Journal of Chromatography, 572 (1991) 1–9
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6058

Analysis of polyunsaturated fatty acids in blood serum after fish oil administration

H. M. LIEBICH*, C. WIRTH and B. JAKOBER

Medizinische Universitätsklinik, 7400 Tübingen (Germany)

(First received April 24th, 1991; revised manuscript received July 8th, 1991)

ABSTRACT

Free and total fatty acids in the blood serum of patients with hyperlipoproteinemia have been analysed as their methyl esters by capillary gas chromatography using an FFAP column. In one-step reactions the free fatty acids in serum react with methanol–acetyl chloride (50:1, v/v) at 25°C, the total fatty acids (free plus esterified) are transesterified with methanol–toluene–acetyl chloride (8:2:1, v/v) at 100°C. The quantification of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is based on an internal standard (13,16,19-docosatrienoic acid) and on calibration standards. Under normal diet the concentrations of EPA and DHA are as follows (mean ± S.D., $n = 27$): free EPA, 0.2 ± 0.1 mg/dl; free DHA, 0.6 ± 0.2 mg/dl; total EPA, 3.6 ± 2.1 mg/dl; total DHA 11.4 ± 3.1 mg/dl. Under a fish oil intake of 9 g per day, free and total EPA concentrations rise by *ca.* five- to six-fold, and free and total DHA concentrations by *ca.* two-fold.

INTRODUCTION

Since the early studies on plasma lipid and lipoprotein patterns in a coastal settlement of Greenland Eskimos [1], a very large number of investigations have been conducted on the health effects of fish oil in humans. Hypolipidemic and potential anti-atherogenic effects have been attributed to $\omega-3$ fatty acids ($\omega-3$ FAs), in particular to 5,8,11,14,17-eicosapentaenoic acid ($C_{20:5\omega-3}$, EPA) and 4,7,10,13,16,19-docosahexaenoic acid ($C_{22:6\omega-3}$, DHA). The analysis of lipoproteins, lipids and FAs has been stimulated by these observations. In principle, the qualitative and quantitative measurement of FAs is relatively easy. However, several pitfalls are involved in the sample preparations, which explain the multitude of procedures described for FA analysis. Methylation of the FAs has been performed, *e.g.* with boron trifluoride in methanol [2], methyl iodide in N,N-dimethylacetamide [2], methanol and hydrochloric acid in dimethoxypropane [3] and boron trichloride in methanol [4]. Some methods have been developed for the determination of free FAs in the presence of lipids, others for the measurement of total FAs either after saponification of the lipids or by transesterification of the lipid-bound FAs.

This paper describes a simple and reliable method for the analysis of free FAs and total FAs (free plus esterified) in serum. It is based on the methods proposed

0378-4347/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

by Lepage and Roy [5,6], and is optimized for the analysis of polyunsaturated FAs. The procedure has been applied to the quantitative determination of free and total EPA and DHA in a clinical study on patients with hyperlipoproteinemia treated with fish oil capsules.

EXPERIMENTAL

Samples

Blood samples were taken from 27 patients with hyperlipoproteinemia during two phases of a study with fish oil supplementation. In the placebo phase the patients received 9 g of an encapsulated oil preparation containing olive oil in medium-chain triglycerides (Hormon-Chemie, Munich, Germany) per day for four weeks, and in the verum phase 9 g of a fish oil preparation rich in $\omega-3$ FAs (twelve capsules of Eicosapen, Hormon-Chemie) per day for four weeks. Blood was drawn in the fourth week of each phase. After centrifugation of the clotted blood samples at 1800 *g* for 10 min, the serum was stored at -20°C prior to analysis.

Sample preparation for the free fatty acids in serum

A modification of a one-step reaction reported by Lepage and Roy [5] was applied. The free FAs were methylated and isolated from 150 μl of serum by adding 5 ml of methanol containing 20 μg of 13,16,19-docosatrienoic acid (Sigma, Deisenhofen, Germany) as internal standard (I.S.) and 100 μl of acetyl chloride. The mixtures were treated at 25°C for 45 min in screw-capped vials (Vetter, Ammerbuch, Germany) using a heating/stirring module (Model Reacti Therm, Bender-Hobein, Heidelberg, Germany) and neutralized with 3 ml of 6% aqueous potassium carbonate. Following the addition of 150 μl of *n*-hexane, the reaction mixture was shaken, and centrifuged (1800 *g* for 10 min), and the supernatants were analysed. All solvents and reagents were purchased from Merck (Darmstadt, Germany) and were of analytical grade.

