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ANALYSIS OF FATTY ACID METHYL ESTERS BY HIGH-RESOLUTION GAS CHROMATOGRAPHY–CHEMICAL IONISATION MASS SPECTROMETRY

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SUMMARY

The potential of support-coated open tubular gas chromatography combined with chemical ionisation mass spectrometry using NH_3, CH_4 and He as reactant gases in the analysis of fatty acid methylesters (FAME) has been investigated. Differentiation between unsaturated FAME and cyclopropane FAME could be made on the basis of retention time and by comparison of the chromatograms obtained using methane and ammonia chemical ionisation. The ammonia chemical ionisation mass spectra of FAME, with the exception of 3-hydroxy-FAME, contained only the $[M + NH_4]^+$ adduct ion. The helium chemical ionisation mass spectra of branched chain FAME were qualitatively similar to reported electron impact mass spectra and enabled the point of branching to be determined.

INTRODUCTION

Gas chromatography–mass spectrometry (GC–MS) has now become the method of choice for the analysis of fatty acid methyl esters (FAME). The most widely used methods have relied on packed column gas chromatography (GC) and electron impact mass spectrometry (EI-MS)[1], although more recently the advantages of wall-coated open tubular columns (WCOT) in combination with EI-MS have been reported[2]. A further refinement has been the use of chemical ionisation mass spectrometry combined with packed column GC[3]. Chemical ionisation (CI) offers the advantage of enhanced abundance of molecular ions compared to electron impact and aids greatly in identifying small or partially resolved GC peaks. Another advantage of chemical ionisation which has not yet been exploited in FAME analysis is that by using different reactant gases significantly different CI mass spectra may be obtained. For example, the use of helium as reactant gas produces spectra which are similar to conventional 70 eV EI mass spectra[4] whereas ammonia gives rise to spectra which usually contain only the adduct ion $[M + NH_4]^+$ (refs 5 and 6).

This paper describes an approach to the analysis of FAME in which the potential of support-coated open tubular GC is combined with CI-MS using a range of reactant gases.

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MATERIALS AND METHODS

Materials

The methyl ester of *cis*-10-heptadecenoic acid was purchased from Nu Chek Prep Inc. (Elysian, MN, U.S.A.), all other methyl esters used in preparation of FAME mixtures 1 and 2 (see Table I) were purchased from Applied Science Labs. (State College, PA, U.S.A.). Extraction of fatty acids from Streptomyces R61 cultures will be described elsewhere.

Gas chromatography–mass spectrometry

Analysis of FAME mixtures was carried out on a Finnigan 3200 quadrupole mass spectrometer fitted with a CI source and interfaced to a Finnigan 9500 gas chromatograph. Data acquisition and processing were carried out on-line using a Finnigan 6110 data system. The GC column (50 m × 0.5 mm) used was a SE 30 support-coated open-tubular (SCOT) glass column (Scientific Glass Engineering, Melbourne, Australia). Helium was used as the carrier gas at a flow-rate of 2.5 ml/min. The column temperature was programmed from 190°C to 230°C at 2°/min and held at 230°C until elution of peaks ceased. Injection port and interface temperatures were kept at 250°C.

Chemical ionisation mass spectra were generated by adding reactant gas (He, CH_4 or NH_3) through a make-up T-piece at the end of the column. The reactant gas flow-rate was adjusted to give a source pressure of 67 Pa for He and 133 Pa for CH_4 and NH_3. A system of solenoid-operated valves enabled rapid between different reactant gases. However, after using NH_3 it was necessary to evacuate the gas lines for 30 min or so before using He or CH_4 as traces of NH_3 tended to persist in the lines. Mass spectra were generated using an electron beam energy of 135 eV and source temperature was kept at 100°C.

RESULTS AND DISCUSSION

Fig. 1 shows typical CI mass spectra of methyl hexadecanoate using He, CH_4 and NH_3 as reactant gases. Although fragmentation is more extensive under helium CI conditions than under EI conditions, the helium CI spectrum is quite similar to published EI spectra. The base peak in the methane CI mass spectrum is the protonated molecular ion (MH^+) at *m/z* 271 and this ion is accompanied by the adduct ions $[M + C_2H_5]^+$ and $[M + C_3H_5]^+$ at *m/z* 299 and 311 respectively. The fragment ions at *m/z* 269 and *m/z* 239 correspond to loss of H_2 and CH_3OH, respectively, from the MH^+ ion.

