the blood pressure of SHR fed the perilla diet was significantly lower than that of the safflower group from 9 weeks of age. The difference was about 10%. In contrast, the blood pressure of the WKY strain was the same throughout the period examined regardless of diet.

Table II

Dietary Groups	Systolic Blood Pressure at Ages of 10 Weeks (mmHg)	18 Weeks (mmHg)
Safflower Diet	211±5 (n=6)	227±14 (n=12)
Perilla Diet	* 187±13 (n=4)	** 206±15 (n=12)

Litter mates were divided into two groups and fed the test diets from 3 weeks of age (weaning). In a separate experiment with different litter mates, the blood pressure of the normal diet group at 18 weeks of age was 226 ± 6 (n=5). *) $p<0.02$, **) $p<0.01$. See text for details.

Platelets were isolated from SHR fed either the safflower diet or the perilla diet. Phospholipid contents (safflower group; 0.28 μmol/mg protein, perilla group; 0.26 μmol/mg protein) and the proportions of the individual phospholipids in platelets were quite similar between the two dietary groups (data not shown). Cholesterol contents were not different (the safflower group; 95 μg/mg protein, the perilla group; 87 μg/mg protein). However, as shown in Table III the 20:5 n-3/ 20:4 n-6 ratios were much higher in the perilla group than in the safflower group, as expected from the difference in the dietary fatty acids.

Discussions

A hypotensive effect on SHR of dietary fish oil, which is rich in 20:5 n-3 and 22:6 n-3, was first reported by Schoene and Fiore (23). We have found that feeding an α-linolenate rich diet (perilla diet) to SHR or SHR-SP resulted in increases in the proportions of 20:5 n-3 and 22:6 n-3 in the phospholipids of some tissues, and that the perilla diet had a hypotensive effect (Fig. 2). A slight but significant fall in systolic blood pressure was observed in volunteers fed a fish-oil concentrate (12,24-26), although plasma renin activity was higher in Eskimos (27). The degree of changes in the 20:5 n-3/20:4 n-6 ratios of platelet lipids observed in SHR (Table III) were enough to induce changes

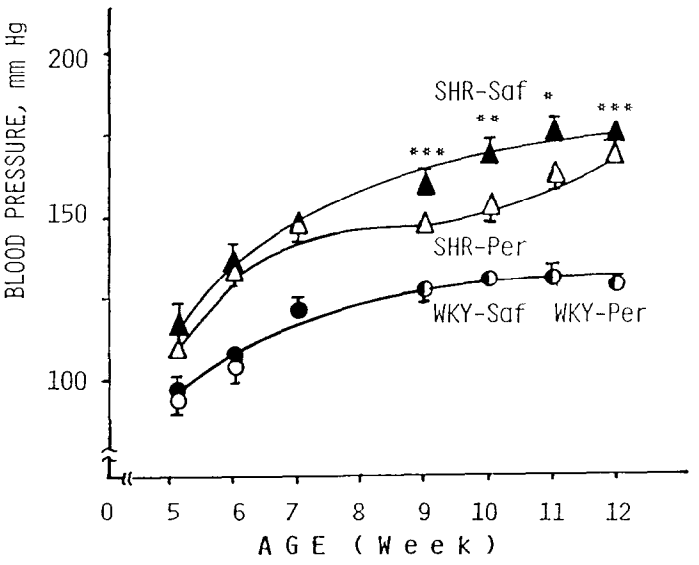

FIG. 2

Male rats were fed the test diets through two generations, and the systolic blood pressure of the second generation was measured. Each point represents the average (±S.D.) of eight rats each determined in triplicate. Statistical significance between the safflower diet group and perilla diet group in SHR ; *) $p<0.1$, **) $p<0.05$, ***) $p<0.01$. WKY-(Safflower diet; ●, Perilla diet; ○), SHR-(Safflower diet; ▲, Perilla diet; △)

Table III

Dietary Groups	20:5 n-3 / 20:4 n-6 ratio				
	PC	PE	PS	PI	Total
Safflower Diet	0.02	0.00	0.01	0.00	0.01
Perilla Diet	0.80	0.61	0.26	0.23	0.54

Platelet phospholipids from male SHR-SP were extracted, and separated by thin-layer chromatography. The fatty acids were analyzed by gas-liquid chromatography as described in the text. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) were analyzed. Values were calculated from the averages of two determinations.

in platelet aggregability by at least 25% as determined in the Sprague-Dawley strain (Watanabe, S. et al., manuscript in preparation). Both the hypotensive effect and the decrease in platelet aggregability are assumed to contribute to the observed increases in the average survival time observed in SHR-SP fed the perilla diet as compared to the safflower diet group.