Sample preparation for the total fatty acids in serum

To 100 μl of serum, 2 ml of methanol–toluene (4:1, v/v) containing 40 μg of the I.S. and 200 μl of acetyl chloride were added. The mixture reacted at 100°C for 60 min [6] on the heating/stirring module, and was then neutralized by shaking with 5 ml of 6% aqueous potassium carbonate and centrifuged (1800 *g* for 10 min). The toluene phases with the FA methyl esters (FAMEs) were directly subjected to gas chromatographic (GC) analysis without concentration.

Sample preparation for the total fatty acids in placebo oil and fish oil

Solutions of 100 μl of placebo oil and fish oil, respectively, in 50 ml of methanol–toluene (4:1, v/v) were prepared, and aliquots were diluted 1:10 with additional methanol–toluene (4:1). A 2-ml volume of this diluted solution, containing

40 μg of the I.S., was mixed with 100 μl of water and 200 μl of acetyl chloride and processed as described for the total FAs in serum.

Gas chromatographic analysis of the free and total fatty acids

The GC separation was performed on a Model 5890 A gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a flame ionization detector and an automatic sampler (Model 7673 A). A 25 m × 0.25 mm I.D. fused-silica column coated with 25-μm FFAP (free fatty acid phase, polyethylene glycol 2-nitroterephthalate, Macherey-Nagel, Düren, Germany) was programmed from 80 to 160°C at 6°C/min, then to 200°C at 0.5°C/min. Nitrogen was used as a carrier gas at a split ratio of 1:25. The sample size was 2 μl. The identities of the peaks were established by spiking with reference compounds (Sigma) and, in part, by mass spectrometry (TSQ 70, Finnigan MAT, Bremen, Germany).

Determination of EPA and DHA

The determination of EPA and DHA was based on calibration curves obtained with reference standards and internal standards. Different amounts of reference EPA and DHA (Sigma) were added to the sample preparation of a pooled serum (1, 2, 4, 10 and 20 μg for free EPA and DHA; 4, 8, 20, 40 and 80 μg for total EPA and DHA calibration curves). For the calculation of the unknown serum concentrations of the $\omega-3$ FAs EPA and DHA, the following formula was used:

$$\omega-3\ \text{FA}\ (\mu\text{g/ml}) = \frac{\text{peak area of } \omega-3\ \text{FAME}}{\text{peak area of I.S. ME}} \times A$$

where A is a calibration factor, derived from the calibration graph.

Determination of the recovery rates

The recovery rates were determined for EPA, DHA and the I.S. by spiking the reaction mixture used in the sample preparation for the total FAs in the pooled serum with 40 μg of each acid. The peak areas of added EPA, DHA and the I.S. obtained after the work-up procedure were compared with the peak areas obtained when the methyl esters were directly subjected to GC analysis. The determination was performed nine-fold.

Determination of the precision of the method

Free and total EPA and DHA were analysed nine-fold in the pooled serum to determine the between-series precision of the method.

RESULTS AND DISCUSSION

Reliability of the method

The sample preparation for the analysis of free and total FAs includes the release of the FAs from their binding locations, the removal of proteins, the

isolation of the acids from the aqueous medium and the derivatization. Binding locations are the albumin for the free FAs, and the binding within the cholesteryl esters, triglycerides, diglycerides, monoglycerides and the different phospholipids for the esterified FAs. The individual lipid classes are bound to proteins to form the various lipoproteins. In all parts of the sample preparation procedure, analyte losses leading to lower recoveries may occur. Therefore the use of internal standards, and calibration standards added to the biological matrix, is essential. The methods of Lepage and Roy [5,6] are one-step sample work-up methods including all parts of the process, that is displacement of the FAs from their binding locations, protein precipitation, derivatization and isolation of the acid derivatives. In contrast to the Lepage method, in this work the acetyl chloride is added to the reaction mixture only shortly before the serum, in order to achieve equal reaction times for serum FAs and the I.S. 13,16,19-docosatrienoic acid is a suitable I.S. for the quantification of EPA and DHA, because it elutes between the two acids, has similar chemical properties and is not detectable in serum. Toluene is used instead of the toxic benzene.

Transesterification with methanol and acetyl chloride has been shown to give very good recoveries for triglycerides, cholesteryl esters and the different classes of phospholipids, including sphingomyelin [6]. The reactions described with methanol–acetyl chloride under the defined conditions allow the analysis of free FAs and of total FAs. However, strict adherence to the protocol is necessary to avoid interferences. Under the described conditions the completeness of esterification of the free FAs is *ca.* 97% without significant hydrolysis of other lipids [5].

