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ANALYSIS OF FATTY ACIDS FROM HUMAN LIPIDS BY GAS CHROMATOGRAPHY

G.J. HAAN*, S. VAN DER HEIDE and B.G. WOLTHERS**

University Hospital Groningen, Central Laboratory for Clinical Chemistry, Oostersingel 59, Groningen (The Netherlands)

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SUMMARY

A rapid, quantitative method is described for the analysis of fatty acids from human lipids, namely serum lipids and lipids from adipose tissue biopsies. The method includes extraction of serum lipids with chloroform—methanol, hydrolysis with tetramethylammonium hydroxide, methylation with methyl iodide and N,N-dimethylformamide and gas chromatographic analysis on a Supelcoport SP-2330 column. Fat biopsies are analysed without extraction. Optimal hydrolysis conditions have been investigated.

INTRODUCTION

Many procedures have been described for the quantitative analysis of fatty acids [1]. All these procedures are time-consuming with regard to extraction, hydrolysis and esterification for gas chromatography (GC). Because of the interest at our hospital in fatty acids of adipose tissue from diabetic children and of serum lipids from children with cystic fibrosis and also from adults, as a part of a population screening programme, we searched for more rapid procedures. Greeley [2] described a new method for alkylation of acidic substances for GC and West [3] modified this method to prepare methyl esters from glycerides, alkyl paint resins and ester plasticizers. We tried this method on our samples and modified it to obtain rapid hydrolysis and methylation of the lipids under mild conditions. Combined with a simple extraction procedure and an appropriate GC procedure, an efficient analysis of fatty acids was achieved for clinical chemical purposes.

*Present address: Independent Regional Laboratories for Clinical Analysis, Nieuwe Gracht 39, Haarlem and Watercirkel 302, Amstelveen, The Netherlands.

**To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade and were purchased from Merck (Darmstadt, G.F.R.) except the following chemicals: fatty acids and methyl esters of the fatty acids were from Applied Science Labs., (State College, Pa., U.S.A.), tetramethylammonium hydroxide, 24% in methanol, was from Fluka (Buchs, Switzerland), trilinolein was from ICN Pharmaceuticals (Plainview, N.Y., U.S.A.), and 10% SP-2330 on Chromosorb W AW (100—120 mesh) was from Supelco (Bellefonte, Pa., U.S.A.). High-performance thin-layer chromatography plates, silica gel 60, 10 × 20 cm, were from Merck.

Procedures

Standard concentrations of fatty acids and their methyl esters were made by dissolving 15—20 mg of each lipid in 100 ml of chloroform. The following fatty acids and their methyl esters were used: myristic acid (C_{14}), pentadecanoic acid (C_{15}), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and arachidonic acid ($C_{20:4}$). Pentadecanoic acid, 130 mg dissolved in 100 ml of chloroform, was used as an internal standard.

To 0.5 ml serum, 0.2 ml of internal standard solution was added in a reagent tube. For the extraction of serum lipids and the free fatty acids, 1.5 ml of water, acidified with concentrated HCl to pH 2, and 5 ml of chloroform—methanol (7:3, v/v) were added to the mixture of serum and internal standard. The tubes were shaken twice for about 30 sec on a vortex mixer. After centrifugation for 5 min at 700 *g*, the chloroform layer was removed and the extraction was repeated with 3 ml of chloroform—methanol. Both the chloroform layers were combined. The chloroform extract was evaporated under a stream of nitrogen at 40° to complete dryness.

For the saponification of the lipids 0.1 ml of tetramethylammonium hydroxide solution and 0.1 ml of methanol were added. The tubes were tightly capped and were allowed to stand for 10 min at 70° in a heating block. After cooling to room temperature the methyl esters were prepared by adding 1 ml of N,N-dimethylformamide and 0.1 ml of methyl iodide. The tubes were shaken and the precipitate was removed by centrifugation. The supernatant was ready for injection into the gas chromatograph. Fat biopsies were weighed and then saponified and methylated as described above.

The gas chromatograph used was a Packard-Becker Model 428 equipped with flame ionization detectors. A 5-μl aliquot of the sample was injected on a 2-m glass column (2 mm I.D.) packed with 10% SP-2330 on Chromosorb W AW, 100—120 mesh. The carrier gas was nitrogen, flow-rate 20 ml/min. The injection port temperature was 220° and the detector temperature 250°. The oven temperature was programmed to run for 5 min isothermally at 130°, followed by a temperature rise of 5°/min to 220°.

