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ANALYSIS OF FATTY ACIDS BY COMBINED APPLICATION OF CHEMICAL, CHROMATOGRAPHIC AND SPECTROSCOPIC METHODS

J. PÖRSCHMANN
Institute of Technical Microbiology, DDR-1017 Berlin (G.D.R.)
T. WELSCH*, R. HERZSCHUH and W. ENGEWALD
Karl-Marx-University, Department of Chemistry, Liebigstr. 18, DDR-7010 Leipzig (G.D.R.)
and
K. MÜLLER
Karl-Marx-University, Institute of Forensic Medicine, DDR-7010 Leipzig (G.D.R.)

SUMMARY

By combined application of chemical pretreatments, and chromatographic and spectroscopic methods, it is possible to coordinate individual species of a complex lipid extract with high safety. In the sample studied, besides a high content of palmitic and *cis*-vaccenic acid, small amounts of unusual fatty acids containing hydroxy groups and cyclopropane rings were detected. The presence of polyunsaturated fatty acids was excluded by structure–retention relationships, which were obtained on phases of different polarity.

On the basis of these studies, and without the use of reference substances, a routine analysis of these unusual fatty acids in biological materials is possible.

INTRODUCTION

Branched, unsaturated, hydroxy, epoxy, cyclopropane, and cyclopropene carboxylic acids containing between 12 and 24 carbon atoms have been found in lipid-containing tissues and oils in small amounts. The qualitative as well as quantitative estimation of the so-called atypical and rare fatty acids is attracting increasing interest for the study of biochemical processes.

Because of the complexity of the samples and lack of appropriate reference substances, we describe here an extensive identification of these atypical fatty acids by the use of capillary gas chromatography (GC) in combination with structure–retention relationships and subtraction techniques, as well as GC–mass spectrometry.

EXPERIMENTAL

Preparation of the sample

The preparation of the sample consists of the isolation of the lipids and their saponification, the liberation of the free fatty acids and their methylation (Fig. 1, upper part).

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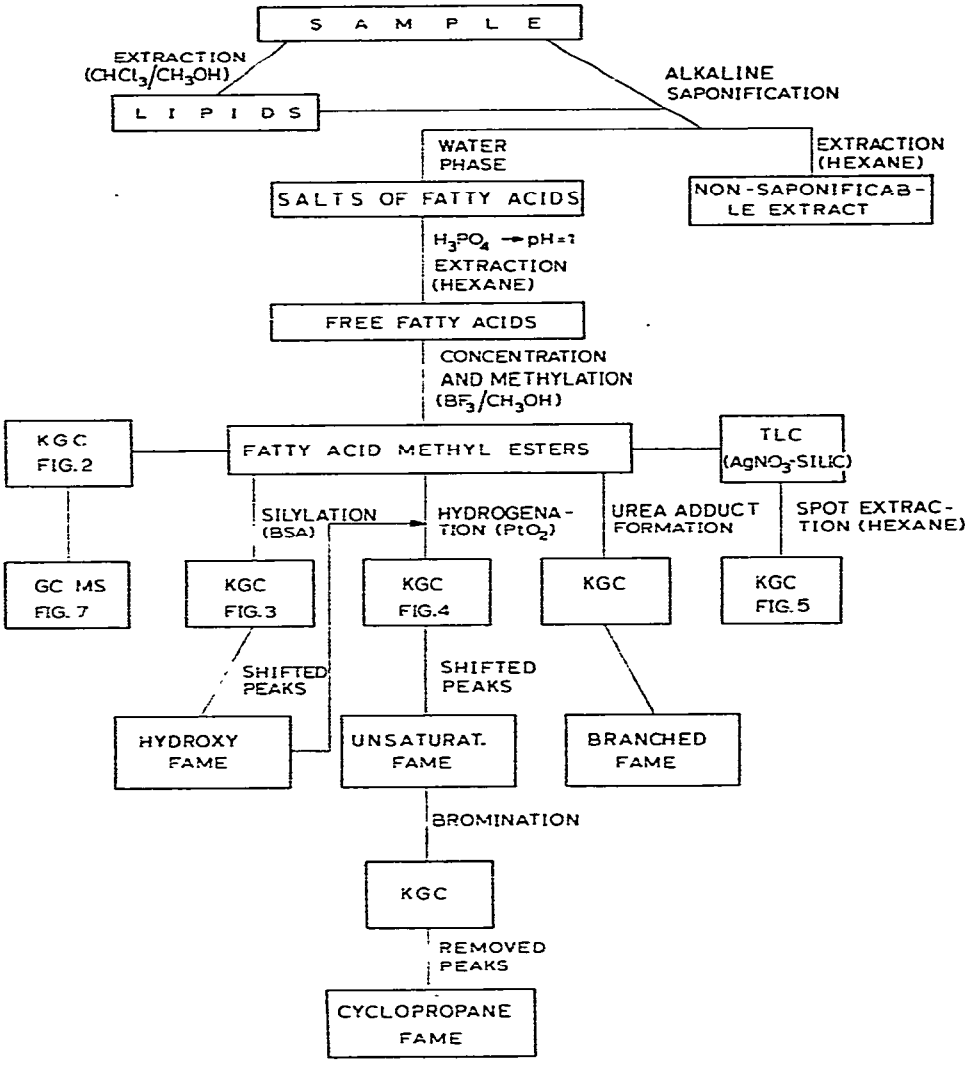

Fig. 1. Sample preparation. KGC = capillary gas chromatography.