In contrast to vegetable oil feeding, the administration of ethyl eicosapentaenoate was reported to decrease the mean survival time of SHR-SP (14). The dose of α-linolenate used in the present experiments was about 20 times greater than the dose of 20:5 n-3 used by others (14). Significant amount of 20:5 n-3 were found in phospholipids of some tissues including platelets from rats fed the perilla diet, although a direct comparison of the amounts of 20:5 n-3 in tissues of rats supplemented with α-linolenate (18:3 n-3) (Table III) and 20:5 n-3 (14) is not possible at present. Possible cardiotoxic activities of the ethylesters of fatty acids are reported by Laposata and Lange (28). However, it seems unlikely that the toxicity of ethyl eicosapentaenoate administration (14) is due to its ethylester form since the dose of ethyl eicosapentaenoate (4 mg as ethanol/rat/d) was much below the cardiotoxic dose (1560 mg as ethanol/rat/d) (29).

It is widely accepted that linoleic acid is beneficial to human health. Therefore, considerable efforts have been made by the food industry to increase the linoleate content of foods. In contrast, very little attention has been paid to α-linolenate, which is relatively rich in the leaves and roots of vegetables. The α-linolenate/ linoleate ratios of the safflower diet, normal diet and perilla diet used in our experiments were 1/100, 1/10 and 5/1, respectively. Beneficial effects of feeding the perilla diet as compared to the normal diet have so far been shown for learning ability (16), electroretinographic responses (30), metastatic potentials of tumor cells (31), allergic responses (32), hypertension and survival time (present experiments) in rats. Furthermore, in a separate experiment, we have obtained the result that the average survival time of a conventional strain of rats (Donryu) fed the perilla diet were longer than those fed the safflower diet by 11% ($p<0.05$)(unpublished data). Thus far,

no unfavorable symptoms have been observed to result from feeding the perilla diet to rats for up to two generations.

One objection to applying these results obtained with rats directly to human nutrition is that the conversion of linoleate and α-linolenate to highly unsaturated fatty acids is relatively slow; it takes time in human tissues to show increased proportions of highly unsaturated 20 carbon fatty acids in response to increases in the dietary supply of 18 carbon unsaturated fatty acids. However, this does not mean that the desaturation-elongation activity in human beings is intrinsically low. Highly unsaturated fatty acids are found in plasma lipids of complete vegetarians, who depend entirely on linoleate and α-linolenate for their supply of ssential fatty acids, usually do not develop essential fatty acid deficiency (33,34). Supplying α-linolenate was sufficient for humans to recover from n-3 fatty acid deficiency symptoms (35,36). Cultured cells of human origin show desaturation-elongation activities comparable to those of animal origin (37,38). These desaturation-elongation enzymes are known to be tightly regulated; the enzyme activities vary depending on the dietary supply of different types of unsaturated fatty acids (39).

We speculate that it is important to try to increase the α-linolenate/ linoleate ratio of our foods. We should not accept any attempts to remove α-linolenate from vegetable oils and oil products (40) simply because α-linolenate is more oxidizable than linoleate.

Acknowledgments

We are grateful to Dr. Atsushi Hashimoto, Dr. Shigeo Murase, Mr. Hiroyuki Kurita, Naoki Segi, Yukiko Kimura, Nobuhiro Yamamoto, Kenichi Izutsu and Shiro Watanabe of our laboratory for their help in keeping animals and for their helpful discussions. This work was supported in part by grants from Takeda Science Foundation and Ito Science Foundation.

References

1. J. DYERBERG, Nutr. Rev. 44 125-134 (1986).
2. N. KROMANN and A. GREEN, Acta Med. Scand. 208 401-406 (1980).
3. W.E.M. LANDS, M. INOUE, Y. SUGIURA and H. OKUYAMA, J. Biol. Chem. 257 14968-14972 (1982).
4. S. MURASE, K. YAMADA and H. OKUYAMA, Chem. Pharm. Bull. 36 2109-2117 (1988).
5. B.R. CULP, B.G. TITUS and W.E.M. LANDS, Prostaglandins and Medicine 3 269-278 (1979).
6. J. DYERBERG and H.O. BANG, Lancet 1 152 (1978).
7. M. HAMBERG, J. SVENSSON and B. SAMUELSSON, Proc. Natl. Acad. Sci. USA 72 2994-2998 (1975).
8. P. NEEDLEMAN, H. SPRECHER, M.O. WHITAKER and A. WYCHE, Prostaglandin Thromboxane Res. 6 61-68 (1980).
9. P. NEEDLEMAN, A. RAZ, M.S. MINKES, J.A. FERRENDELLI and H. SPRECHER, Proc. Natl. Acad. Sci. USA 76 944-948 (1979).
10. J. DYERBERG, H.O. BANG, E. STOFFERSEN, S. MONCADA and J. R. VANE, Lancet 2 117-119 (1978).
11. H.O. BANG and J. DYERBERG, Dan. Med. Bull. 27 202-205