Methyl ester standards in chloroform of all the acids were injected before and after each series of sample analyses. When differences of less than 5% in the peak heights of all the fatty acids, relative to the internal standard (I.S.), were observed between the first and the last standard run, the first standard run was

used for calculating the amounts of the individual fatty acids (FA) in terms of unmethylated acids. Calculations were made according to the equation

$$\mu g \text{ FA per sample} = \frac{\text{peak height FA} \times \mu g \text{ I.S.}}{\text{peak height I.S.}} \times \text{factor}$$

where factor (standard) is

$$\frac{\mu g \text{ FA} \times \text{peak height I.S.}}{\text{peak height FA} \times \mu g \text{ I.S.}}$$

Thin-layer chromatography was performed according to the method of Van Gent [4] using high-performance silica-gel plates with consecutive development in chloroform and hexane—chloroform (3:1). The slides were sprayed with the charring agent, 10% sulphuric acid in acetone—water (1:1).

NMR spectra were made on a Brucker spectrometer Model HX-360 (360 MHz). Further details are given in the figures. Mass spectra were made on a Varian gas chromatograph—mass spectrometer combination Model MAT 112, on a 2-m column (1.2 mm I.D.) packed with 10% SP-2330 on Chromosorb W AW, 100—120 mesh. The carrier gas was helium, flow-rate 9 ml/min. The oven temperature was 190°, separator temperature 250°, injection port temperature 250° and the source temperature 200°. The ionization energy was 70 eV and the scan speed 200 a.m.u./sec.

Methylation with diazomethane as a reagent was performed according to the procedure described by Schenk and Gellerman [5].

RESULTS

The methylation procedure was checked by methylating a fatty-acid mixture of known composition in chloroform (see Table I). Of this mixture 1 ml was evaporated under a stream of nitrogen at 40°. To the dry fatty acids, all the saponification—methylation reagents were added as described above, omitting heating for saponification. The 5 μl of the sample were injected, followed by 5 μl of the standard mixture of methyl esters of the fatty acids. To see whether the methylation was complete the amount of each acid, including pentadecanoic acid, was calculated, considering pentadecanoic acid not as an internal standard. The methylation was also compared to the procedure using diazomethane as reagent. As can be seen in Table I, the methylation is almost complete for all fatty acids, and gave results comparable with the diazomethane procedure.

The original method of West [3] described the saponification at 100°. Because loss of the polyunsaturated fatty acids is possible at this temperature, we performed the same procedure with the fatty acid mixture at different saponification temperatures to see whether temperature effects were present. The results are given in Table II. Recoveries of both polyunsaturated acids $C_{18:2}$ and $C_{20:4}$ are temperature dependent. Performing the procedure with linoleic acid at high saponification temperature resulted in the appearance of two new peaks in the gas chromatograms immediately after the partly disappeared linoleic acid peak. Mass spectrometric analysis of these peaks revealed that they were due to compounds with the structure and molecular weight of methyl lino-

TABLE I

METHYLATION OF A STANDARD MIXTURE OF FATTY ACIDS WITH THE REPORTED PROCEDURE AND WITH DIAZOMETHANE AS METHYLATING REAGENT

Figures are the mean recovery values for 24 estimates (duplicate injections of three samples on four different days), expressed in µg per sample and as percentages of the original standard values. Recoveries were calculated by dividing average peak heights of the methylated free fatty-acid standard by those of the average peak heights of methyl ester standard.

	C_{14}	C_{15}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Standard mixture								
Amount present (µg)	160	199	161	195	168	161	163	169
Reported procedure								
Amount recovered (µg)	161	193	176	203	167	164	164	154
Percentage	101	97	109	104	99	102	101	91
Diazomethane								
Amount recovered (µg)	150	179	166	196	161	158	165	160
Percentage	94	90	103	101	96	98	101	95

TABLE II

EFFECT OF DIFFERENT SAPONIFICATION TEMPERATURES ON THE FATTY-ACID STANDARD MIXTURE

Figures are the mean values for 6 estimates, expressed in µg per sample and as percentages (in parentheses) of the original standard values.