Extraction of lipids[1]. The solid sample (10 g) is stirred for 1 h in 100 ml of chloroform–methanol (2:1, v/v), containing 0.2 ml of concentrated hydrochloric acid. After filtration, 25 ml of a 1% NaCl solution is added to the extract. The mixture separates into two phases. The upper phase, consisting of methanol–water and salt, is discarded. The procedure is repeated twice. The combined lipid phases are evaporated to dryness.

Alkaline saponification of lipids. Absolute methanol–diethyl ether (3:1, v/v) (50 ml) is added to 1 g of the lipid extract, followed by the addition of 1 ml of 5 *n* NaOH. Saponification is performed by refluxing for 5 h under chemically pure nitrogen. Water is added and the unsaponificable fraction is extracted with *n*-hexane.

Direct alkaline saponification of the sample[2]. The solid sample (20 g) is saponi-

fied with 5 g of solid NaOH in 200 ml of absolute methanol by refluxing for 5 h under chemically pure nitrogen. For the separation of the unsaponificable fraction the mixture is diluted with 50 ml of water, and after filtration extracted with *n*-hexane several times.

Liberation of fatty acids. The water phase containing the sodium salts of the fatty acids is acidified to pH 1 with concentrated phosphoric acid. The free fatty acids are extracted several times with *n*-hexane. The combined hexane phases are washed with water, dried by anhydrous Na_2SO_4 and evaporated to dryness.

Esterification of fatty acids[3]. The fatty acids (100 mg) are refluxed with 3 ml of a methanolic solution of BF_3 (14%) for 5 min. After dilution with water the fatty acid methyl esters (FAME) are extracted by *n*-hexane. The hexane phase is washed with water, dried and concentrated by partial refluxing[4].

Chemical pretreatment of the FAME mixture

For structure group classification several modifications were performed (see Fig. 1, lower part):

Hydrogenation. The FAME mixture (100 mg in 10–20 ml of ethyl acetate–acetic acid) is hydrogenated at room temperature with stirring for 8 h using freshly prepared platinum oxide as a catalyst.

Bromination[5]. To 100 mg of the hydrogenated FAME mixture, 2 ml of diethyl ether are added. After cooling to 0°C, 1 ml of a 20% solution of bromine in diethyl ether is added and reaction is performed for 3 h. The excess bromine is removed by introduction of nitrogen.

Silylation. The FAME mixture (100 mg) is dissolved in 2 ml of acetone* and three drops of N,O-bis(trimethylsilyl)acetamide (BSA) are added. The mixture is heated for 6 h at 80°C in a sealed vial. After reaction and dilution with water the product of silylation is extracted with *n*-hexane. After washing with water the hexane phase is dried by anhydrous Na_2SO_4.

Urea adduct formation[7]. Adduct formation is performed by a column technique with urea as stationary phase. The sample is dissolved in urea-saturated methanol and poured onto the column. The column is eluted with urea-saturated methanol. The branched components, which are unable to form urea adducts, are eluted. After dilution with water the FAME are extracted with *n*-hexane. This extract is concentrated under partial refluxing and oxidized by performic acid to eliminate the unsaturated compounds.

Pre-separation by thin-layer chromatography (TLC)

The FAME mixture was separated by TLC according to its polarity using silica gel H plates impregnated with silver nitrate, and light petroleum (b.p. 60–80°C)–diethyl ether (9:1, v/v) as solvent[8].

Equipment

A capillary gas chromatograph of type HP 5840 A was used. The mass spectra were recorded using a GC–MS system type Finnigan 4021.

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* The excellent properties of acetone as a solvent for silylation have been reported recently[6].

Capillary columns

For the separation of FAME mixtures capillaries were prepared according to Table I.

RESULTS AND DISCUSSION

Preparation of the sample

For the preparation of the sample one can select between two principal possibilities (see Fig. 1): (i) direct alkaline saponification followed by the separation of saponificable and unsaponificable fractions, (ii) extraction of the lipids with special solvents or mixtures of solvents followed by saponification or transesterification.

In contrast to the direct alkaline saponification the second method does not include covalently linked lipids (*e.g.* lipopolysaccharides). A comparison of capillary gas chromatograms of samples prepared by both methods shows no significant differences. This indicates the absence of covalently linked lipids or the sole presence of hydrophobically associated and membrane-associated lipids in the extract. Nevertheless, we opted for direct alkaline saponification because of its simplicity and speed.

Among the methods of the methylation we preferred the BF_3 method to esterification with methanol catalyzed by H_2SO_4 or HCl[11], because of the short reaction time and the almost quantitative esterification. In the BF_3 method the lipid extract must not contain sensitive fatty acids, *e.g.* epoxy acids, because of the opening of the epoxy ring by BF_3. In this case it is advisable to use a mild methylation by methyl iodide via the silver salts[11].

In many cases a direct transesterification of the extracted lipids without previous saponification is unfavourable, because the unsaponificable fraction (hydrocarbons, sterols, etc.) is not removed. This disturbs the analysis of FAME. Moreover, during transesterification free fatty acids inactivate the basic catalyst needed (alkali metals, alkali alcoholates).

Pre-chromatographic and chromatographic analysis

As shown in Fig. 2 a FAME sample obtained by direct alkaline saponification can be separated into the individual components nearly completely using a SE-54 coated capillary of high efficiency.

The presence of hydroxy acids in our native FAME sample must be assumed on the basis of their IR spectra. Therefore, we had to use well-deactivated capillaries (*e.g.* high-temperature silylated—see Table I).

With the help of authentic fatty acids we detected myristic, palmitic, stearic and iso-stearic acid. Further elucidation of the components was possible by the application of pre-chromatographic derivatizations of the obtained FAME mixture, such as hydrogenation, bromination, and silylation, the use of relationships between structure and GC retention, and GC–MS.