As shown previously [7], in the GC analysis of FAMEs, columns coated with FFAP give very good separations. FFAP appears to be superior to other phases, such as SP-2330 [6], SP-2331 [5] and DB-225 [8]. Using the sample preparation procedures described, hundreds of biological samples can be injected without column deterioration.

The mean recovery rates (n = 9) are 91.5% for EPA, 93.7% for DHA and

TABLE I

BETWEEN-SERIES PRECISION

Compound	Mean concentration (n = 9) (mg/dl)	Coefficient of variation (%)
EPA		
Free	0.2	5.7
Total	2.1	1.4
DHA		
Free	0.5	5.5
Total	5.6	1.5

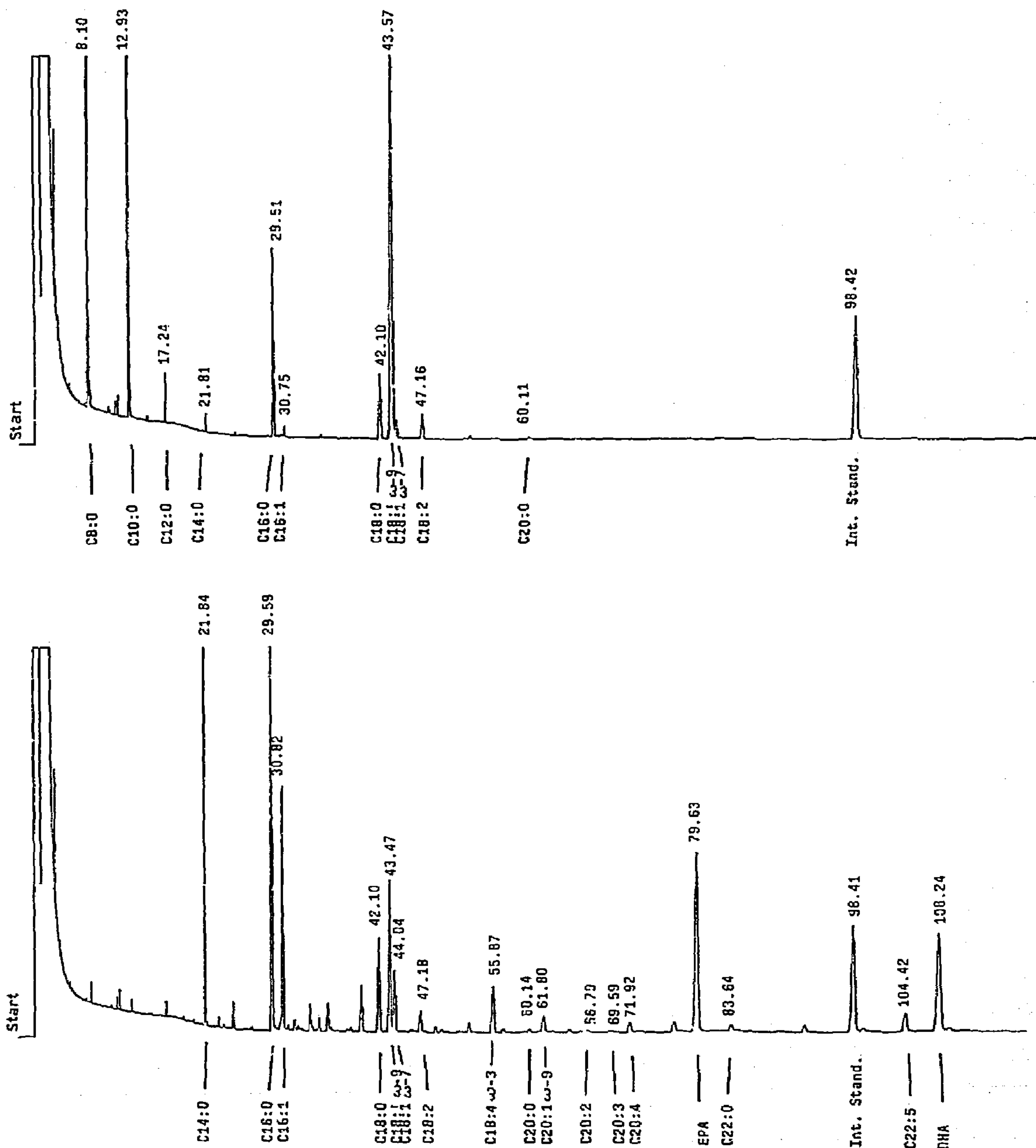

Fig. 1. Gas chromatograms of FAMEs: (top) placebo oil; (bottom) fish oil. Retention times shown in minutes.

