CHROMBIO. 5132

# Rapid analysis of non-esterified fatty acids as methyl esters from different biological specimens by gas chromatography after one-step esterification

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## SUMMARY

A rapid gas chromatographic method for the determination of medium-chain and long-chain free fatty acids ($C_{14\,0}$ to $C_{24\,0}$ fatty acids) from different biological specimens is presented. After a rapid one-step transesterification method in methanol–acetyl chloride (50 1, v/v), fatty acid methyl esters were extracted into *n*-hexane and analysed on a 15-m Durabond-Wax column within a 12-min chromatographic run. The detection limit is 500 pg per injection.

## INTRODUCTION

The determination of fatty acids (FAs) has been the target of many investigations (for a review see ref. 1). Attention has also been paid to the deter-

mination of both saturated and polyunsaturated non-esterified fatty acids (NEFAs) in biological fluids [2–4].

Different FA derivatization procedures [5–7] and various techniques such as high-performance liquid chromatography [8,9], gas chromatography (GC) [10] and gas chromatography–mass spectrometry (GC–MS) [11,12] have been described for the determination of FAs. To achieve further selectivity for the determination of NEFAs only, improvements in methodology led to direct transesterification methods [13,14]. The one-step extractive methylation procedure for FAs gained much attention, reducing as it does both the time required and the extraction losses [15–17]. Most of the pioneering work in the development of this procedure was carried out by Lepage and Roy [14,16,17].

However, the decrease of time-consuming steps during esterification was in general not accompanied by an increase in the speed of the analytical technique and by a concomitant baseline resolution of fatty acid methyl esters (FAMEs). The present study was designed to improve the rapidity of GC analysis of FAMEs from different biological fluids and standard mixtures without affecting the baseline separation.

EXPERIMENTAL

*Reagents and chemicals*

Tetradecanoic ($C_{14\ 0}$), hexadecanoic ($C_{16\ 0}$), heptadecanoic ($C_{17\ 0}$), octadecanoic ($C_{18\ 0}$), 9-octadecaenoic ($C_{18\ 1,\omega-9}$), 9,12-octadecadienoic ($C_{18\ 2,\omega-6}$), 6,9,12-octadecatrienoic ($C_{18\ 3,\omega-6}$), 5,8,11,14-eicosatetraenoic ($C_{20\ 4,\omega-6}$), 5,8,11,14,17-eicosapentaenoic ($C_{20\ 5,\omega-3}$), docosanoic ($C_{22\ 0}$), 16-docosaenoic ($C_{22\ 1,\omega-6}$), 4,7,10,13,16,19-docosahexaenoic ($C_{22\ 6,\omega-3}$) and tetracosanoic ($C_{24\ 0}$) acids and trioleate were from Sigma (Munich, F.R.G.). Fatty acid methyl ester standards (RM-3, PUFA-1, and PUFA-2) were from Supelco (Bellefonte, PA, U.S.A.). Fish oil capsules (EPAX-5000 EE) were from *HOL*phar (Sulzbach, F.R.G.). Cholesterol arachidate was from NU-CHEK-PREP (Tokyo, Japan). 2,6-Di-*tert.*-butyl-4-methylphenol (BHT) and all other chemicals and solvents were purchased as analytical-grade reagents from Merck (Darmstadt, F.R.G.).

*Tissue samples*

Blood was taken by antecubital venipuncture from normolipemic volunteers and anticoagulated as described previously [18,19]. After centrifugation at 2500 $g$ for 20 min, platelet-poor plasma was stored at $-70°C$. For preparation of rat liver microsomes, unfrozen liver biopsies were minced and homogenized in Tris–HCl (50 m$M$, pH 7.4) as described previously [20]. The final microsomal fraction was suspended in 0.1 $M$ phosphate buffer (pH 7.4).