Temperature (°C)	C_{14}	C_{15}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
60	161	193	176	202	165	163	166	157
	(101)	(97)	(109)	(104)	(98)	(101)	(102)	(93)
70	165	197	177	204	164	163	166	149
	(103)	(99)	(110)	(105)	(98)	(101)	(102)	(88)
80	165	195	175	202	161	162	160	120
	(103)	(98)	(109)	(104)	(96)	(100)	(98)	(71)
90	159	191	175	202	167	165	152	44
	(99)	(96)	(109)	(104)	(99)	(103)	(93)	(26)
100	163	195	177	204	169	168	70	4
	(102)	(98)	(110)	(105)	(100)	(105)	(43)	(2)

leate (Fig. 1). NMR spectral analysis showed that these compounds were the result of migration of the double bonds to conjugated positions during the saponification step (Fig. 2). The double bonds are probably located in the 9—10-*cis*, 11—12-*trans* and 10—11-*trans*, 12—13-*cis* positions instead of the 9—10-*cis*, 12—13-*cis* positions, as in linoleic acid. We expect that a similar process occurs during saponification of arachidonic acid, at an even faster rate (Table II). Losses by oxidative processes during the analytical steps were not observed because the sum of the amounts of the $C_{18:2}$ acids was the same as the original amount of linoleic acid. To prevent the migration of double bonds, saponification at lower temperatures, where losses of linoleic acid and arachidonic acid are minimal, is necessary. To test the saponification at lower temperatures, trilinolein was used. Trilinolein was dissolved in chloroform (19.9 mg/100 ml) with pentadecanoic acid as an internal standard (15.3 mg/100 ml). Of this solution, 0.5, 1, 2 and 3 ml were evaporated under a stream of nitrogen at 40°. Saponification was carried out as described above at 70° for 10 min. Under these conditions the saponification is almost complete with no losses of linoleic acid (Table III).

To determine whether hydrolysis of serum lipids is complete under these circumstances, aliquots of pooled serum were analyzed, using different tempera-

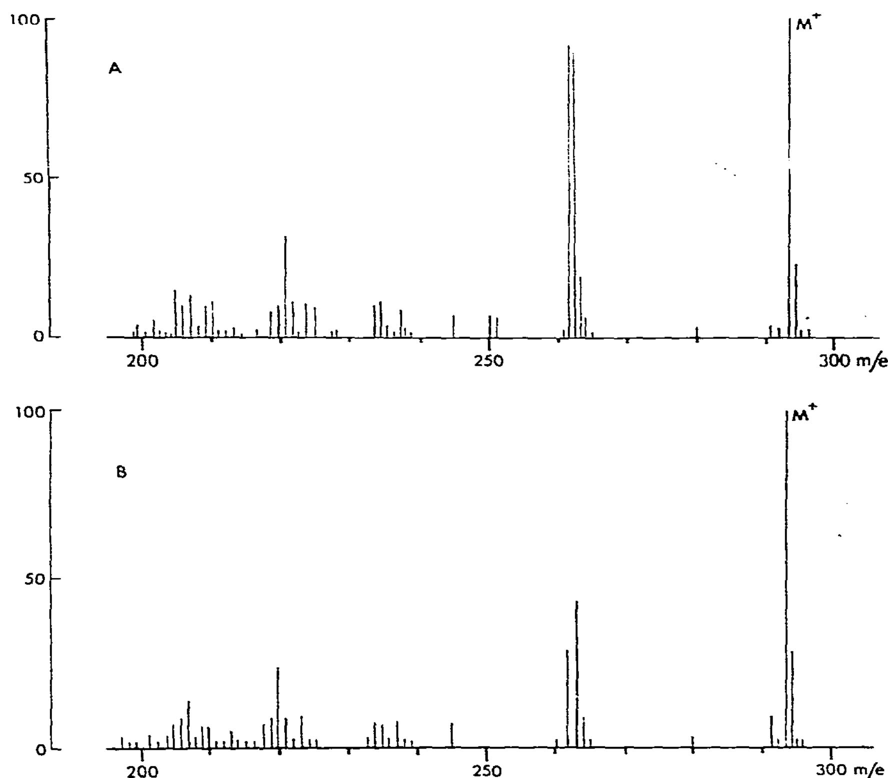

Fig. 1. Parts of the mass spectra of: (A) methyl linoleate and (B) the formed compounds.