By the pre-chromatographic derivatizations we intended not only to obtain important preliminary information on the structural type of the individual components, but also to verify the structural data obtained by chromatographic and spectroscopic methods.

After chemical modification and urea-adduct formation, the mixtures were separated by capillary GC analysis under identical conditions (Figs. 3 and 4). By comparison of these chromatograms with a chromatogram of an unmodified FAME

TABLE I

CAPILLARIES PREPARED

The capillaries were made of soda-lime glass in all cases.

Stationary phase	*Dimensions of capillaries*	*Pretreatment of the surface*	*Coating*	*Film thickness*
OV-1	38 m × 0.28 mm I.D.	Combined leaching and high-temperature silylation by hexamethyldisilazane or	static	0.20 μm
SE-54	22 m × 0.28 mm I.D.	tetraphenyl dimethyldisilazane, respectively[9]	static	0.20 μm
Carbowax 20M	60 m × 0.25 mm I.D.	Barium carbonate deposit[10]	dynamic	

mixture (Fig. 2) conclusions concerning the assignment of peaks to the fatty acid structural types were drawn (see Table II).

One result is that the lipid sample studied contains three saturated hydroxy fatty acids (ECL 15.16; 15.45; 17.17). The positions of these peaks, which like the peaks at ECL 18.91 and 19.90 can be assigned definitely to the hydroxy acid type by comparison of the native and the silylated sample (Figs. 2 and 3), is changed neither by hydrogenation (Figs. 2 and 4) nor by bromination of the trimethylsilyl derivatives.*

The peak at ECL 18.91 shifts to higher retention times after hydrogenation (Figs. 2 and 4). This demonstrates that it was originally an unsaturated hydroxy acid. After silylation, hydrogenation, and bromination of the sample, analogous considerations lead to the conclusion that in the case of the peak with ECL 19.90 (Fig. 2) it is a cyclopropane-hydroxy FAME.

Because of the complexity of the FAME mixture an assignment of all the individual components separated on columns of different polarity, and thereby the application of structure–retention relationships, is difficult. Therefore we pre-separated the FAME mixture by TLC into three fractions of different polarity. Thereafter these fractions were separated on OV-1, SE-54 and Carbowax 20M capillaries.

The fraction with the highest polarity should contain polyunsaturated and hydroxy FAME[19]. The fraction with the highest polarity of our sample without pretreatment and after silylation contained only hydroxy FAME, as indicated by a comparison of their chromatograms.**

The fraction of the middle polarity (R_F 0.38–0.78), according to ref. 19, contains the mono-unsaturated and the di-unsaturated compounds. This is confirmed by hydrogenation of this fraction. This fraction contains only two peaks which elute on SE-54 at ECL-values of 15.82 and 17.82 (see Fig. 2 and Table II). The identification of these two compounds as mono-unsaturated fatty acids results from the fact that they

* By bromination of the trimethylsilyl derivatives of hydroxy FAME coordination to the type of cyclopropane-hydroxy fatty acids is possible (see Fig. 1). Bromination of the free hydroxy FAME destroys all hydroxy compounds.

** By addition of linolenic and arachidonic methyl esters to the native sample, followed by TLC and capillary GC, we confirmed the results reported in literature that the fraction of the highest polarity contains the polyunsaturated FAME.

