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GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF C_1–C_{20} FATTY ACID BENZYL ESTERS

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SUMMARY

Benzyl esters of saturated normal and some branched fatty acids (C_1–C_{12}) and of saturated and unsaturated even-numbered long chain fatty acids (C_{14}–C_{20}) were prepared by reaction with phenyldiazomethane. Mass spectra of the individual esters were recorded. A mixture of all the prepared saturated esters was separated on an SE-30 column and identified in the μg range by a combination of gas chromatography and mass spectrometry. Separation of saturated and unsaturated long chain fatty acid benzyl esters was achieved on an EGSS-X column at 210°. A general fragmentation scheme for this class of substances is proposed and the formation of the main fragments is discussed.

INTRODUCTION

For the gas chromatographic (GC) separation of short and medium chain fatty acids (FA), the corresponding benzyl esters have proved to be particularly suitable[1]. Quantitative benzylation with N,N'-dicyclohexyl-O-benzylisourea or phenyldiazomethane was achieved under mild conditions. Short and medium chain FA benzyl esters (FABE) could be readily separated on normal columns, within a temperature range of 100–200°.

This paper reports that this method is also applicable to GC of long chain FA, thereby permitting analysis of mixtures of FA from C_1 to C_{20}. The basis for GC–mass spectrometric (MS) analysis of these compounds was therefore established. Mostly FA methyl esters or free FA have been used for MS of FA[2–7]. A few short and medium chain FA have been analysed by MS as their ethyl, propyl, butyl, and hexyl esters by Sharkey *et al.*[8] Except for the published spectra of C_1–C_4 FABE by Emery[9], FABE have not yet been investigated.

The mass spectra of saturated C_1–C_{20} FABE, as well as those of some branched and unsaturated FABE, are recorded subsequent to GC separation and the efficiency of the GC–MS method is checked by analysis of a mixture of FABE. The significance of this method is seen in its application to complex mixtures of lipids and biological materials, the components of which cannot be definitely identified by GC

retention volumes alone. By MS, FABE are well distinguished from other classes of compounds by characteristic fragments and, in addition, each individual FABE can usually be identified by its peculiar fragmentation pattern.

MATERIALS

Carboxylic acids (C_3–C_{12}, $C_{16:1,n-7}$, $C_{18:3,n-6}$, $C_{18:3,n-3}$, $C_{20:1,n-9}$ and $C_{20:4,n-6}$) were obtained from Sigma (St. Louis, Mo., U.S.A.); formic acid, acetic acid and $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1,n-9}$, $C_{18:2,n-6}$ and $C_{20:0}$ from E. Merck (Darmstadt, G.F.R.); and isobutyric acid, 2-methyl-*n*-butyric acid and 4-methyl-*n*-valeric acid from Fluka (Buchs, Switzerland). All solvents (reagent grade) and chemicals were purchased from E. Merck.

The gas–liquid chromatograph (Packard Model 419, Becker, Delft, The Netherlands) was equipped with a flame ionization detector and recorder (Kipp & Zonen, BD 8, Delft, The Netherlands). Glass columns (2 m long × 3 mm I.D.) were packed with 3% EGSS-X on Gas-Chrom Q, 100–120 mesh, obtained from Applied Science Labs. (State College, Pa., U.S.A.). For GC–MS measurements a MAT 111 Model GC–MS system (Varian-MAT, Bremen, G.F.R.) was used. Gas chromatograms were recorded with a Hitachi/Perkin-Elmer Model 159 recorder (Perkin-Elmer, Überlingen, G.F.R.), mass spectra with an Oscilloport model light point recorder (Siemens, Erlangen, G.F.R.). GC separations were achieved on a glass column packed with 3% SE-30 on Gas-Chrom Q, 125–150 μm (Applied Science Labs.).

METHODS

Preparation of FABE

FABE were synthesized by esterification of FA with phenyldiazomethane[1]. Due to a faster rate of reaction, long chain FA were prepared in chloroform–methanol (2:1).

Gas chromatography of FABE

C_1–C_{12} FABE were separated by GC as described previously[1]. Long chain FABE were separated on an EGSS-X column using the following conditions: column temperature, 210°; helium flow-rate, 18 ml/min; injection port and flame ionization detector temperatures, 260°.