(1983).
12. J.Z. MORTENSEN, E.B. SCHMIDT, A.H. NIELSEN and J. DYERBERG, Thromb. Haemostasis 50 543-546 (1983).
13. J.H. BROX, J-E. KILLIE, S. GUNNES and A. NORDOY, Thromb. Haemostasis 46 604-611 (1983).
14. T. YASUGI, J. Jpn Atheroscler. Soc. 15 379 (1987).
15. K. OKAMOTO and K. AOKI, Jpn. Cir. J. 27 282-293 (1963).
16. N. YAMAMOTO, M. SAITOH, A. MORIUCHI, M. NOMURA and H. OKUYAMA, J. Lipid Res. 28 144-151 (1987).
17. L.K. DAHLE, R.T. HOLMAN, Anal. Chem. 33 1960-1961 (1961).
18. M.M. BILLAH, E.G. LAPETINA and P. CUATRECASAS, J. Biol. Chem. 256 5399-5403 (1981).
19. E.G. BLIGH and W.J. DYER, Can. J. Biochem. Physiol. 37 911-917 (1959).
20. A. BENSADOUN and D. WEINSTEIN, Anal. Biochem. 70 241-250 (1976).
21. H. EIBL, E.E. HILL and W.E.M. LANDS, Eur. J. Biochem. 9 250-258 (1969).
22. O.H. LOWRY, N.J. ROSEBROUGH, A.L. FARR and R.J. RANDALL, J. Biol. Chem. 193 265-275 (1951).
23. N. W. SCHOENE and D. FIORE, Prog. Lipid Res. 20 569-570 (1982).
24. T.A.B. SANDERS, M. VICKERS and A.P. HAINES, Clin. Sci. 61 317-324 (1981).
25. P. SINGER, M. WIRTH, S. VOIGT, S. ZIMONTKOWSKI, W. GODICKE and H. HEINE, Biomed. Biochem. Acta 43 421-425 (1984).
26. P. SINGER, M. WIRTH, S. VOIGT, E. RICHTER-HEINRICH, W.C. TAUBE, Atherosclerosis 56 223-235 (1985).
27. K.A. JORGENSEN, A.H. NIELSEN and J. DYERBERG, Acta Med. Scand. 219: 473-479 (1986).
28. E.A. LAPOSATA and L.G. LANGE, Science 231 497-499 (1986).
29. L.D. SEGEL, S.V. RENDIG, V. CHOQUET, K. CHACHO, E.A. AMSTERDAM and D.T. MASON, Cardiovasc. Res. 9 649-663 (1975).
30. I. WATANABE, M. KATO, H. AONUMA, A. HASHIMOTO, Y. NAITO, A. MORIUCHI and H. OKUYAMA, Adv. Biosci. 62 563-570 (1987).
31. T. HORI, A. MORIUCHI, H. OKUYAMA, T. SOBAJIMA, K. TAMIYA-KOIZUMI and K. KOJIMA, Chem. Pharm. Bull. 35 3925-3927 (1987).
32. A. HASHIMOTO, M. KATAGIRI, S. TORII and H. OKUYAMA, Jpn. J. Allergol. 37 157-165 (1988).
33. T.A.B. SANDERS, and K.M. YOUNGER, Br. J. Nutr. 45 613-616 (1981).
34. S. RENAUD and A. NORDOY, Lancet 2 1169 (1983).
35. R.T. HOLMAN, S.B. JOHNSON and T.F. HATCH, Am. J. Clin. Nutr. 35 617-623 (1982).
36. K.S. BJERVE, S. FISCHER and K. ALME, Am. J. Clin. Nutr. 46 570-576 (1987).
37. R.R. ISSEROFF, V.A. ZIBOH, R.S. CHAPKIN and D.T. MARTINEZ, J. Lipid Res. 28 1342-1349 (1987).
38. M. MAEDA, O.DOI and Y. AKAMATSU, Biochim. Biophys. Acta 530 153-164 (1978).
39. I.N.T. DE GOMEZ DUMM, M.J.T. DE ALANIZ and R.R. BRENNER, Lipids. 18 781-788 (1983).
40. ANONYMOUS, J. Amer. Oil Chem. Soc. 59 882A-884A (1982).