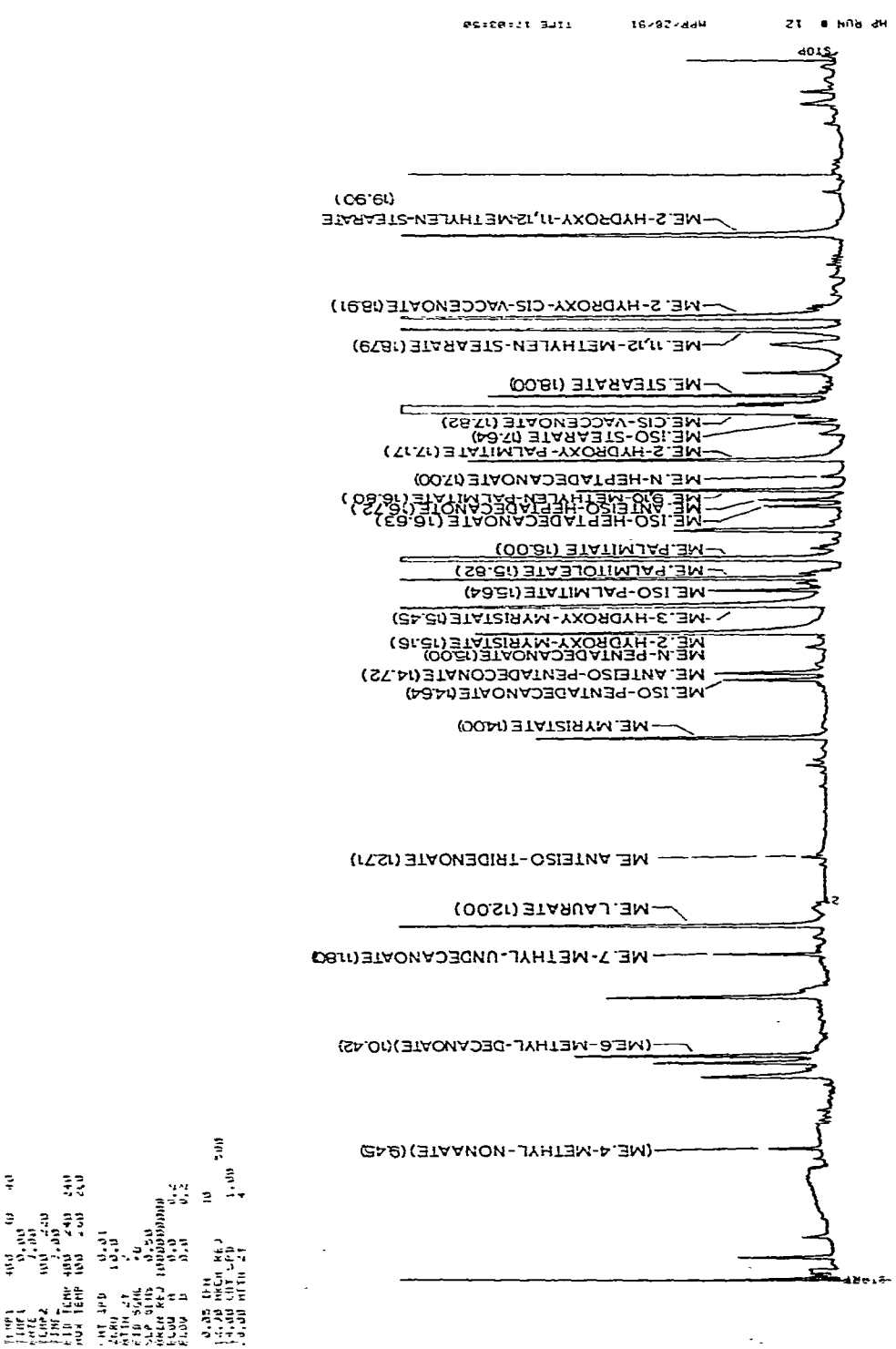


Fig. 2. FAME on an SE-54 capillary column. Sample: untreated.

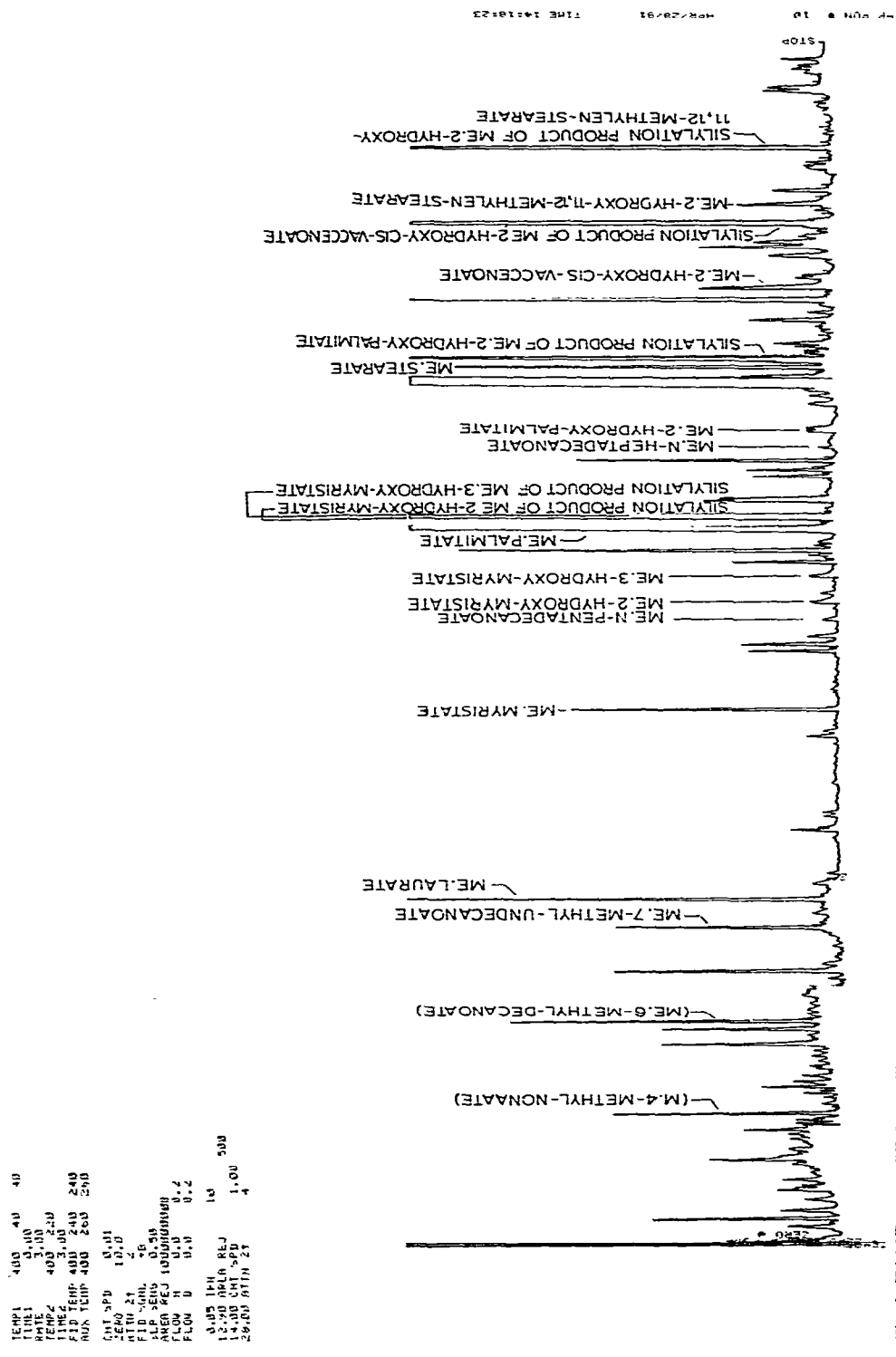


Fig. 3. FAME on an SE-54 capillary column. Sample: silylated.