In contrast to the helium and methane mass spectra the ammonia CI mass spectrum contains only one ion, the adduct ion, $[M + NH_4]^+$, at *m/z* 288. Of all the FAME studied in this work only 3-hydroxy-FAME gave ammonia CI mass spectra in which fragment ions were observed (Fig. 2a). These fragmentation processes parallel those observed in the methane CI spectrum of 3-hydroxy-FAME (Fig. 2b) and are summarised for methyl 3-hydroxytetradecanoate in Fig. 3.

Methane chemical ionisation also provides an excellent method of distinguishing between 2-hydroxy- and 3-hydroxy-FAME (Fig. 2). Fragmentation of 3-hydroxy-FAME is much more extensive than fragmentation of 2-hydroxy-FAME

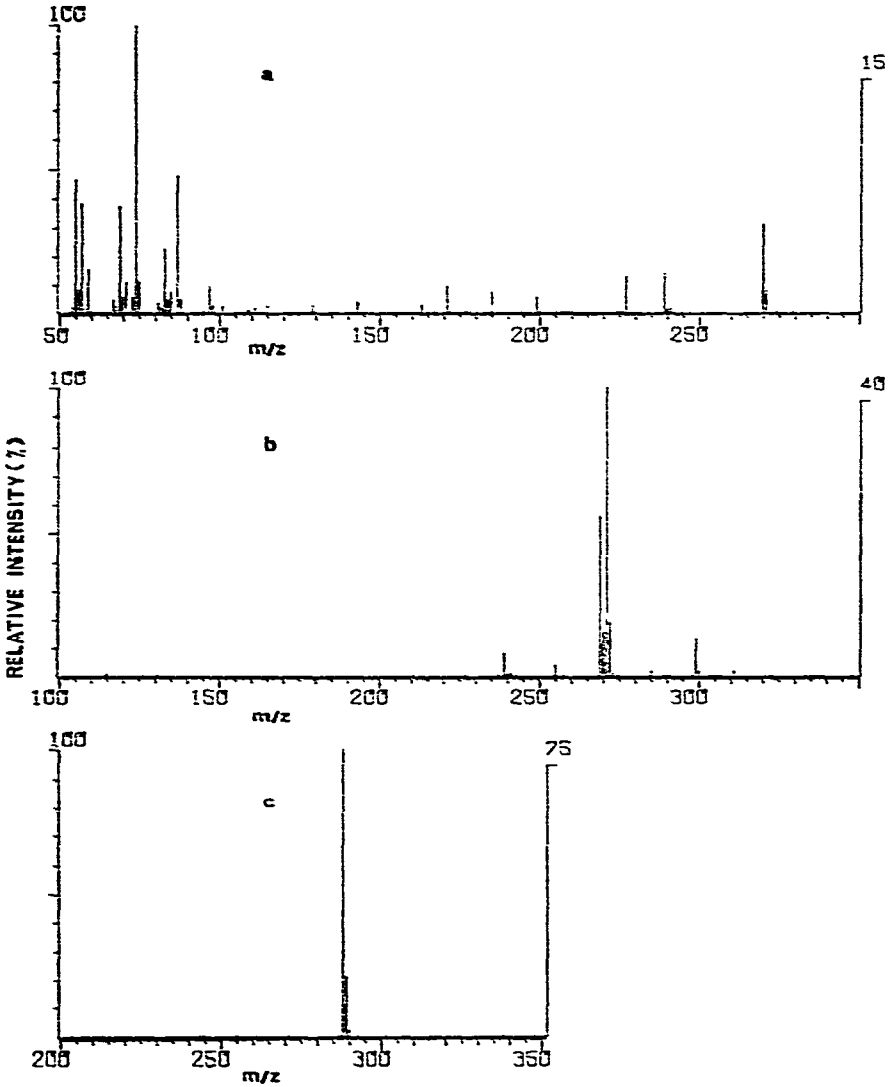

Fig. 1. Chemical ionisation mass spectra of methyl hexadecanoate using different reactant gases: (a) He, (b) CH_4 and (c) NH_3.