GC–MS of single FABE

FABE (0.5 μl of a 1% *n*-pentane solution) were separated from solvent by a MAT 111 GC–MS system and the mass spectra were recorded. Conditions: helium flow-rate, 20 ml/min (measured at 25° without vacuum); inlet pressure, 1.6 kp/cm²; column temperature, 140° (C_1–C_6), 180° (C_7–C_{12}), 250° (C_{14}–C_{20}); injection port temperature, 240°, ionizing voltage, 80 V; current, 270 μA; ion source temperature, 300°; inlet tube temperature, 220°.

GC–MS of a mixture of C_1–C_{12} FABE

One microlitre of a 1% *n*-pentane solution of C_1–C_{12} FABE was injected. Condi-

tions; column temperature, 130° (isothermal, 5 min), temperature programme, to 130 170° at 2°/min, 170 to 240° at 4°/min. Other conditions as described above.

Evaluation of mass spectra

Intensities of fragments were measured relative to the peak with greatest intensity (100%).

RESULTS

According to the previously described[1] GC separation of short and medium chain FABE on SE-30, C_{14}–C_{20} FABE were well separated only by changing the temperature to 250°. Saturated and unsaturated FA of equal carbon number could not be differentiated on this stationary phase. Therefore a mixture of saturated and unsaturated C_{14}–C_{20} FABE was separated on EGSS-X (Fig. 1). All FABE of the mixture used were separated except for the pair $C_{18:3,n-3}$ and $C_{20:1,n-9}$. The chromatogram of FABE is similar to that of FA methyl esters.

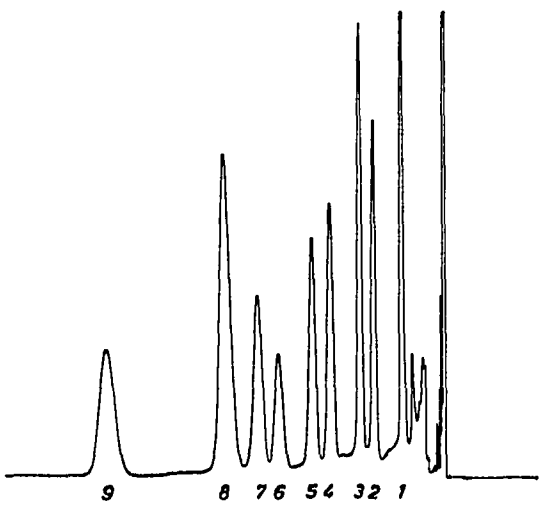


Fig. 1. Chromatogram of benzyl esters of the following fatty acids: 1, $C_{14:0}$; 2, $C_{16:0}$; 3, $C_{16:1,n-7}$; 4, $C_{18:0}$; 5, $C_{18:1,n-9}$; 6, $C_{18:2,n-6}$; 7, $C_{20:0}$; 8, $C_{18:3,n-3}$ + $C_{20:1,n-9}$; 9, $C_{20:4,n-6}$. Stationary phase, EGSS-X on Gas-Chrom Q; temperature, 210°.

Fig. 2 shows the mass spectra of C_1–C_{12} FABE. The intensity of molecular ions diminished with increasing alkyl residue (C_2–C_{12}). The molecular ion of C_{12} FABE was still detectable at 1.5% relative intensity. Ions of *m/e* 91 (tropylium cations) and *m/e* 108 (protonated benzyloxy radicals) appeared with high intensity in all mass spectra. Further characteristic fragments were acylium cations, produced by cleavage of a benzyloxy radical from M^+ (M^+ — 107). Fragments M^+ — 91 together with fragments M^+ — 91 — 18 appeared from C_6 FABE upwards. The relative intensities of these two fragments were similar and decreased from about 22% for C_6 to about 7% for C_{12}. Fragments M^+ — 14 were observed in mass spectra of