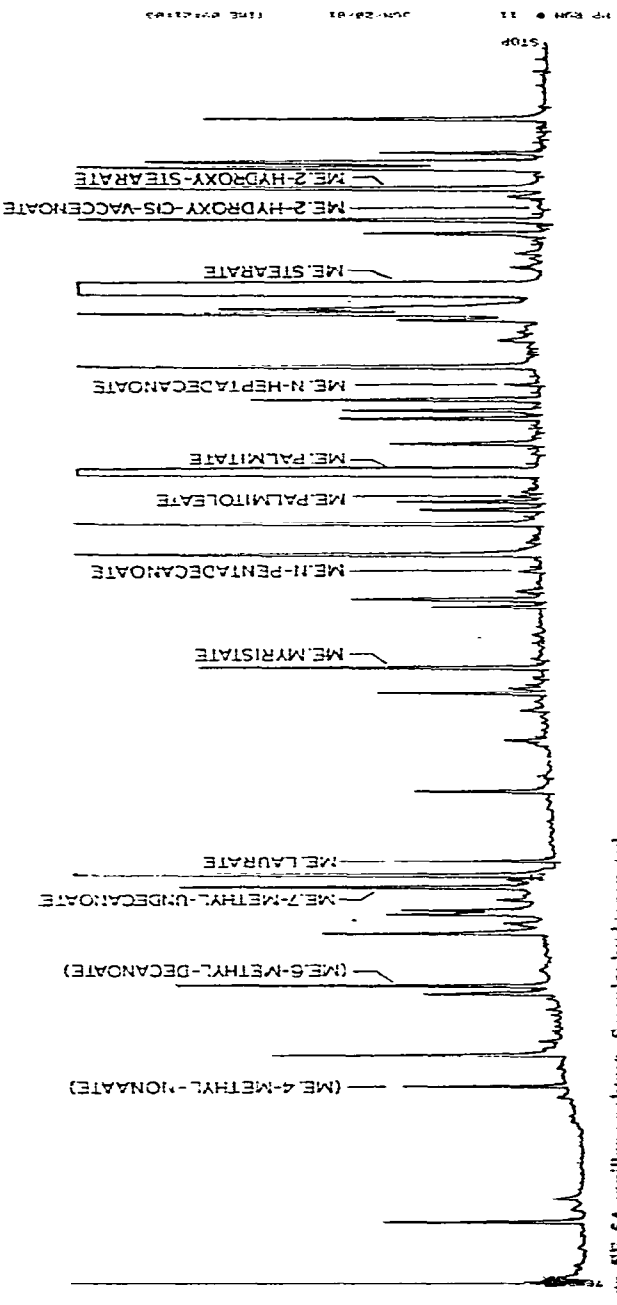


Fig. 4. FAME on an SE-54 capillary column. Sample: hydrogenated.

TABLE II

IDENTIFICATION OF FAME MIXTURES BY CHEMICAL PRETREATMENTS AND STRUCTURE-RETENTION RELATIONSHIPS

Symbols: s, saturated FAME; us, unsaturated FAME; b, branched FAME; nb, non-branched FAME; h, hydroxy FAME; c, cyclopropane FAME.

ECL OV-1 at 200°C	*ECL SE-54 at 190°C (Fig. 4)*	*Information from*				
		Silylation	*Hydrogenation*	*Bromination*	*Urea-adduct formation*	*Structure-retention relationships*
9.42	9.45		s		b	10 C
10.40	10.42		s			11 C
11.79	11.80		s		b	12 C, s
12.01	12.00	>	s		nb	12 C, s
12.70	12.71		s		b	13 C, s
14.00	14.00		s		nb	14 C
14.64	14.64		s		b	15 C, s, *iso*
14.72	14.72		s		b	15 C, s, *anteiso*
15.00	15.00		s			15 C, s, nb
15.12	15.16	h	s			14 C
15.40	15.45	h	s			14 C
15.64	15.64		s		b	16 C, s, *iso*
15.76	15.82		us			16 C, mono-unsaturated (palmitoleic type)
16.00	16.00		s		nb	16 C
16.64	16.63		s		b	17 C, s, *iso*
16.72	16.72		s		b	17 C, s, *anteiso*
16.77	16.80		s	c		17 C
17.00	17.00		s			17 C
17.14	17.17	h	s			16 C
17.64	17.64		s		b	17 C, *iso*
17.76	17.82		us			18 C, mono-unsaturated (palmitoleic type)
18.00	18.00		s		nb	18 C
18.75	18.79		s	c		19 C
18.81	18.91	h	us			18 C
19.82	19.90	h	s	c		19 C

elute on Carbowax 20M at ECL-values of 16.18 and 18.18. This excludes[13] the possibility of their being di-unsaturated fatty acids (see below).

A capillary gas chromatogram of the fraction obtained by TLC with the lowest polarity (R_F of 0.82 to 0.89) is shown in Fig. 5. As chromatograms of the hydrogenated and the hydrogenated/brominated fraction of lowest polarity show, it contains only saturated and cyclopropane FAME.

A comparison of Figs. 2 and 5 shows that TLC pre-separation produces simplified gas chromatograms. On this basis we succeeded in assigning the majority of the peaks obtained on OV-1, SE-54, and Carbowax 20M. After determination of the ECL-values on the different phases it was possible to apply structure–retention relationships.