*Esterification procedure*

Heptadecanoic acid (150 $\mu$l) in *n*-hexane (0.1 $\mu$g/$\mu$l) was added to 5 ml of methanol–acetylchloride (50:1, v/v) as described previously [17], followed by the addition of 150 $\mu$l of plasma, rat liver microsomal suspension or fish oil capsule extract. Teflon screw-capped glass tubes were tightly closed and incubated at 25°C for 45 min. The tubes were shaken vigorously for periods of 5
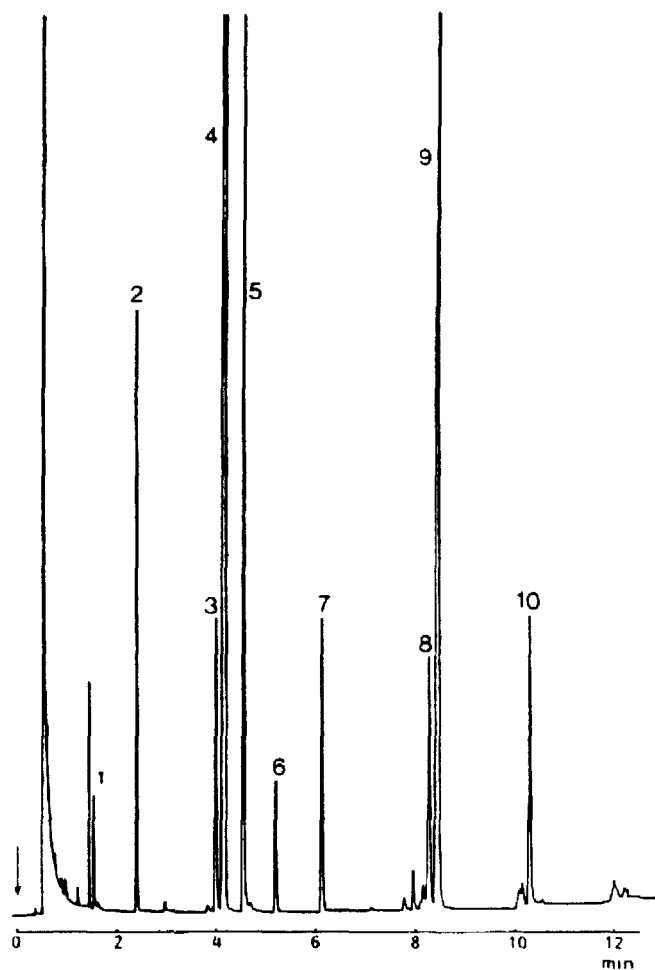


Fig. 1. GC profile of a standard FAME mixture (RM-3, Supelco). Chromatographic conditions: column, Durabond-Wax (15 m×0.32 mm I.D.); film thickness, 0.15 $\mu$m; carrier gas, hydrogen; flow-rate, 50 cm/s; column temperature, 1 min at 160°C, then to 185°C at 5°C/min, then to 240°C at 8°C/min and 10 min at 240°C; detection, flame ionization at 240°C; sensitivity, 4·10$^{10}$ attenuation full scale; sample size, 0.1 $\mu$l, split ratio, 1·186; injector temperature, 250°C Peaks: $1=C_{14\ 0}$, $2=C_{16\ 0}$; $3=C_{18\ 0}$; $4=C_{18\ 1}$; $5=C_{18\ 2}$; $6=C_{18\ 3}$; $7=C_{20\ 4}$; $8=C_{22\ 0}$; $9=C_{22\ 1}$; $10=C_{24\ 0}$.

min. Esterification was stopped with 2.5 ml of 6% potassium carbonate solution slowly added to neutralize the reaction mixture. After addition of $n$-hexane (150–200 $\mu$l), two 2-min periods of vortexing and centrifugation (10 min at 1500 $g$), 50 $\mu$l of the organic layer were transferred to precooled screw-capped conical vials and kept at $-20\degree$C until GC analysis. The injection volumes were 0.1–0.3 $\mu$l.
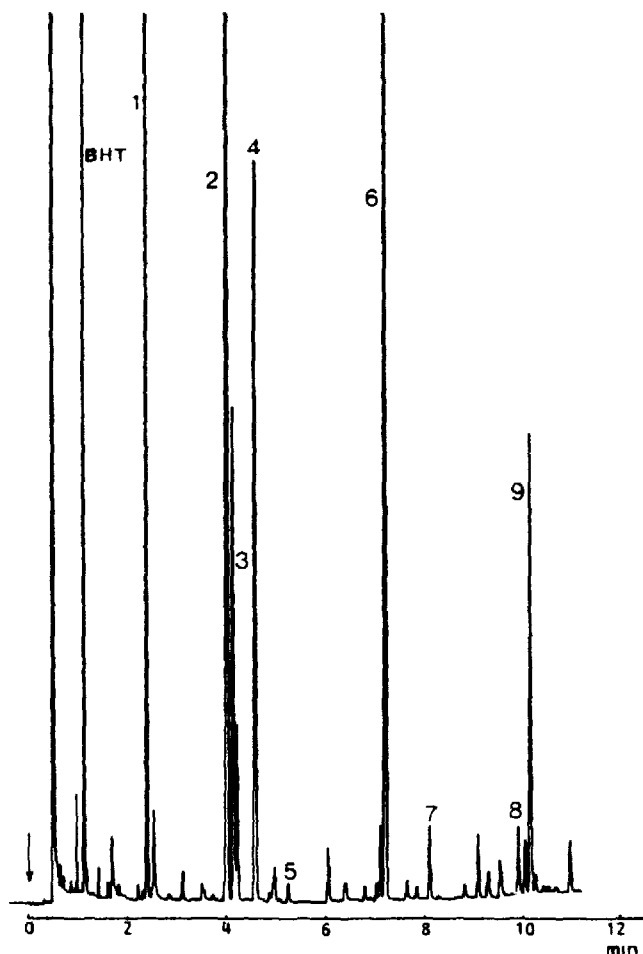