because of favourable mechanistic pathways available for 3-hydroxy-FAME (Fig. 3) but not for 2-hydroxy-FAME (Fig. 4). Loss of 74 a.m.u. is characteristic of a 3-hydroxyl group as this reaction involves transfer of a hydrogen via a six-membered ring transition state (ion a, Fig. 3). In addition, loss of a molecule of water from the protonated molecular ion is favourable for a 3-hydroxyl group but not for a 2-hydroxyl group because in this case the charge on the product ion (ion b, Fig. 4) is on the carbon atom adjacent to the carbonyl carbon atom. This is a very unfavourable arrangement because of the polarisation of the carbonyl bond.

By using the three CI reactant gases in turn it was possible to maximise the

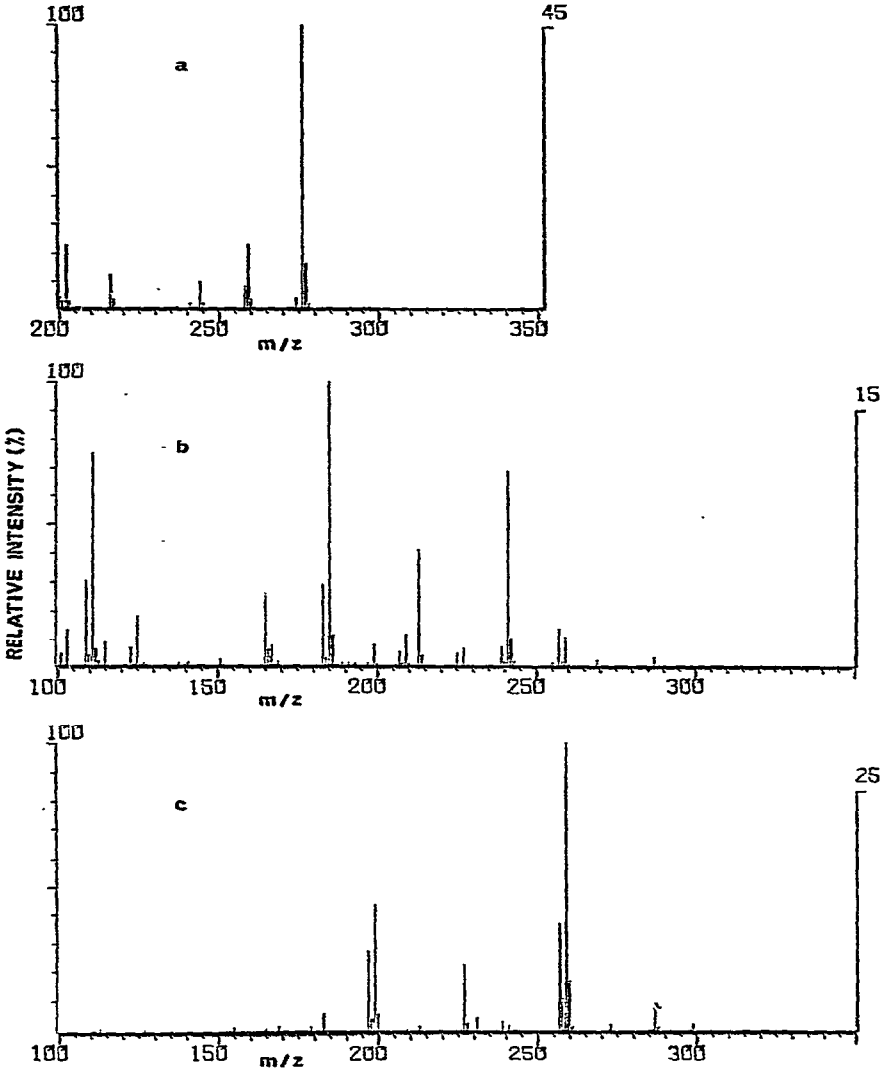

Fig. 2. Chemical ionisation mass spectra of the methyl esters of hydroxytetradecanoic acids: (a) methyl 3-hydroxytetradecanoate (ammonia CI), (b) methyl 3-hydroxytetradecanoate (methane CI) and (c) methyl 2-hydroxytetradecanoate (methane CI).