92.2% for the I.S. Less than 100% recoveries are due to a number of reasons, *e.g.* incomplete methylation and incomplete extraction into the toluene phase.

The data for the between-series precision of the quantification of EPA and DHA are summarized in Table I. The coefficients of variation (C.V.) refer to the low EPA and DHA levels in a pooled serum from individuals under normal diet conditions, demonstrating that with the analytical procedure described precise quantifications are achieved. The calibration curves obtained by spiking serum with reference standards showed a linear relationship over the entire measurement range. The correlation coefficients invariably exceeded 0.999.

Analogous to the determination described of the $\omega-3$ FAs, the other FAs can be quantified as well using corresponding reference FA standards for the calibration graphs, for example phytanic acid, which is a marker for Refsum's disease. The method can be also applied to the analysis of the FA composition of the oil preparations. In Fig. 1 the FA patterns of the placebo oil and fish oil administered in this study are shown. The main components in the placebo oil preparation (Table II) are caprylic acid, capric acid and oleic acid. EPA and DHA are not detected. The fish oil preparation (Table II) contains high amounts of EPA and DHA (18.2 and 11.6%, respectively, of the total FAs).

EPA and DHA in serum under normal diet and under fish oil administration

Figs. 2 and 3 show typical patterns of the free FAs and total FAs in serum under normal diet with placebo oil supplementation and under fish oil supplementation. The concentrations of free and total EPA and DHA have been quan-

TABLE II

FATTY ACID COMPOSITION OF THE PLACEBO OIL AND FISH OIL PREPARATIONS

Values are percentages of the total FAs; acids below 1% are not included.

		Placebo oil	Fish oil
$C_{8:0}$	Caprylic acid	37.0	–
$C_{10:0}$	Capric acid	29.0	–
$C_{14:0}$	Myristic acid	–	7.6
$C_{16:0}$	Palmitic acid	4.7	18.0
$C_{16:1\ \omega-7}$	Palmitoleic acid	–	8.2
$C_{18:0}$	Stearic acid	2.8	4.9
$C_{18:1\ \omega-9}$	Oleic acid	22.0	8.1
$C_{18:1\ \omega-7}$	*cis*-Vaccenic acid	1.0	3.2
$C_{18:2\ \omega-6}$	Linoleic acid	1.1	1.2
$C_{18:4\ \omega-3}$	6,9,12,15-Octadecatetraenoic acid	–	3.0
$C_{20:1\ \omega-9}$	11-Eicosenoic acid	–	1.3
$C_{20:5\ \omega-3}$	EPA	–	18.2
$C_{22:5\ \omega-3}$	7,10,13,16,19-Docosapentaenoic acid	–	1.8
$C_{22:6\ \omega-3}$	DHA	–	11.6