Fig. 2. GC profile of total FAMEs from a rat liver microsomal preparation. Transesterification was performed with boron fluoride-methanol with BHT as antioxidant [20]. For chromatographic conditions see Fig. 1. Peaks: $1=C_{16\ 0}$; $2=C_{18\ 0}$; $3=C_{18\ 1}$; $4=C_{18\ 2}$; $5=C_{18\ 3}$; $6=C_{20\ 4}$; $7=C_{20\ 5}$; $8=C_{22\ 5}$; $9=C_{22:6}$.

*GC conditions*

FAs were chromatographed as methyl esters on a $15\ m \times 0.32\ mm$ I.D. Durabond-Wax (J&W Scientific, Rancho Cordova, MI, U.S.A.) with a film thickness of $0.15\ \mu m$ and a polyethylene glycol phase (the polarity is comparable with that of a CB-Wax 20M column). Analysis was performed on a Dani 6800 gas chromatograph (with a PTV system) equipped with a flame ionization detector and a Shimadzu C-R1B integration system. Hydrogen was used as carrier gas at a flow-rate of $50\ cm/s$ and nitrogen as make-up gas at $40\ ml/min$. The split rate was $45\ ml/min$ $(1:186)$. After an initial isothermal period of 1 min at $160°C$ the temperature was programmed to rise to $185°C$ at $5°C/min$ and then to $240°C$ at $8°C/min$, with a hold at $240°C$ for 10 min. The detector temperature was $240°C$ and the injector temperature $250°C$.

RESULTS

The analytical procedure for the analysis of FAMEs using the GC conditions described was first applied to commercially available FAME standards. Fig. 1 displays a typical GC trace of the reference mixture RM-3 (commercially available). After an early solvent peak (injection volume $0.1\ \mu l$ of the organic layer) baseline separation of the FAMEs of RM-3 standard is obtained in 12 min. Fig. 2 shows the total FA pattern of a rat liver microsomal preparation under the same GC conditions. The microsomal FAMEs were prepared ac-

TABLE I

INTRA-ASSAY AND INTER-ASSAY VARIATION OF FAMEs $(n=5)$

Using different concentrations of individual FAs, intra-assay and inter-assay variations were calculated from the peak areas of the FAMEs after GC separation with $15\ \mu g$ of heptadecanoic acid as internal standard.

| FAME | Concentration (mg/ml of *n*-hexane) | |
|---|---|---|
| | Intra-assay | Inter-assay |
| $C_{14\ 0}$ | $2.21 \pm 0\ 0191$ | $0.993 \pm 0.009$ |
| $C_{16\ 0}$ | $2.16 \pm 0.0216$ | $0.995 \pm 0.037$ |
| $C_{18\ 0}$ | $2\ 32 \pm 0.0216$ | $1.213 \pm 0\ 006$ |
| $C_{18\ 1,\omega-9}$ | $2.16 \pm 0.0141$ | $1\ 054 \pm 0\ 039$ |
| $C_{18\ 2,\omega-6}$ | $3.80 \pm 0.0236$ | $1.253 \pm 0.016$ |
| $C_{18\ 3,\omega-6}$ | $2.26 \pm 0.0149$ | $0.996 \pm 0.009$ |
| $C_{20\ 4,\omega-6}$ | $2.35 \pm 0.0174$ | $1.154 \pm 0.010$ |
| $C_{20\ 5,\omega-3}$ | $2.12 \pm 0.0193$ | $1.242 \pm 0.015$ |
| $C_{22\ 0}$ | $2.15 \pm 0.0219$ | $1.234 \pm 0.018$ |
| $C_{22\ 1,\omega-6}$ | $2.23 \pm 0.0204$ | $1.042 \pm 0.015$ |
| $C_{22\ 6,\omega-3}$ | $2.56 \pm 0.0184$ | $0.994 \pm 0.008$ |
| $C_{24\ 0}$ | $2.13 \pm 0.0183$ | $1.002 \pm 0.011$ |