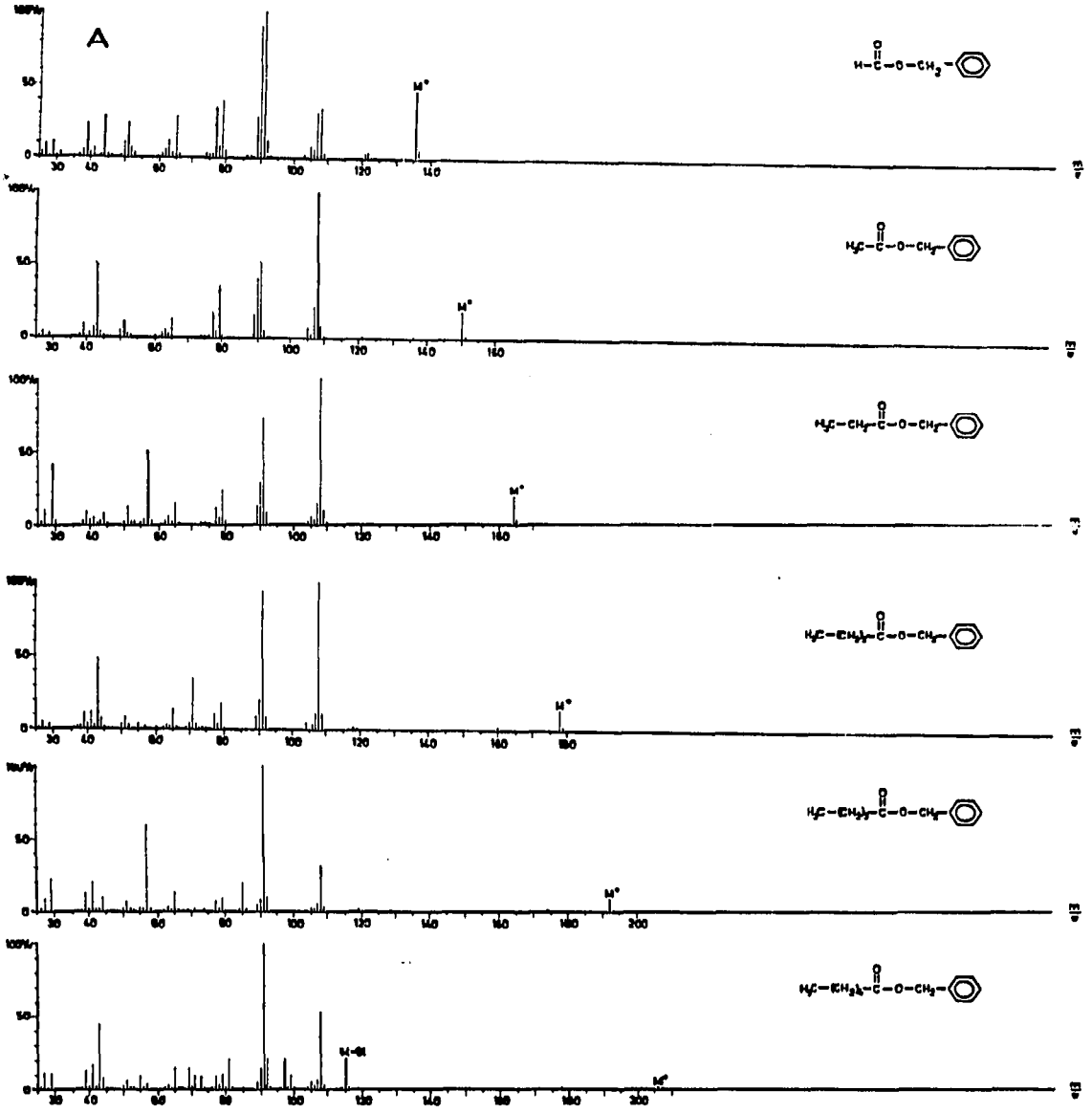