amount of structural information obtainable from each sample. Initially the sample was analysed using ammonia CI to produce a chromatogram which is essentially a molecular weight profile of the FAME present. The chromatogram obtained for FAME mixture 1 is shown in Fig. 5. All of the components of this mixture including the two $C_{18:1}$ isomers, methyl *cis*-9-octadecenoate and methyl *cis*-11-octadecenoate, were resolved under the GC conditions used. Relative retention times are given in Table I.

The GC–MS run was then repeated using CH_4 and He as reactant gases in turn to obtain spectra in which increased fragmentation could be observed. The

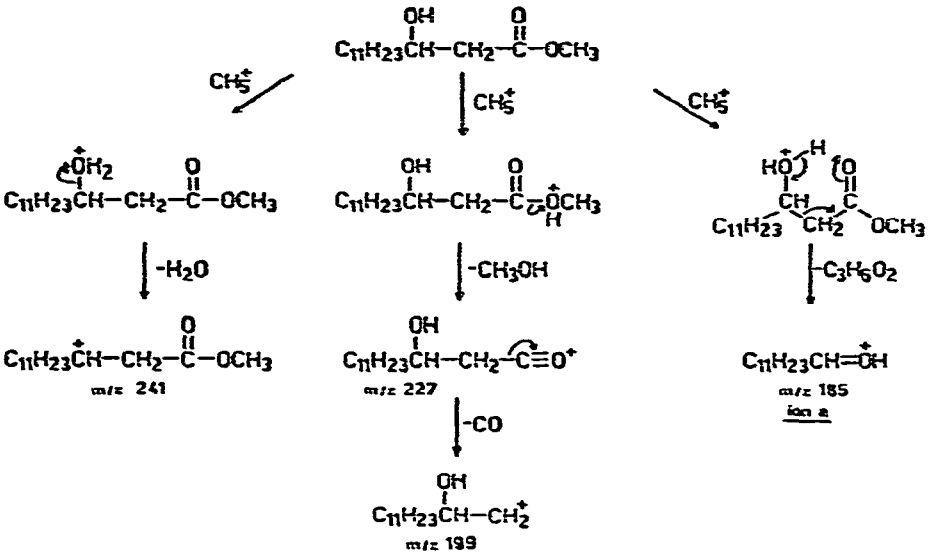

Fig. 3. Methane chemical ionisation-induced fragmentation of methyl 3-hydroxytetradecanoate.

chromatogram obtained using methane CI is shown in Fig. 5. Branched chain FAME could be easily identified by their helium CI mass spectra which show the characteristic enhancement of bond cleavage at branched sites observed in EI mass spectra[1,2].

In studies of FAME derived from bacterial lipids a recurrent problem is to distinguish between FAME containing a double bond and FAME of the same molecular weight but containing a cyclopropane ring. These compounds cannot be resolved on packed columns and their mass spectra (EI or CI) are usually so similar