cording to Esterbauer et al. [21], with boron fluoride–methanol as an esterification reagent and BHT as antioxidant. The percentage FA composition found is in good agreement with previous data [22].

For qualitative analysis, different FAs (ranging from $C_{14\ 0}$ to $C_{24\ 0}$, Table I) were methylated with methanol–acetyl chloride and spiked with 15 $\mu$g of heptadecanoic acid as internal standard. For quantitative analysis of these FAs as methyl esters after transesterification, different concentrations of individual FA standards (ranging from 20 $\mu$g/ml to 3.8 mg/ml in $n$-hexane) were pre-
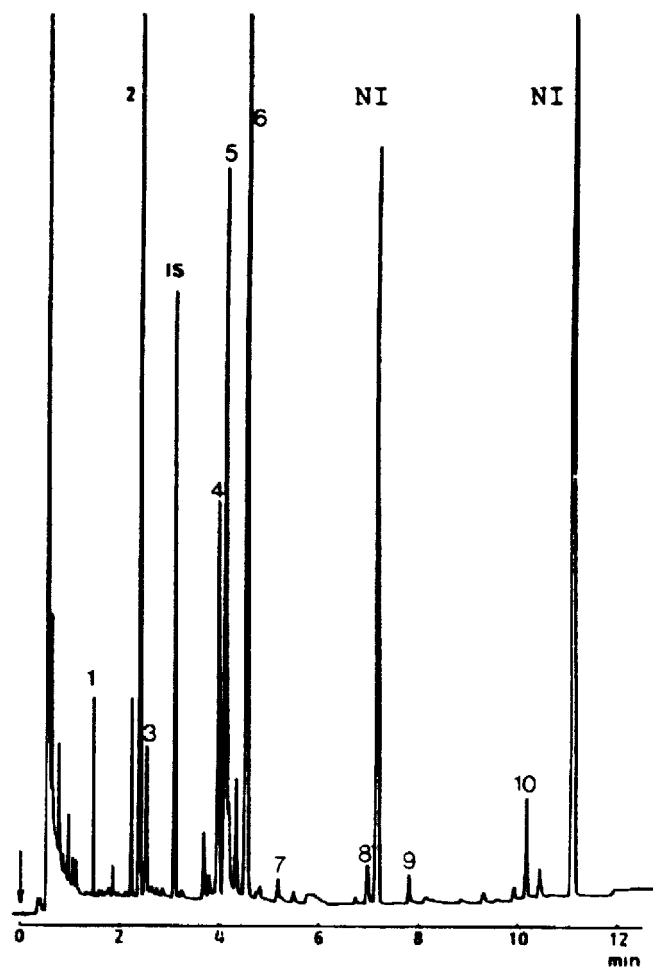