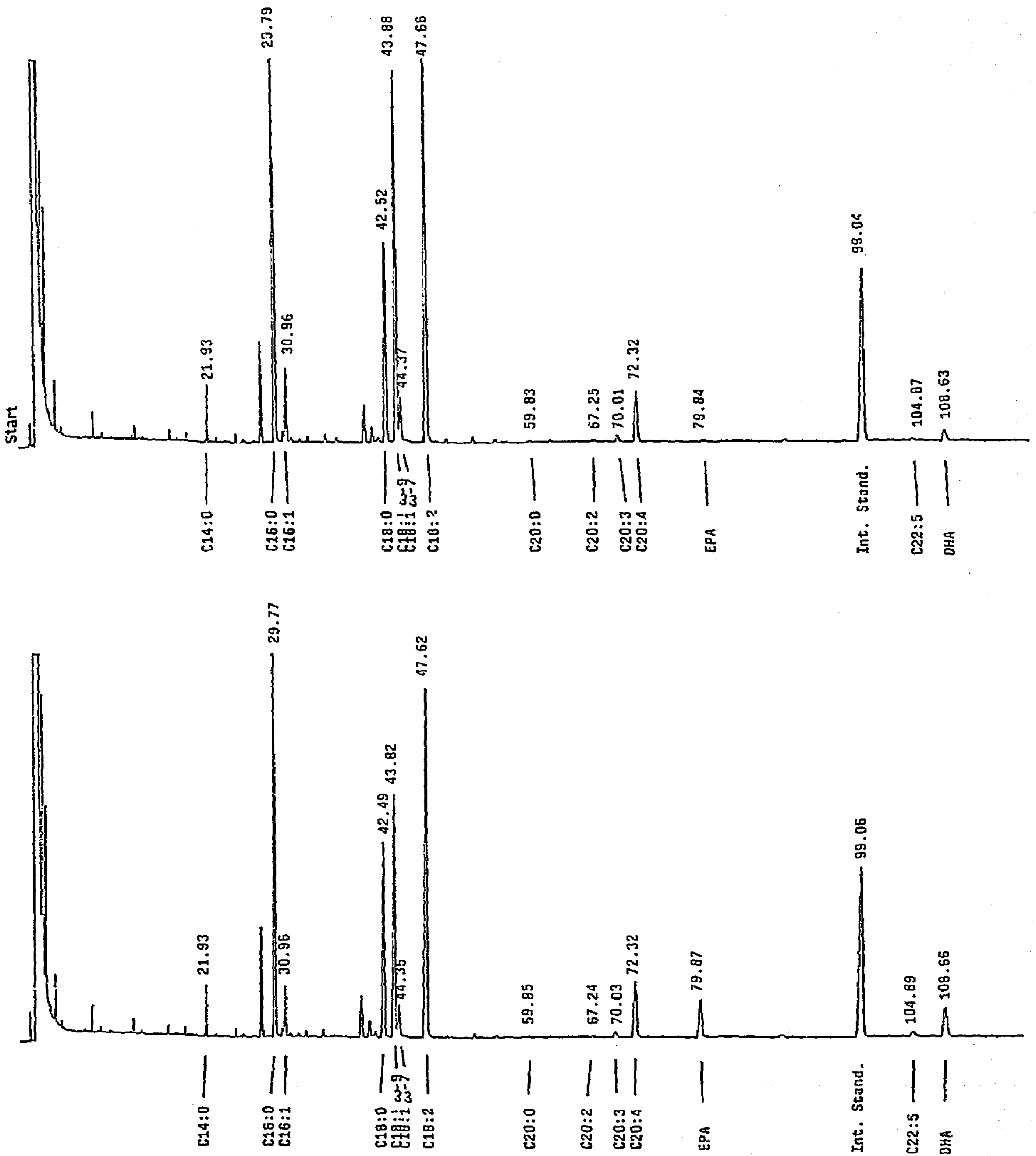

Fig. 2. Gas chromatograms of free FAMEs from serum: (top) normal diet plus placebo oil; (bottom) fish oil supplementation.