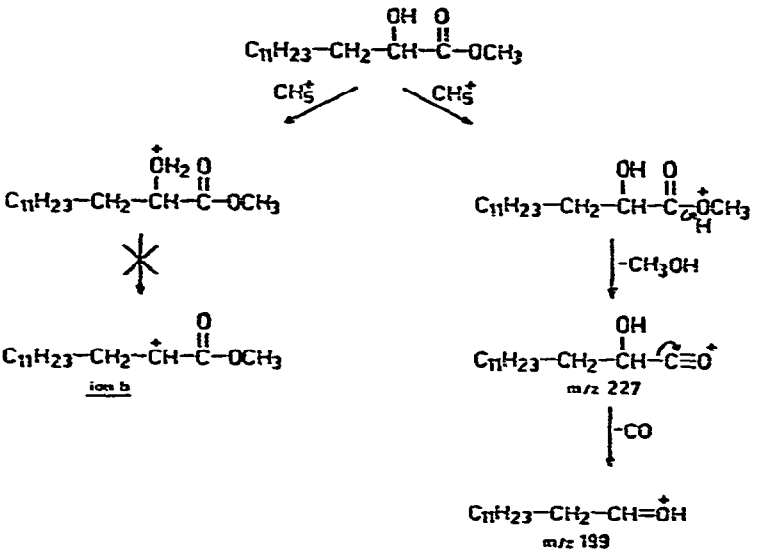

Fig. 4. Methane chemical ionisation-induced fragmentation of methyl 2-hydroxytetradecanoate.