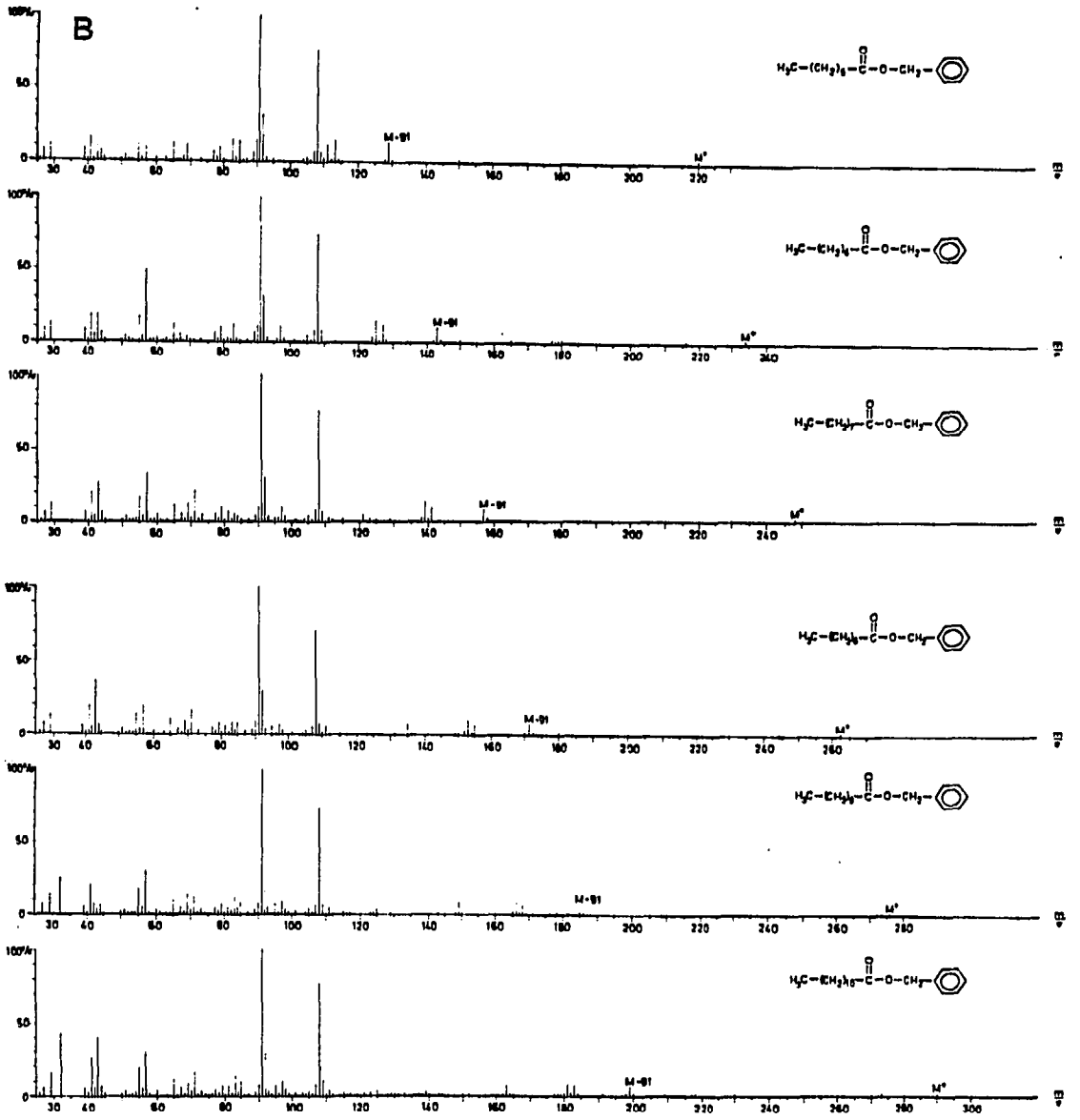