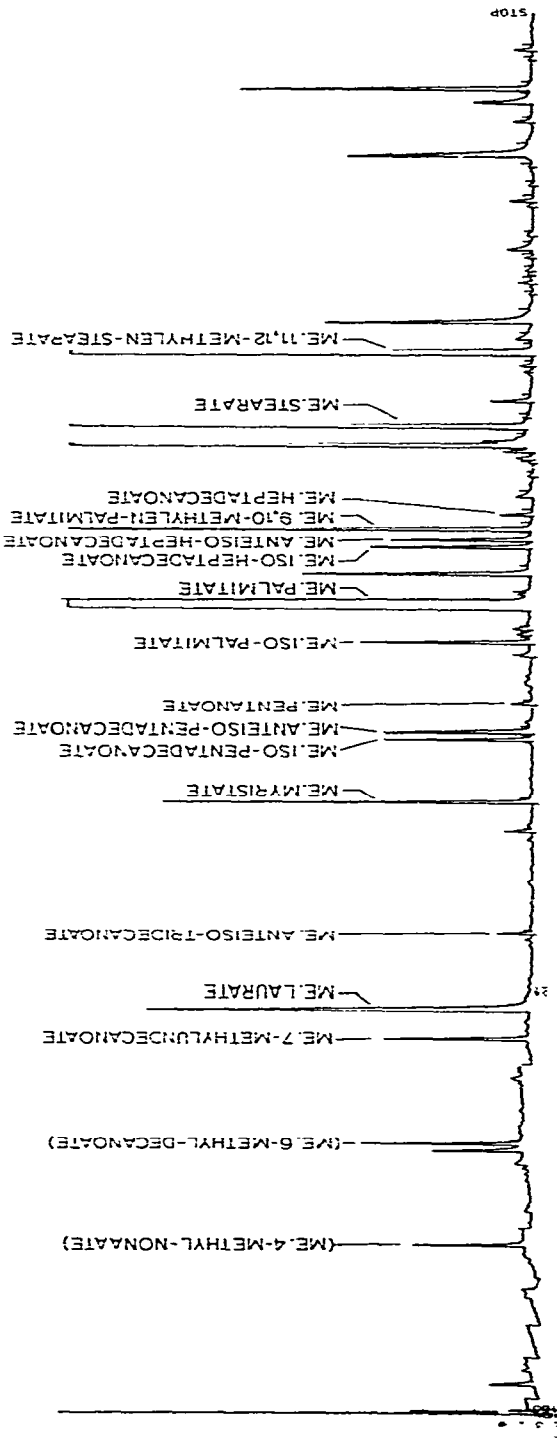


Fig. 5. FAME on an SE-54 capillary column. Sample: saturated TLC fraction.

A comparison of the chromatogram of the TLC fraction with the lowest polarity (Fig. 5), as well as that of the hydrogenated sample (Fig. 4), with the chromatogram of the native sample demonstrates that the main peak in Fig. 2 with an ECL-value of 17.82 also contains small amounts of saturated branched compounds.

For identification we used the following structure-retention relationships[12–15].

(1) In a diagram of the equivalent chain lengths obtained on a non-polar and a polar column it is possible to obtain separate lines for saturated, mono-unsaturated, di-unsaturated, etc. FAME.

(2) FAME with the same degree of saturation and the same end-carbon chain length on a particular phase show a linear relationship between ECL-values and total carbon number. Separate straight lines result, *e.g.*:

14:0, 16:0, 18:0

14:1ω7C, 16:1ω7C, 18:1ω7C

14:1ω9C, 16:1ω9C, 18:1ω9C

(3) Cyclopropane FAME behave in GC like unsaturated compounds. On polar phases they show a higher, and on non-polar phases a lower retention time than their homomorphs.

(4) The nearer the double bond is to the methyl end the later the corresponding ester elutes.

By the application of the first-mentioned relationship, in agreement with the results obtained by TLC pre-separation, it was shown that the lipid extract did not contain polyunsaturated compounds.

Information of special value was obtained by comparison of the ECL-values of the compounds measured on OV-1 and the data reported in the literature. Fig. 6 shows an ECL-total carbon number diagram for unbranched (*n*), *iso*-branched (*i*) and *anteiso*-branched (*a*) saturated and for unsaturated compounds of the palmitoleic type (*n*1ω7C) on SE-30, according to the values of Golovnya *et al.*[13]. Because the polarity of SE-30 is nearly the same as that of OV-1 we used these lines for purposes of identification.

On the basis of the information concerning the structural type obtained up to now we could introduce some of our calculated ECL-values into this diagram (see points introduced into Fig. 6). By this method it was possible to obtain information about the length of the carbon chain, the probable kind of branching*, and the degree of saturation.

In this way it was possible to assign the two unsaturated FAME (ECL 15.82 and 17.82; see Fig. 2) because the straight lines were parallel to the other lines to one type of the mono-unsaturated fatty acids. Besides *iso*- and *anteiso*-branched compounds could be identified with great probability.

A comparison of the values on OV-1 obtained by us with data on SE-30 in the literature[13] for the palmitoleic type (*n*1ω7C) shows complete coincidence. Therefore we concluded that the two unsaturated compounds are palmitoleic and *cis*-vaccenic acid. The presence of unsaturated unbranched compounds is supported by the capillary gas chromatogram of the hydrogenated sample (Fig. 4). Both peaks are absent

* A specification of the type of branching is limited because of the great number of fatty acids[16] and because of the error sources of ECL-estimation. Errors of ECL-estimation occur especially in the case of minor components close to large peaks in the chromatogram.