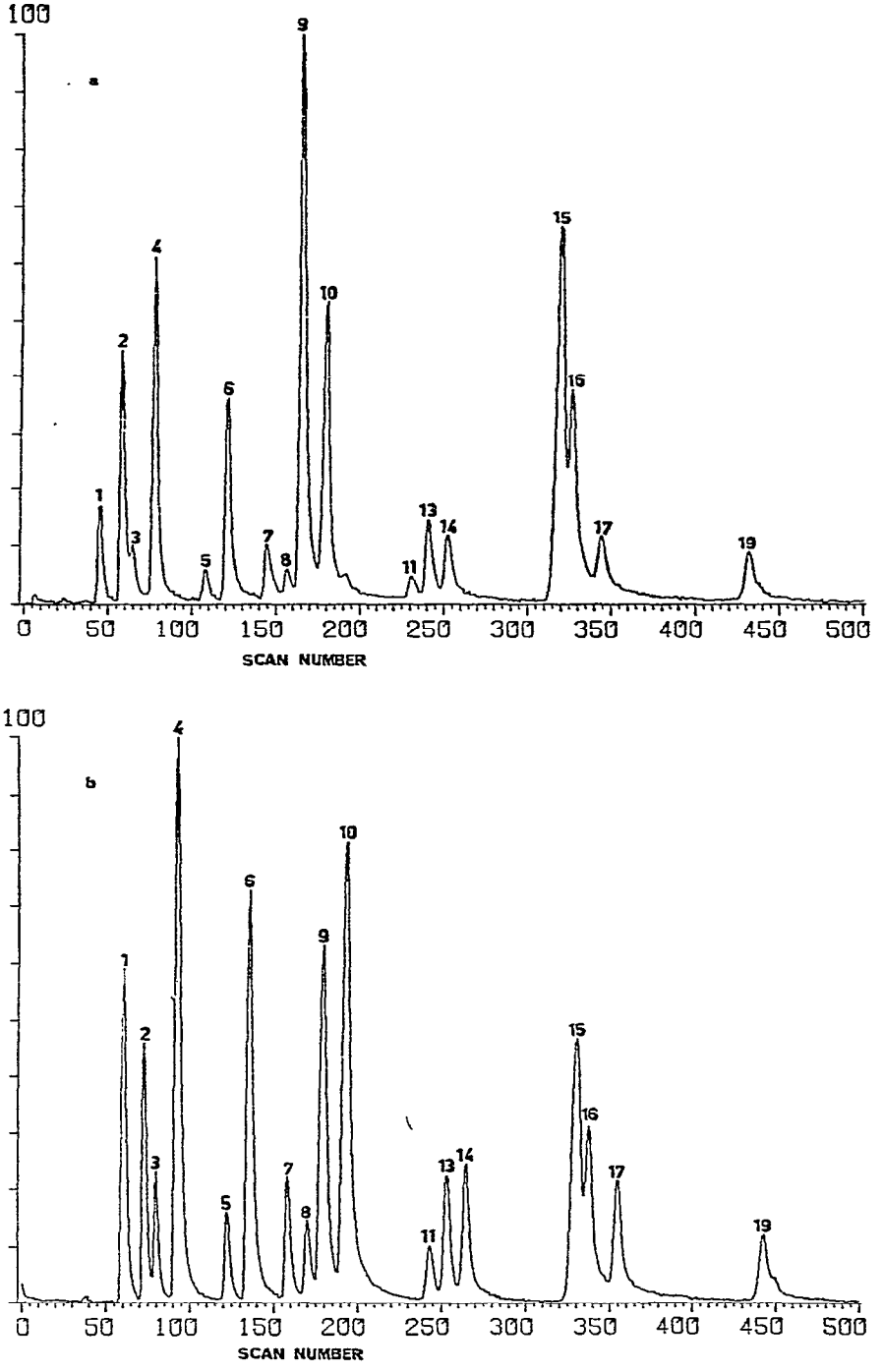

Fig. 5. Gas chromatograms obtained by GC–CI-MS analysis of FAME mixture 1: a, ammonia chemical ionisation; b, methane chemical ionisation. Peak numbers refer to FAME listed in Table I. See text for GC–CI-MS conditions.

TABLE I

RETENTION TIMES OF FATTY ACID METHYL ESTERS RELATIVE TO DOCOSANE

Peaks 1–11, 13–17 and 19 appear in Fig. 5, using FAME mixture 1, peaks 12, 13 and 15–19 appear in Fig. 6 using FAME mixture 2. Δ = Cyclopropane ring; Me = methyl.

GC peak no.	*Fatty acid methyl ester*	*Relative retention time*
1	$C_{13:0}$	0.388
2	3-OH-$C_{12:0}$	0.408
3	13-Me-$C_{14:0}$ (*iso*)	0.419
4	$C_{14:0}$	0.439
5	13-Me-$C_{15:0}$ (*anteiso*)	0.489
6	$C_{15:0}$	0.508
7	3-OH-$C_{14:0}$	0.542
8	15-Me-$C_{16:0}$ (*iso*)	0.561
9	9-*cis*-$C_{16:1}$	0.577
10	$C_{16:0}$	0.597
11	15-Me-$C_{17:0}$ (*anteiso*)	0.681
12	10-*cis*-$C_{17:1}$	0.687
13	9-*cis*-$C_{17:\Delta}$	0.695
14	$C_{17:0}$	0.712
15	9-*cis*-$C_{18:1}$	0.815
16	11-*cis*-$C_{18:1}$	0.826
17	$C_{18:0}$	0.853
18	10-*cis*-$C_{19:1}$	0.974
19	9-*cis*-$C_{19:\Delta}$	0.991
	Docosane	1.00

that a positive identification cannot be made. To investigate the potential of SCOT columns in identifying such compounds, FAME mixture 2, which contained two such pairs of FAME, was analysed and the chromatogram obtained is shown in Fig. 6. All of the components of the mixture were resolved by the column and the relative retention times of the components of this mixture are shown in Table I (peaks 12, 13 and 15–19). Owing to a lack of suitable authentic samples of compounds of this type, it was not possible to determine how the position of the double bond or cyclopropane ring influences retention time. However, such standards are currently being sought by synthesis.

This work does suggest that resolution of the more commonly occurring unsaturated and cyclopropane FAME may be achieved on SCOT columns.

Having established the potential of the techniques described in this work analysis of FAME obtained from a culture of Streptomyces R61 was attempted. The chromatogram of the FAME-containing extract is shown in Fig. 7.

Ammonia CI enabled the molecular weight of all FAME to be quickly established. The simplicity of the ammonia CI mass spectra facilitated determination of the composition of partially resolved and unresolved GC peaks. For example, the presence of two $[M + NH_4]^+$ ion peaks at *m/z* 298 and 296 in the spectrum obtained from peak 12 (Fig. 7) clearly shows that this peak represents a cochromatographing mixture of $C_{17:0}$ and $C_{17:1}$ respectively. This was not at all obvious when using methane CI, as the MH^+ ion of $C_{17:1}$ has the same *m/z* value as the M — 1 fragment ion of $C_{17:0}$. In the same way the partially resolved peaks 14 and 15 were clearly shown, by ammonia CI, to correspond to $C_{18:1}$ and $C_{18:0}$ respectively.