Fig. 2. Mass spectra of short and medium chain FABE.

C_1–C_7 FABE. *n*- and iso-C_4–C_6 FABE had similar, but distinguishable, fragmentation patterns.

Molecular ions were absent in mass spectra of long chain FABE (C_{14}–C_{20}) (Fig. 3). In the mass spectra of saturated C_{14}–C_{20} FABE, *m/e* 108 had a high relative intensity (31–74 %) whereas this fragment appeared with lower relative intensity (2.4–13 %) in mass spectra of unsaturated long chain FABE. This difference can be used to distinguish saturated from unsaturated FA. Although no molecular peak was ob-



served in these mass spectra, the molecular weight could be determined from the characteristic fragment $M^+ - 91$ that occurs in all mass spectra of long chain FABE. However, this peak was present only in low relative intensity (0.5–5.6%). Medium and long chain FABE showed the typical fragments of hydrocarbons C_nH_{2n+1} of *m/e* 69, 83 and 97. These fragments occurred with remarkably high intensity in mass spectra of monoenoic FABE $C_{16:1}$, $C_{18:1}$ and $C_{20:1}$. Mono-unsaturated fragments C_nH_{2n-1} of *m/e* 67, 81 and 95 had maximum intensity with $C_{18:2,n-6}$ FABE. The MS