Fig. 3. GC profile of FAMEs from human plasma. One-step esterification was performed with 150 $\mu$l of plasma in methanol–acetyl chloride (50:1, v/v, 45 min, 25 °C). Chromatographic conditions as in Fig 1. Peaks: $1 = C_{14\ 0}$; $2 = C_{16\ 0}$; $3 = C_{16\ 1}$; $4 = C_{18\ 0}$; $5 = C_{18\ 1}$; $6 = C_{18\ 2}$; $7 = C_{18\ 3}$; $8 = C_{20\ 4}$; $9 = C_{20\ 5}$, $10 = C_{22\ 6}$; NI = not identified $C_{17\ 0}$ (15 $\mu$g) was used as internal standard (IS).
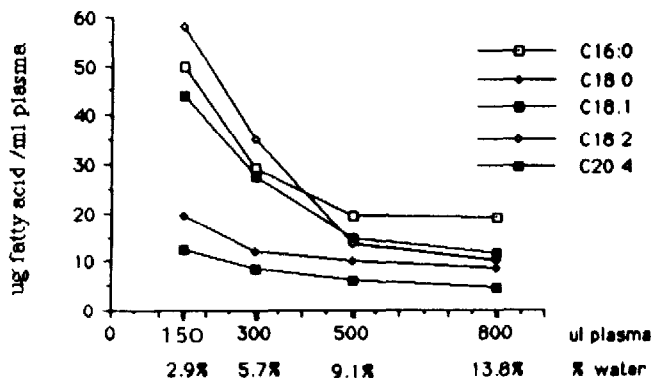
Fig. 4. Effect of increasing amounts of water (2.9% to 13.8%, v/v) on the recovery of NEFAs ($C_{16\ 0}$; $C_{18\ 0}$; $C_{18\ 1}$; $C_{18\ 2}$; $C_{20\ 4}$) from human plasma. Esterification was performed with methanol–acetyl chloride (50 1, v/v); 150, 300, 500 or 800 $\mu$l of plasma were added to 5 ml of the derivatization mixture. For GC conditions see Fig. 1.

pared. Following their GC separation, calibration curves for FAMEs were estimated using 15 $\mu$g of heptadecanoic acid as internal standard. The correlation coefficients for the calibration curves of twelve fatty acids (Table I) were in all cases higher than 0.998. Intra-assay variations ($n = 5$, lower than 1%) and inter-assay variations ($n = 5$, lower than 4%) are listed in Table I.

Based on the retention times of these FAs the identification of NEFAs from human plasma was performed. Fig. 3 shows the free fatty acid (FFA) profile of 150 $\mu$l of plasma (15 $\mu$g of $C_{17\ 0}$ as internal standard) after the one-step esterification procedure using methanol–acetyl chloride as methylating agent [17]. Fig. 3 also shows two peaks with retention times of ca. 7.2 and 11 min, which could not be clearly identified. Other authors have also reported artifact formation at similar retention times after esterification of FFAs from human plasma with diazomethane [15] or methanol–acetyl chloride [17]. Using analytical-grade cholesterol arachidate as a substrate for methylation in methanol–acetyl chloride, we found that cholesterol arachidate could account for one of these two unidentified peaks with a retention time of ca. 11 min under the GC conditions described. Lepage and Roy [17] have also reported peaks that were suggested to be cholesterol ester degradation products.

Plasma volumes greater than 150 $\mu$l in the incubation mixture resulted in a drastic decrease of the recovery for the FAMEs (Fig. 4). A final water content (derived from plasma) of 13.8% (v/v) in the derivatization mixture resulted in a loss of ca. 65% of FFAs acids from plasma. This was confirmed by GC analysis with $C_{17.0}$ (15 $\mu$g) as internal standard, when added to the methanol–acetyl chloride prior to plasma samples.