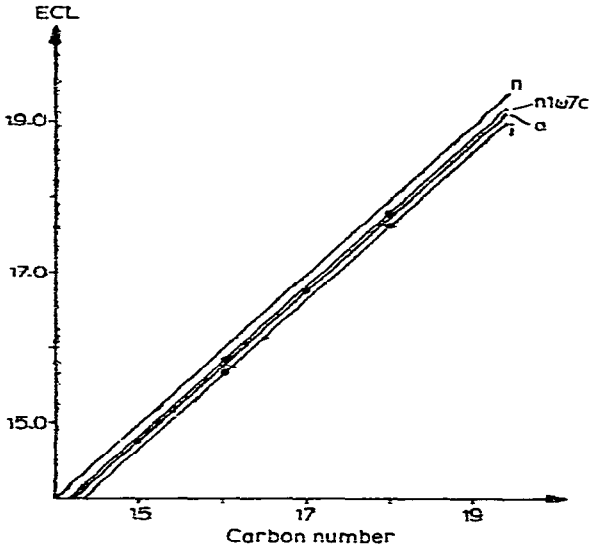


Fig. 6. ECL-values of FAME *versus* carbon number on OV-1. *n*. Saturated non-branched FAME: *a*. saturated *anteiso*-branched FAME; *i*, saturated *iso*-branched FAME; *n*: 1ω7C, mono-unsaturated FAME of the palmitoleic type.

after hydrogenation and the peaks of palmitic and stearic acid are increased. The position of the double bond could be confirmed by addition of oleic acid methyl ester (18:1ω9C). This compound elutes[13] on SE-30 at ECL 0.07 before *cis*-vaccenic acid methyl ester. This agrees with our results on OV-1.

Table II summarizes all the data for chemical pretreatment and structure–retention relationships.

GC–MS analysis

In Fig. 7 a part of a total ion chromatogram of the native sample is presented. It corresponds quite well with its capillary gas chromatogram (Fig. 2).

By interpretation of mass spectra for the different fatty acids the following main fragmentations result (*cf.* refs. 17 and 18).

Saturated unbranched FAME. The base peak is at *m/e* 74 (McLafferty rearrangement)—see explanation 1. By elimination of one propyl radical the (M−43) peak occurs; in relation to the molecular ion peak its intensity decreases with increasing chain length.

Saturated branched FAME. In addition a preferred splitting on both sides of the tertiary carbon atom occurs. Compounds with *anteiso*-branching show peaks at $(M-C_2H_5)$ as well as $(M-C_2H_5-CH_3OH)$ and $(M-C_2H_5-CH_3OH-H_2O)$. *Iso*-branches can be recognized by the peaks $(M-CH_3-CH_3OH)$ and $(M-CH_3-CH_3OH-H_2O)$ as well as by the very low intensity of $(M-CH_3)$.

Cyclopropane FAME. In their mass spectrometric behaviour these compounds are similar to mono-unsaturated FAME. Mono-unsaturated and cyclopropane FAME differ in the ratio $M/(M-CH_3OH)$. The position of the cyclopropane ring within the molecule results from characteristic alkyl splittings at the ring.

Hydroxy FAME. From the fragments $(M-H_2O)$, $(M-CH_3OH-H_2O)$ and

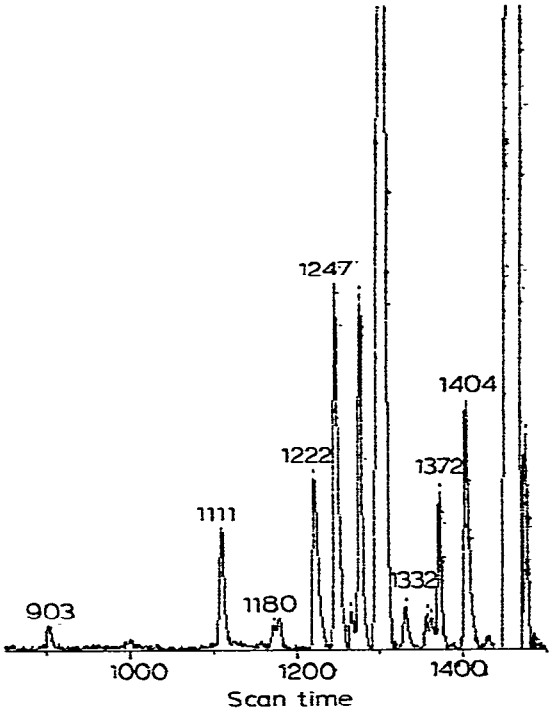


Fig. 7. Total ion chromatogram.