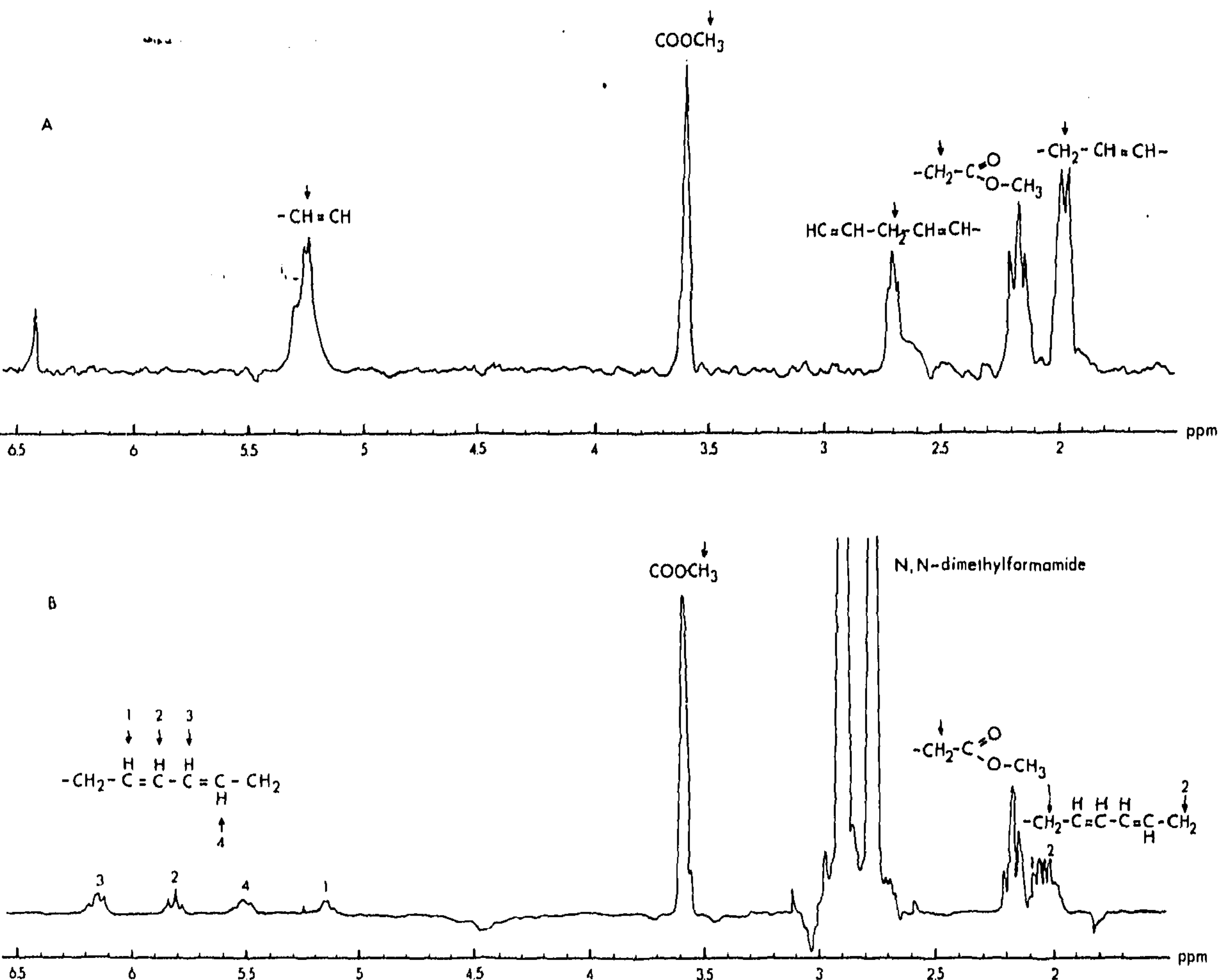

Fig. 2. NMR spectra of: (A) methyl linoleate and (B) the formed compounds. Solvents: (A) tetrachloromethane and (B) N,N-*dimethylformamide*. Internal standard, *trimethylsilane*; sweep width, 3000 Hz; temperature, 25°; number of scans, 100.

tures and incubation times during the saponification step. The results are shown in Table IV. Silica-gel thin-layer chromatography [4] revealed that saponification of the sample at 100° was complete after 30 min because no fatty-acid esters were visible in the chromatogram (Fig. 3).