The GC procedure described was also used to identify the FA content of fish
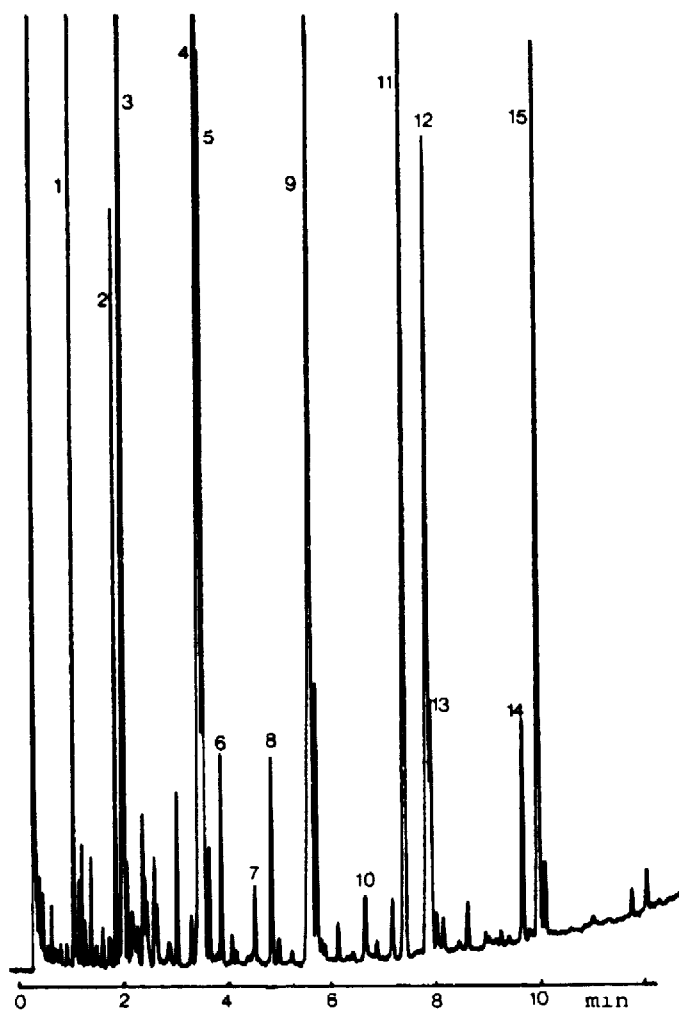
Fig. 5. GC profile of a standard FAME mixture (PUFA 1, marine source, Supelco). For GC conditions see Fig. 1. Peaks: $1=C_{14\ 0}$; $2=C_{16\ 0}$; $3=C_{16\ 1}$; $4=C_{18\ 1,\omega-9}$; $5=C_{18\ 1\ \omega-7}$; $6=C_{18\ 2}$; $7=C_{18\ 3}$; $8=C_{18\ 4}$, $9=C_{20\ 1}$; $10=C_{20\ 4}$; $11=C_{20\ 5}$, $12=C_{22\ 1,\omega-11}$; $13=C_{22\ 1,\omega-9}$; $14=C_{22\ 5}$, $15=C_{22\ 6}$

oil capsules containing both saturated and unsaturated FAs (rich in $\omega-6$ and $\omega-3$ PUFAs). FAMEs were identified by comparing the retention times with those of FAME standard mixtures (RM-3, PUFA 1, and PUFA 2). Fig. 5 shows the FAME chromatogram of a reference mixture (PUFA 1). The FA content of fish oil capsules (EPAX-5000 EE) is shown in Fig. 6.
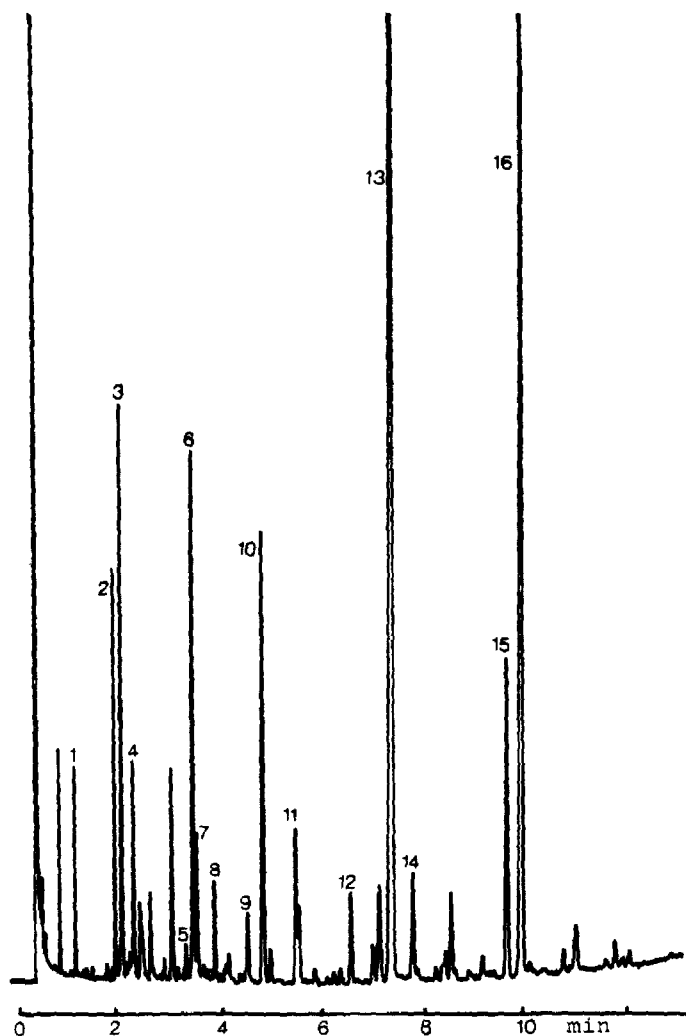
Fig. 6. GC profile of free FAMEs of encapsulated fish oil (EPAX-5000 EE, HOLphar, F.R.G.). One-step esterification was performed with methanol–acetyl chloride (50 1, v/v). Chromatographic conditions as in Fig. 1. Peaks. $1 = C_{14\ 0}$; $2 = C_{16\ 0}$; $3 = C_{16\ 1}$; $4 = C_{16\ 2}$, $5 = C_{18\ 0}$; $6 = C_{18\ 1,\ \omega-9}$; $7 = C_{18\ 1,\ \omega-7}$; $8 = C_{18\ 2}$; $9 = C_{18\ 3}$; $10 = C_{18\ 4}$; $11 = C_{20-1}$; $12 = C_{20\ 4}$; $13 = C_{22\ 5}$, $14 = C_{22\ 1}$; $15 = C_{22\ 5}$; $16 = C_{22\ 6}$.