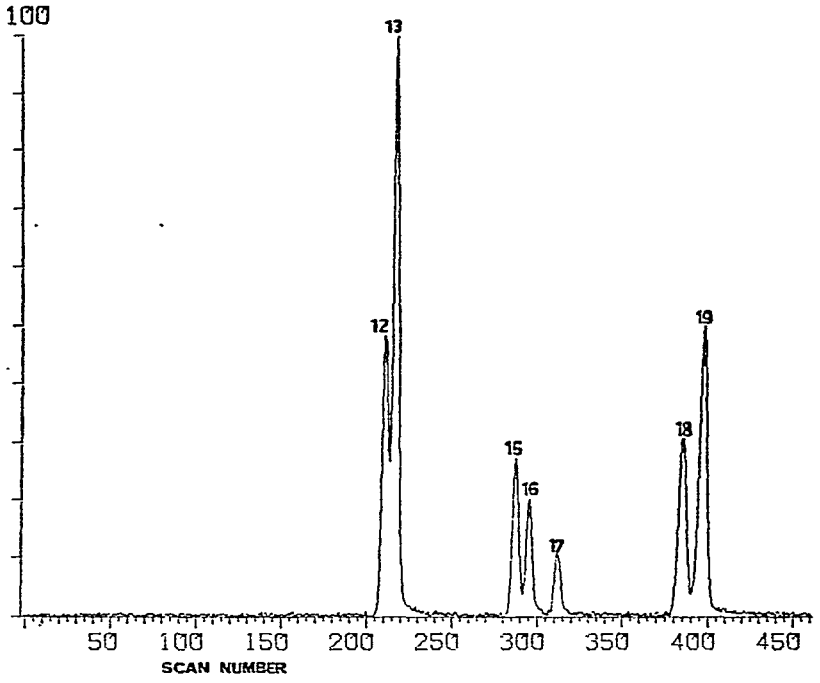

Fig. 6. Gas chromatogram obtained by GC–CI-MS (methane) analysis of FAME mixture 2. Peak numbers refer to FAME listed in Table I. See text for GC–CI-MS conditions.

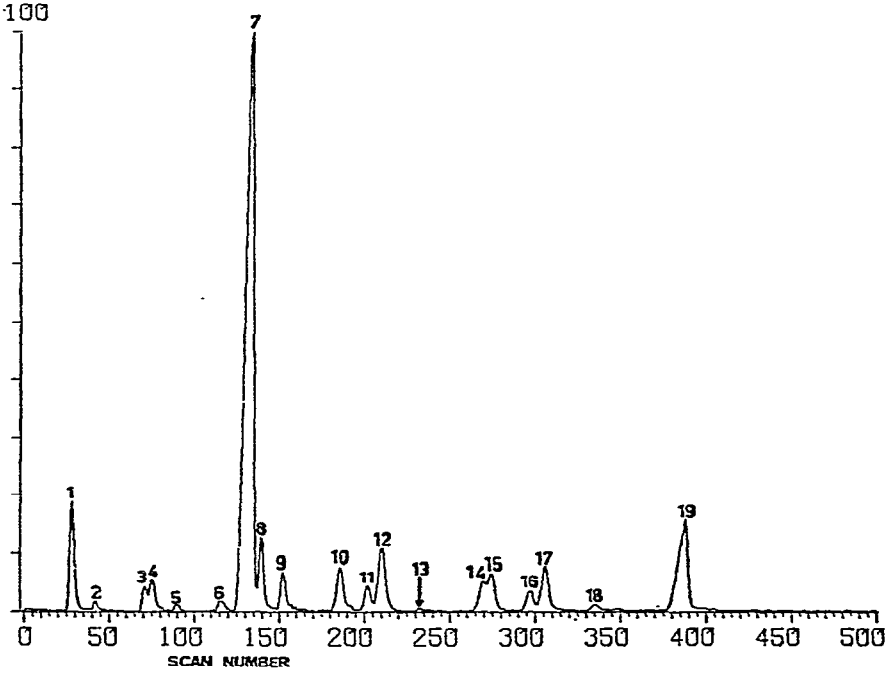

Fig. 7. Gas chromatogram obtained by GC–CI-MS (methane) analysis of FAME from Streptomyces R61. See text for GC–CI-MS conditions.