Moreover, the presence of cholesterol in the serum samples is clearly shown, as should be expected. However, its presence does not interfere in the GC analysis. This was proved by injecting a solution of free cholesterol. After 1 h,

TABLE III

SAPONIFICATION OF TRILINOLEIN FOR 10 MIN AT 70°

Figures are the mean recoveries of duplicate injections of duplicate samples expressed in μg linoleic acid per sample and in percentages of the amount present.

Sample (ml)	Linoleic acid (μg per sample)		
	Present	Found	Recovery (%)
0.5	95	93	98
1.0	190	193	102
2.0	380	383	101
3.0	570	576	101

TABLE IV

EFFECT OF TEMPERATURE AND TIME ON THE SAPONIFICATION OF SERUM LIPIDS

Pooled serum (0.5 ml) was used according to the procedure described in the text, including internal standard. Figures are the mean values of duplicate injections of duplicate samples expressed as percentages of the highest yield found.

Time (min)	Temperature (°C)	C_{14} (%)	$C_{16:0}$ (%)	$C_{16:1}$ (%)	$C_{18:0}$ (%)	$C_{18:1}$ (%)	$C_{18:2}$ (%)	$C_{20:4}$ (%)
10	70	89	85	83	89	83	87	87
10	80	87	88	83	90	87	91	60
10	90	89	89	84	92	89	100	43
10	100	95	92	92	95	93	18	0
30	70	89	88	83	89	86	92	78
60	70	94	89	93	91	88	98	77
90	70	100	90	99	93	90	100	54
30	60	84	90	80	92	87	85	77
60	60	88	89	86	94	89	93	100
90	60	92	91	93	97	93	96	98
30	100	100	100	100	100	100	4	0

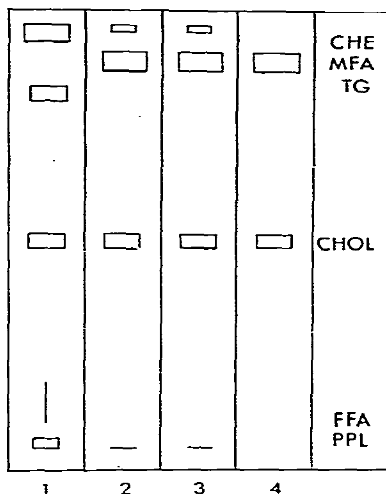

Fig. 3. Thin-layer chromatography on silica gel of a serum lipid extract in chloroform (1), and of serum extracts saponified for 10 min at 70° (2), for 90 min at 60° (3) and for 30 min at 100° (4). CHE = cholesterol esters; MFA = methyl fatty acids; TG = triglycerides; CHOL = cholesterol; FFA = free fatty acids; PPL = phospholipids.

TABLE V

EXTRACTION RECOVERY OF FATTY ACIDS AND TRINOLEIN ADDED TO SERUM

All figures are the mean values of duplicate injections of duplicate samples, expressed in µg per sample and as percentages of the values of the pure standards.

	C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Fatty-acid standard mixture	160	179	215	171	171	172	155
Serum	8	357	48	71	329	271	53
Serum with added fatty-acid standard mixture	168	541	267	241	509	475	217
Amount recovered	160	184	219	170	180	204	164
Recovery percentage	100	103	102	99	105	119	106
Trinolein standard	—	—	—	—	—	191	—
Serum with added trinolein standard	8	364	48	73	335	470	56
Amount recovered	—	—	—	—	—	199	—
Recovery percentage	—	—	—	—	—	104	—

keeping the oven at 220°, no peak was observed. Most likely cholesterol has a very large retention time at this temperature or is completely retained on the column. The samples saponified at 70° and 60° still contained traces of cholesterol esters and phospholipids. Triglycerides were not seen. As can be seen in Table IV, no ideal conditions, in which complete hydrolysis and no loss of polyunsaturated acids occur together, can be found. However, results for 10 min at 70° and 90 min at 60° are quite satisfactory.