## DISCUSSION

Methylation is the most common procedure for the derivatization of esterified FAs and NEFAs prior to their analysis by various techniques [2,10,12,14,16,17,20]. The complete recovery of NEFAs as methyl esters during the methanol–acetyl chloride (50:1, v/v) procedure (45 min, 25°C) [17],

in contrast to other techniques, must be appreciated. The advantage of a more rapid methylation procedure (15 min) using diazomethane is accompanied by a cumbersome preparation of methylating reagents [15,20].

The goal of the present study was to focus on a rapid analysis of FAMEs both for qualitative and quantitative analysis in different biological specimens. The GC technique described provides both a time-saving FA analysis and a rapid methylation procedure. For analysis of medium-chain and long-chain fatty acids from standard lipid mixtures, such as RM-3 (Fig. 1), PUFA 1 (Fig. 5) and PUFA 2 (chromatogram not shown), and from biological samples such as plasma, microsomal preparations and fish oil (Figs. 2, 3 and 6), a baseline separation of FAMEs is achieved within 12 min after injection. The short retention time of a 12-min GC fatty acid profile allows the determination of fatty acid isomers, i.e. $C_{18\ 1}$ ($\omega-9$ and $\omega-7$; Fig. 5) as well as $C_{22\ 1}$ ($\omega-11$ and $\omega-9$; Fig. 5). Under the GC conditions described by other groups [15,17,21], the retention times for a total FA profile ($C_{12\ 0}$ to $C_{22\ 6}$) ranged from 35 min [21] up to 55 min [15–17]. Another advantage of the GC method presented is the high chemical stability of the column used. Even after injection of more than 200 FAME samples of standard mixtures or biological samples, no alteration in the baseline peak resolution has been observed.

Lepage and Roy [17] found 45 min to be suitable incubation time for the one-step methylating procedure of FFAs from biological specimens. The same authors extensively investigated the conditions necessary for quantitative derivatization of FFAs with methanol–acetyl chloride without significant hydrolysis of triglycerides, cholesterol esters and phospholipids. We also found that trioleate, cholesterol arachidate and lecithin are transesterified to a neglectible amount (less than 3%) under the conditions described.

As found in our experiments, plasma volumes higher than 150 $\mu$l (more than 2.9% water in the incubation mixture) resulted in a considerable decrease of recovery of FAMEs from biological fluids such as plasma (Fig. 3). On the other hand, chicken egg sphingomyelin standards were reported to be unaffected by the presence of 5% water using even lower amounts of acetyl chloride [14]. About the same percentage of water (5.7%) derived from plasma resulted in a recovery of $66\pm5\%$ for free FAMEs (Fig. 4). Even the addition of 200 $\mu$l of plasma (3.4% water content in the derivatization mixture) led to a maximum esterification rate of $85\pm4\%$ (Fig. 4). Similar results were observed for other biological specimens with respect to the water content in the methanol–acetyl chloride mixture.

In conclusion, a rapid method for the determination of both saturated and unsaturated FAMEs by GC is presented. FAMEs (ranging from $C_{14\ 0}$ to $C_{24\ 0}$ fatty acids) can be separated with baseline resolution within 12 min using the GC conditions described.

ACKNOWLEDGEMENTS

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