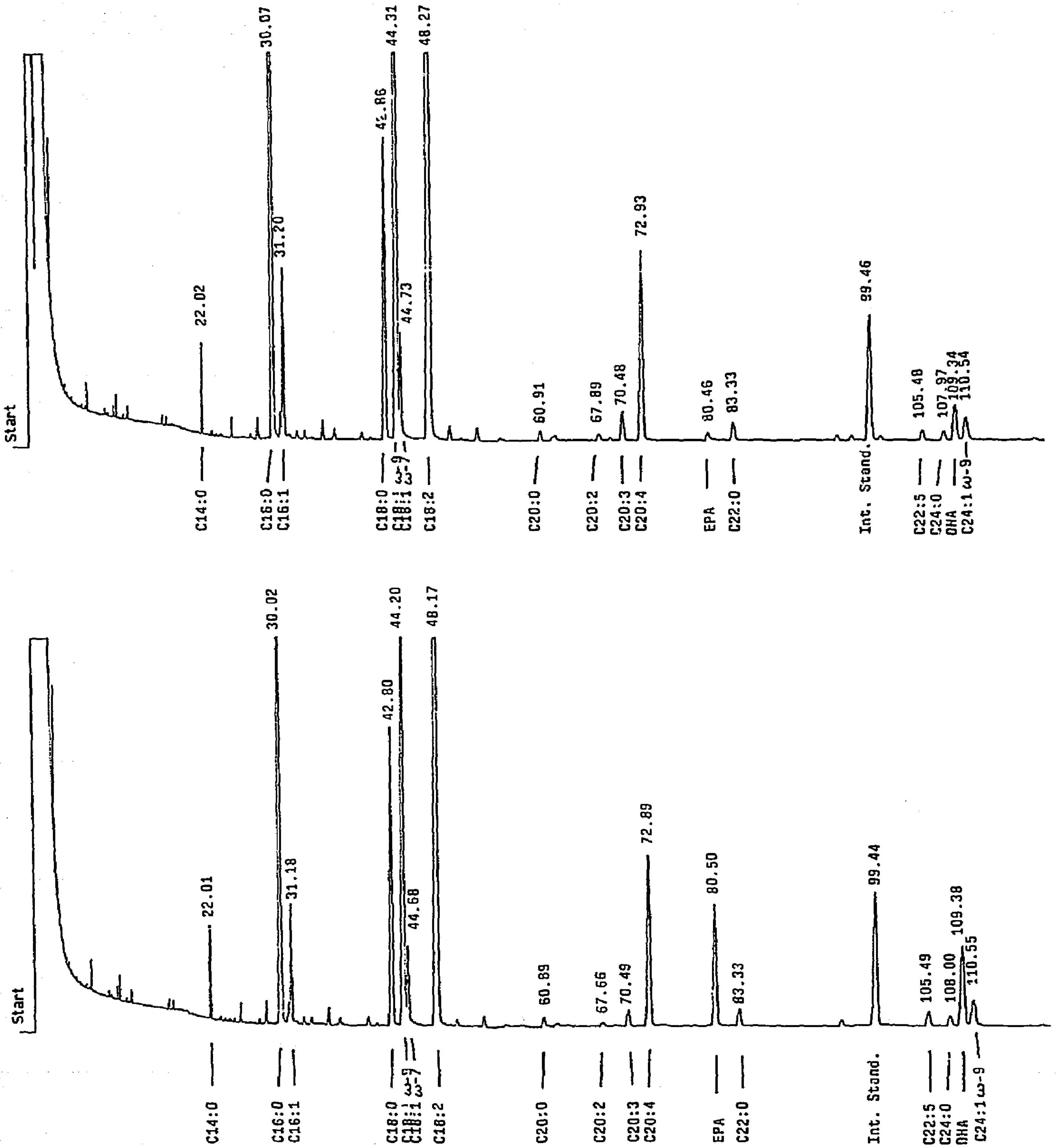

Fig. 3. Gas chromatograms of total FAMEs from serum: (top) normal diet plus placebo oil; (bottom) fish oil supplementation.

TABLE III

EPA AND DHA IN SERUM OF PATIENTS WITH HYPERLIPOPROTEINEMIA UNDER NORMAL DIET PLUS PLACEBO OIL AND AFTER FOUR WEEKS OF DAILY INTAKE OF FISH OIL

Compound	Concentration (mean ± S.D., n = 27) (mg/dl)	
	Placebo oil	Fish oil[a]
EPA, free	0.2 ± 0.1	1.2 ± 0.3
DHA, free	0.6 ± 0.2	1.2 ± 0.3
EPA, total	3.6 ± 2.1	18.7 ± 5.2
DHA, total	11.4 ± 3.1	19.2 ± 5.7

[a] Daily intake 9 g.

tified and are summarized in Table III. During fish oil treatment, the free and total EPA levels rise by *ca.* five- to six-fold. The increase of DHA is less pronounced and amounts to *ca.* two-fold for both free and total DHA. The extent of the shifts in EPA and DHA concentrations is dependent on the dosage of fish oil.

REFERENCES

1 H. O. Bang, J. Dyerberg and A. Nielsen, *Lancet*, i (1971) 1143.
2 K. G. Allen, J. Mac Gee, M. E. Fellows, P. A. Tornheim and K. R. Wagner, *J. Chromatogr.*, 309 (1984) 33.
3 K.-Y. Tserng, R. M. Kliegman, E.-L. Miettinen and S. C. Kalhan, *J. Lipid Res.*, 22 (1981) 852.
4 W. Onkenhout, P. F. H. van der Poel and M. P. M. van den Heuvel, *J. Chromatogr.*, 494 (1989) 31.
5 G. Lepage and C. C. Roy, *J. Lipid Res.*, 29 (1988) 227.
6 G. Lepage and C. C. Roy, *J. Lipid Res.*, 27 (1986) 114.
7 H. Jaeger, H. U. Klör, H. Ditschuneit and H. Frank, in H. Jaeger (Editor), *Glass Capillary Chromatography in Clinical Medicine and Pharmacology*, Dekker, New York, Basel, 1985, p. 271.
8 B. J. Holub and C. M. Skeaff, *Methods Enzymol.*, 141 (1987) 234.