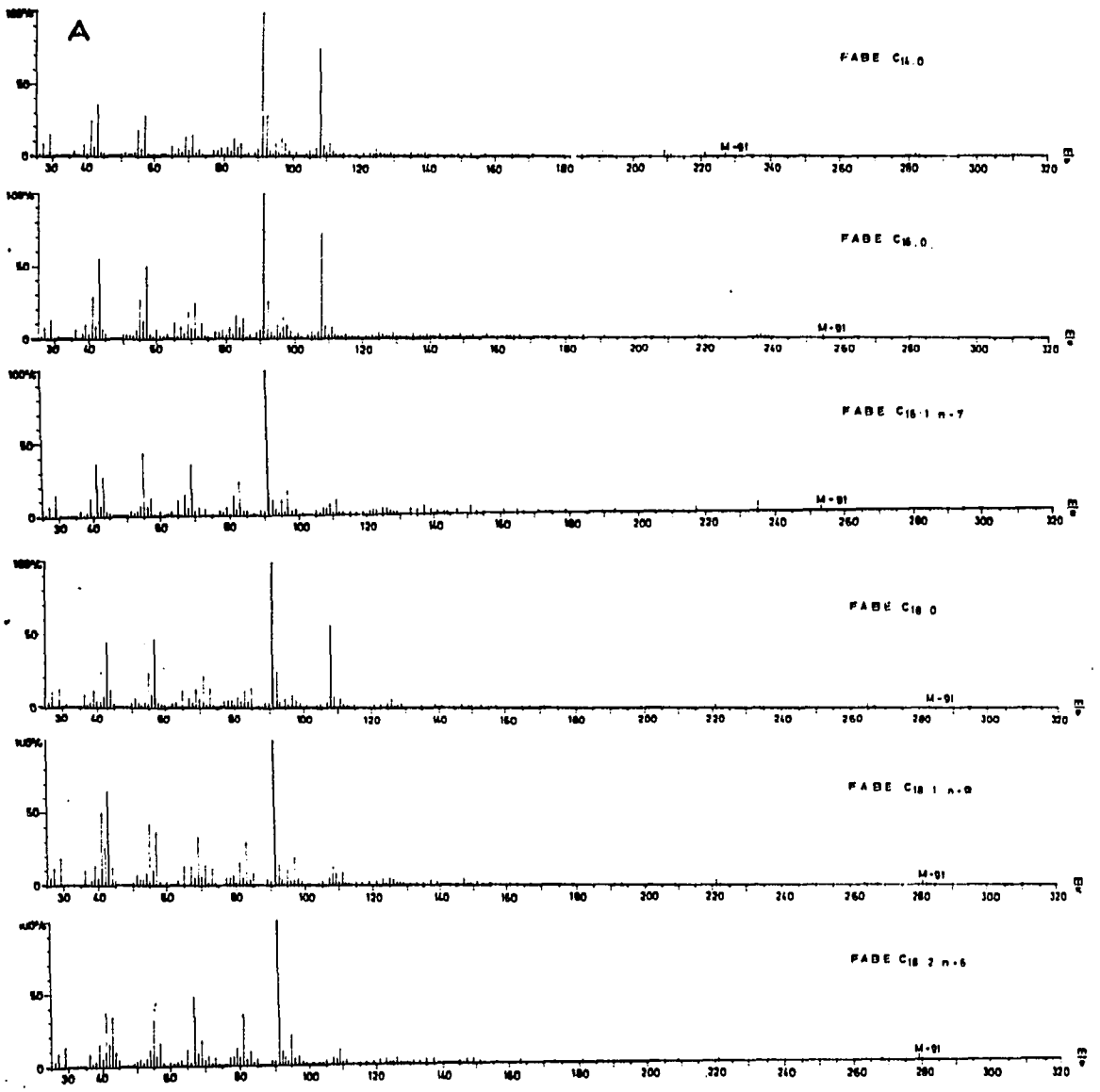
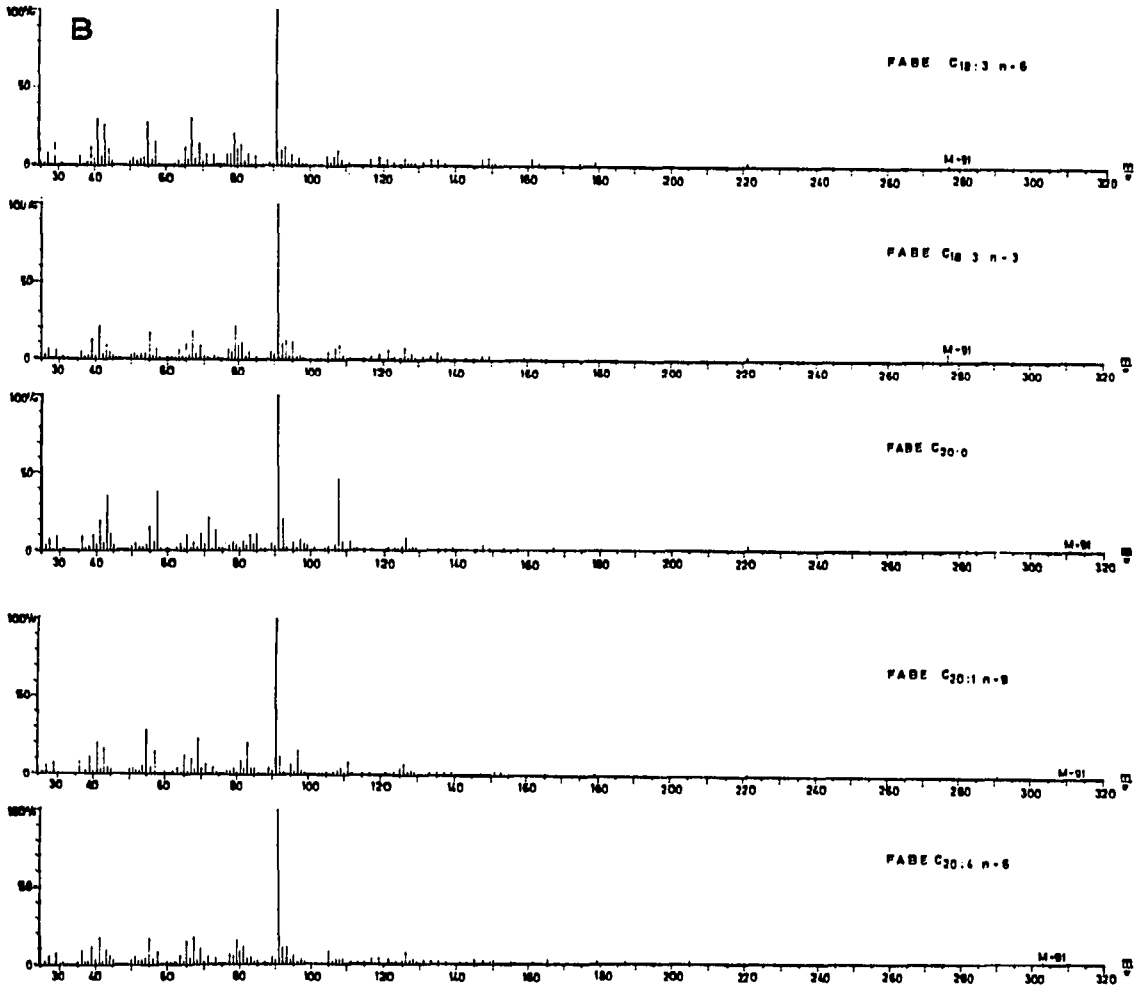