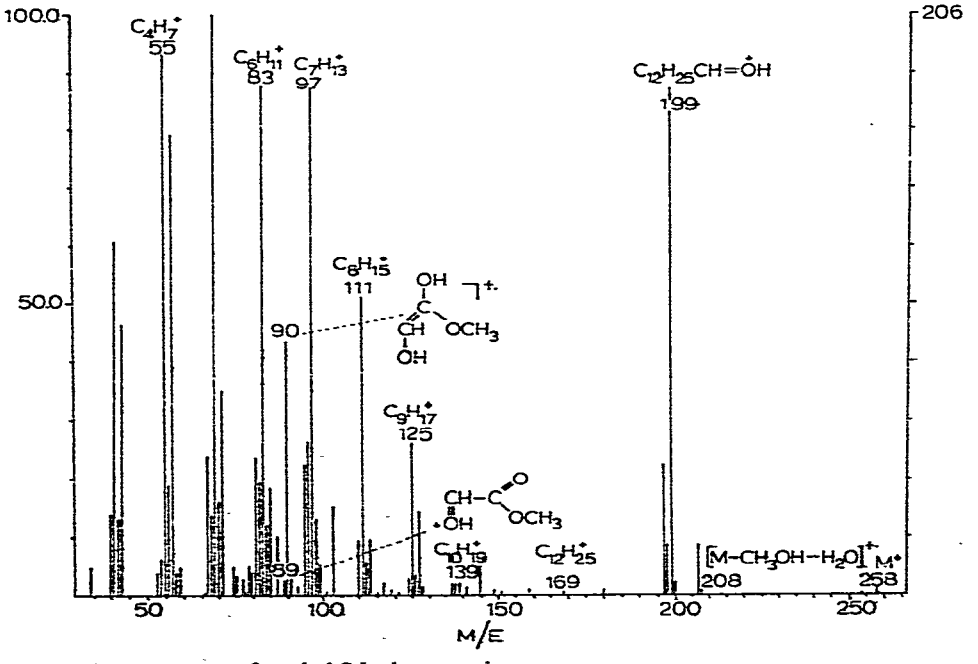


Fig. 8. Mass spectrum of methyl 2-hydroxymyristate.

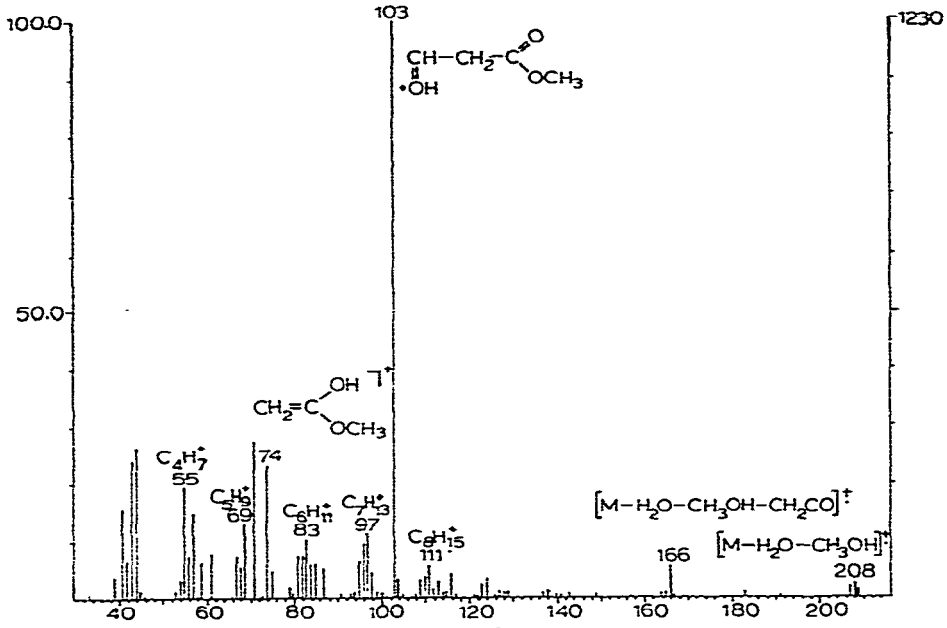
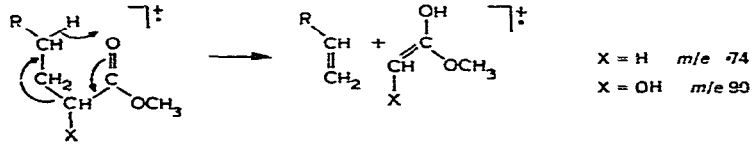


Fig. 9. Mass spectrum of methyl 3-hydroxymyristate.

$(M-CH_3OH-H_2O-CH_2CO)$ the molecular weight can be estimated. The position of the hydroxy group can be estimated from the intensive ions originating from onium-splitting (explanation 1) as well as from the McLafferty peaks (explanation 2). In Figs. 8 and 9 characteristic fragmentations of methyl 2-hydroxymyristate and methyl 3-hydroxymyristate are demonstrated.

Explanation 1



In the case of all esters with the characteristic structure element $R–CH_2–CO–OCH_3$ a peak at *m/e* 74 occurs. Thus occurrence of the peak at *m/e* 90 in Fig. 8 is understandable.

Explanation 2

$$CH_3\text{-}(CH_2)_m\text{-}\underset{\displaystyle OH}{\underset{|}{CH}}\text{-}(CH_2)_n\text{-}CO\text{-}OCH_3$$

$\rightarrow$ $CH_3\text{-}(CH_2)_m\text{-}\overset{+}{C}H{=}OH$
m=11 (*e.g.* methyl 2-hydroxymyristate) $\rightarrow$ peak at *m/e* 199

$\rightarrow$ $CH\text{-}(CH_2)_n\text{-}CO\text{-}OCH_3$
$\|$
$+OH$
n=0 (2-hydroxy-FAME) $\rightarrow$ *m/e* 89 (Fig. 8)
n=1 (3-hydroxy-FAME) $\rightarrow$ *m/e* 103 (Fig. 9) etc.

CONCLUSIONS

It is demonstrated that by a combined application of classical chemical reactions and pre-separations, as well as of the known structure–retention relationships, valuable analytical results concerning the structural type and the chain length of fatty acids in complex lipid extracts can be obtained, which generally are obtainable only by computer-supported GC–MS.

This is important for all laboratories that possess the means of separating a mixture of fatty acids into its individual components by a high-performance separation, but not an expensive GC–MS system.

Our data, which were obtained by classical chemical methods and by gas chromatographic identification, were confirmed by GC–MS especially in respect to the position of the functional group and the branching of the carbon chain. By summarizing all the different results a precise assignment of the individual components is possible.

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