TABLE II

BRANCHED CHAIN FAME FROM STREPTOMYCES R61

GC peak no. (Fig. 5)	*FAME*	*m/z values of characteristic ions* (He CI)*
2	*iso*-$C_{14:0}$	227 ($M - CH_3$), 199 ($M - C_3H_7$)
3	*iso*-$C_{15:0}$	213 ($M - C_3H_7$)
4	*anteiso*-$C_{15:0}$	227 ($M-C_2H_5$), 199 ($M-C_4H_9$)
7	*iso*-$C_{16:0}$	255 ($M-CH_3$), 227 ($M-C_3H_7$)
10	10-Me-$C_{17:0}$	171 ($M-C_8H_{17}$), 191 ($M-C_6H_{13}$)
11	*iso*-$C_{17:0}$	241 ($M-C_3H_7$)
12	*anteiso*-$C_{17:0}$	255 ($M-C_2H_5$), 227 ($M-C_4H_9$)
15	10-Me-$C_{18:0}$	171 ($M-C_9H_{19}$), 199 ($M-C_7H_{15}$)
16	*iso*-$C_{18:0}$	283 ($M-CH_3$), 255 ($M-C_3H_7$)
19	10-Me-$C_{19:0}$	171 ($M-C_{10}H_{21}$), 199 ($M-C_8H_{17}$)

* These are ions whose abundances are enhanced relative to their abundances in the spectrum of the corresponding straight chain FAME.

The identity of the straight chain saturated FAME, and some of the branched chain FAME (*iso*-$C_{14:0}$, -$C_{16:0}$ and *anteiso*-$C_{15:0}$, -$C_{17:0}$), were established by comparison of their GC retention time and mass spectra with those of authentic samples. Other branched chain FAME were identified by inspection of their helium CI mass spectra. Enhanced fragmentation at the site of branching enables these FAME to be readily identified[1]. The results are summarised in Table II. Identification of the other components of this mixture will be published elsewhere.

During the analysis of FAME mixtures 1 and 2 it was observed that the relative size of certain FAME GC peaks was greater with ammonia CI than with methane CI. The FAME which showed this enhanced response were those which contained a double bond. The relative peak sizes of saturated and cyclopropane FAME showed little change. Measurement of peak areas showed that for FAME containing a double bond there was an approximately two-fold increase in sensitivity when using ammonia CI compared to methane CI. The reason for this enhancement of response is presumably a result of the fact that a double bond provides an extra localised electron-rich site in the molecule for bond formation with the ammonium ion. The results for FAME mixture 1 are summarised in Table III.

TABLE III

RELATIVE GC PEAK AREAS IN THE CHROMATOGRAM OF FAME MIXTURE 1 USING AMMONIA AND METHANE CI

GC peak no. (Fig. 5)	*FAME*	*Relative peak areas*	
		CH_4CI	*NH_3CI*
10	$C_{16:0}$	1.0	1.0
9	$C_{16:1}$	0.9	1.89
13	$C_{17:1}$	0.31	0.26
14	$C_{17:0}$	0.28	0.22
15	$C_{18:1}$ (*cis* 9)	0.65	1.25
16	$C_{18:1}$ (*cis* 11)	0.42	0.71
17	$C_{18:0}$	0.20	0.17
19	$C_{19:1}$	0.18	0.16

This observation provides an additional method of determining whether an unknown peak corresponds to an unsaturated FAME or a cyclopropyl FAME. If the GC traces obtained using ammonia CI and methane CI are compared, peaks corresponding to unsaturated FAME will be revealed by their different relative areas in the two chromatograms. In this manner it was possible to show that the FAME extract obtained from Streptomyces R61 did not contain cyclopropane acids. Peaks 6, 8, 12 (after correction for the contribution of $C_{17:0}$ in this peak), 14 and 17 (Fig. 7) all showed an approximate doubling in size in the ammonia CI chromatogram compared to the methane CI chromatogram and therefore correspond to unsaturated FAME.

The techniques described here are currently being applied to a number of studies involving the identification of FAME from bacterial sources.

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