Fig. 3. Mass spectra of long chain FABE.

detection limit was 0.5 μg of FABE. In GC–MS, a complete GC separation of a mixture of C_1–C_{12} FABE and of even-numbered saturated C_{12}–C_{20} FABE was achieved. GC separation gave fractions which showed the characteristic mass spectra of the individual FABE.

For the decomposition of FABE the following mechanism is possible (Fig. 4).



(a) Cleavage of an alkylcarboxylic radical from M^+ produces a tropylium cation and is energetically favoured due to mesomeric stabilization of the positive charge.

(b) Cleavage of aldoketenes from M^+ and simultaneous rearrangement of an H atom produces the radical cation *m/e* 108 (Bz–$\dot{O}^+$–H), where Bz = benzyl.

(c) Cleavage of benzyloxy radicals (Bz-$\dot{O}$) from the molecular ions (C_1–$C_{18:0}$) M^+ — 107 leads to mesomerically stabilized acylium cations. Tropylium and acylium ions decompose in a known manner to give low molecular weight fragments.

(d) By cleavage of benzyl radicals from the molecular ions (M^+ — 91), alkyl carboxonium ions appear in the mass spectra of higher FABE (from C_6 upwards). Elimination of H_2O gives fragments M^+ — 91 — 18.

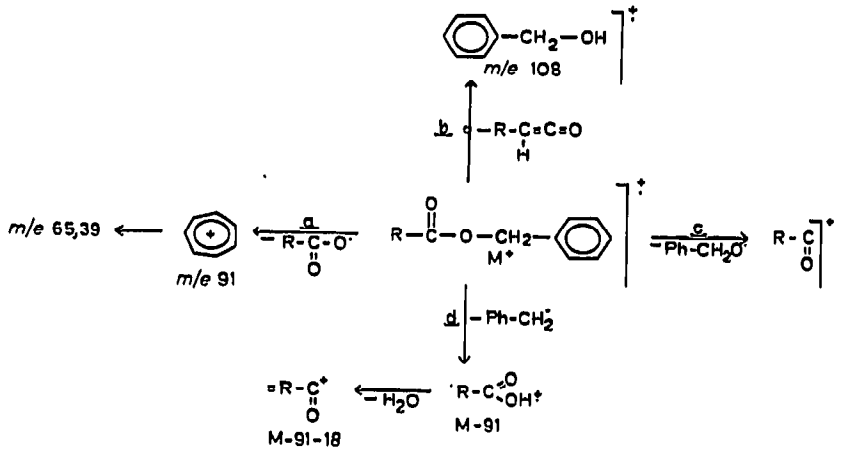


Fig. 4. Fragmentation mechanism.

DISCUSSION

The method of benzylation and gas chromatographic separation of short and medium chain FABE, as previously described, is now also shown to be applicable to long chain FA. Whereas the procedure for preparation and GC of short and medium chain FABE was an analytical improvement, the GC of long chain FABE was not superior to that of FA methyl esters (FAME). The advantage of this method was that it allowed derivatization of C_1–C_{20} carboxylic acids and their separation. In Mass spectra the appearance of fragments *m/e* 91 (tropylium cation) was characteristic of FABE. If the molecular ions were absent in the mass spectra of the higher FA, the fragments $M^+ - 91$ identified the individual FABE. FABE up to C_{20} were clearly identified from the mass spectra. However, there was less information about the hydrocarbon chain than is found in studies of FAME. FAME exhibited not only a clear molecular peak, but also fragments in the higher mass range. The McLafferty product *m/e* 74 appeared as the base peak in the mass spectra of long chain FAME. The corresponding fragment *m/e* 150 or 151, respectively, was observed only in low intensity in most spectra of FABE. Fragmentation $M^+ - 31$, cleavage of methoxyl radicals from the molecular ion of FAME, corresponded to cleavage of benzyloxy radicals ($M^+ - 107$) from that of FABE. The fragments $[(-CH_2)_n\text{–COOR}]^+$ characteristic of FAME occurred only rarely and with low intensities in spectra of FABE. Fragments $M^+ - 14$, present in spectra of C_1–C_7 FABE with low intensity, were not observed for FAME. They were also absent in the spectra of C_1–C_4 FABE published by Emery[9]. Due to prior GC separation, contamination by the lower homologue is excluded. McCloskey[7] considered such contamination to be the most probable explanation for such an observation. Protonated acids appeared in the spectra of FABE only rarely and with low intensity. In the spectrum of stearic acid butyl ester, the most abundant ion was the protonated acid *m/e* 285 (ref. 3). As Sharkey *et al.*[8] observed with short chain FA esters, the relative intensity of fragments of the protonated acid increased with increasing chain length of the unbranched alcoholic component. In spite of mass spectral similarity, a differentiation between short chain *n*- and iso-FABE was possible in

a manner similar to long chain iso-FAME[4,5]. Saturated and unsaturated FA of equal chain-length could be distinguished by GC and MS by the fragments $M^+ - 91$ and the different distribution of intensities in the low mass range. As with the methyl esters of polyenoic FA having equal number, but differently positioned, double bonds, the mass spectra of benzyl esters of $C_{18:3,n-6}$ and $C_{18:3,n-3}$ were clearly differentiated.

The GC–MS analysis of FABE is of importance especially for the investigation of biological materials and of complex lipid mixtures, because of quantitative derivatisation of free FA under mild conditions without isomerisation or hydrolysis of ester bonds. For application to biological material, it is necessary that the derivatisation occurs at the beginning of preparation. FABE can be clearly distinguished by the characteristic fragmentation pattern from other substances having similar gas chromatographic behaviour.

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