The extraction procedure was checked by adding fatty acids and trilinolein to serum and determining the recovery. Dried aliquots (1 ml) of both a fatty-acid standard mixture and trilinolein were determined without the extraction step and also after dissolving them in 0.5 ml serum, followed by extraction. Recoveries are shown in Table V.

The reproducibility of the method was tested by analysing pooled serum ten times according to the above procedure (Table VI).

The fatty-acid composition of the pooled sera of 40 healthy laboratory technicians (aged 18—55 years) together with some values from the literature are listed in Table VII.

Some examples of the usefulness of the method for clinical purposes are given in Tables VIII and IX. Table VIII shows the composition of the serum

TABLE VI

REPRODUCIBILITY OF TEN ANALYSES OF THE SAME SERUM

	C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Mean (mg/l)	22.4	661.7	53.1	197.1	641.6	740.3	131.5
Standard deviation	1.36	22.95	3.61	12.29	31.78	47.52	20.88
Coeff. of variation (%)	6.0	3.5	6.8	6.2	5.0	6.4	15.9

TABLE VII

FATTY-ACID COMPOSITION OF SERUM LIPIDS OF THE POOLED SERA OF 40 LABORATORY TECHNICIANS COMPARED WITH SOME VALUES FROM THE LITERATURE

Figures represent percentages of the total amount of the fatty acids considered.

	C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$	Others
This method	1.2	27.8	2.3	8.1	22.7	32.9	5.0	—
Ref. 6 (calculated)	1.5	29.5	7.0	8.0	29.3	18.6	6.0	—
Ref. 7	1.5	23.5	5.3	9.5	29.3	27.0	—	3.9
Ref. 8	1.6	26.7	4.4	9.0	25.9	27.8	3.9	—

TABLE VIII

FATTY-ACID COMPOSITION OF SERA OF HEALTHY CHILDREN COMPARED WITH CHILDREN SUFFERING FROM CYSTIC FIBROSIS

Figures are expressed as percentages of the total amount of the fatty acids considered.

		C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Normal (age 0.5—12 years, $n = 21$)								
	%	1.9	29.5	3.9	9.8	26.2	25.2	4.6
	S.D.	0.88	3.92	1.04	2.14	3.85	5.17	1.26
Cystic fibrosis (age 1—16 years, $n = 22$)								
	%	1.9	30.5	7.1	9.0	31.7	17.6	2.7
	S.D.	0.85	3.10	2.15	2.26	5.51	6.99	1.70

TABLE IX

FATTY-ACID COMPOSITION OF FAT BIOPSIES OF DIABETIC CHILDREN COMPARED WITH VALUES FROM THE LITERATURE

Figures are expressed as percentages of the total amount of the fatty acids considered.

		C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$	Others
Diabetic children (n=23)									
	%	2.8	20.9	6.7	4.0	46.7	15.8	2.3	—
	S.D.	0.77	2.09	1.40	1.24	2.58	3.31	0.82	—
Ref. 11									
	%	2.7	24.0	5.0	8.4	46.9	10.2	—	2.5

fatty acids of children with cystic fibrosis compared with healthy children. Our results are in agreement with the results of others [9, 10], i.e., higher values for the monounsaturated and lower values for the polyunsaturated acids. The mean values of the fatty acid composition of fat biopsies compared with literature values are given in Table IX.

DISCUSSION

The method presented in this paper has many advantages in comparison with other procedures. The extraction is simple because no further extractions to purify the samples from non-lipid material are necessary. Saponification for 10 min at 70° and the subsequent methylation are very simple and rapid and

occur under mild circumstances without oxidation or double-bond migration in the polyunsaturated acids. However, the saponification can not be totally complete without loss of polyunsaturated acids, but the same probably holds true for other saponification procedures. For practical reasons we have taken a saponification procedure of 10 min at 70°, which will give about 85% saponification, although about 92% saponification can be achieved with the conditions 90 min at 60°.

Reproducibility is good and the results of determinations of the fatty-acid composition of 40 laboratory technicians agree fairly well with values found in the literature. Any differences could be due to dietary circumstances. Furthermore, the method is very well suited to the determination of the fatty-acid composition of fat biopsies.

In a laboratory with good automated equipment for GC, about 50 samples can be analysed daily by one technician. This method should also be suitable for the assay of other fatty acids and for the analysis of other types of sample.

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