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Application of triple quadrupole tandem mass spectrometry to the analysis of pyridine-containing derivatives of long-chain acids and alcohols

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ABSTRACT

An improved derivatization procedure for the preparation of nicotinate and 3-picolinyl esters from mixtures of fatty alcohols and acids has been developed. The derivatives can be analysed by capillary gas chromatography on an SE-54 type column, which affords separation of the acid and alcohol derivatives with the same carbon chain. Detection with tandem mass spectrometric techniques on a triple quadrupole instrument is feasible, and yields informative spectra devoid of coeluting interferences.

INTRODUCTION

Among the several known analytical derivatives of fatty acids and alcohols, those carrying a linked pyridine functional group have gained great importance, since their source electron impact ionization (EI) spectra contain regularly spaced ions that allow the positions of methyl branching, cyclopropane and epoxide rings, and double bonds in the hydrocarbon chain to be determined. These derivatives were recently introduced by Harvey (for a comprehensive review, see ref. 1), following preliminary work by Vetter *et al.* [2,3], and they have proved valuable for the characterization of minor constituents of lipid secretions in animals and humans [4–10].

Their utility in the mass spectrometric (MS) mapping of hydrocarbon chains stems from the isomerization of the initially formed pyridine-centred cation radical to a distonic species the radical site of which triggers the fission on the fatty backbone (Fig. 1). The main disadvantage of these derivatives is that the pyridine derivatizing "head" causes peak tailing and delays elution from a capillary gas chromatographic (GC) column.

Because they carry a basic function, these derivatives can also form MH^+ species under chemical ionization (CI) or fast atom bombardment, the charge being tightly localized at the pyridine nitrogen. Charge-remote processes can therefore be triggered by high-energy collision-induced decomposition (CID), which allows mapping of the hydrocarbon chain, as demonstrated by Deterding and Gross [11].

This paper reports further MS information on these derivatives, obtained by the application of tandem mass spectrometry (MS–MS) to the detection and structural elucidation of long-chain aliphatic compouds in biological matrices.

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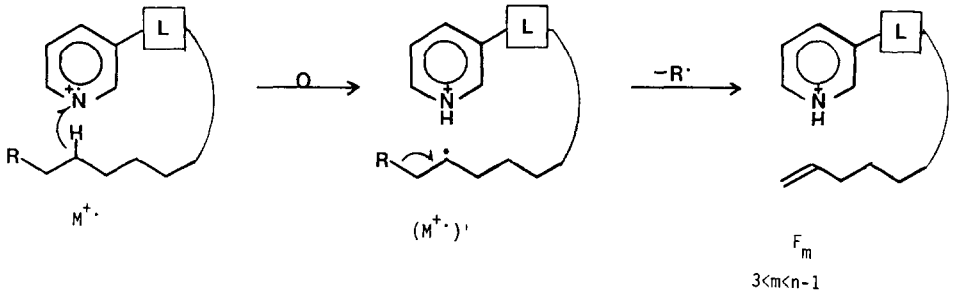

Fig. 1. Isomerization of the initially generated cation radical to the distonic parent of the backbone-related fragment ions. L is the functional group that connects the hydrocarbon chain to the pyridine ring. L = CH_2–O–CO for the picolinyl ester fatty acid derivatives. L = CO–O for the nicotinate ester fatty alcohol derivatives.

EXPERIMENTAL

Chemicals

All solvents were of analytical purity. Straight-chain C_{12}–C_{24} even-carbon fatty acids were of synthetic grade (Fluka, Buchs, Switzerland). The corresponding alcohols were prepared by lithium aluminum hydride reduction of a mixture of the free acids. Nicotinic acid, 3-hydroxymethyl pyridine, N,N-carbonyldiimidazole and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from either Fluka or Aldrich (Belgium).

Derivatization

A mixture of acids and alcohols was converted into the 3-picolinyl and nicotinate esters, respectively, under very mild conditions by employing N,N′-carbonyldiimidazole as the condensing agent, partially according to previously reported methods [12,13]. A 0.1 *M* solution of nicotinoyl imidazolide was prepared by dissolving equimolecular amounts of nicotinic acid and N,N′-carbonyldiimidazole in methylene chloride. An aliquot (20 μl) of this solution was added to the sample (containing *ca.* 100–500 ng of each component) and heated at 40°C for 15 min, then 20 μl of a 0.1 *M* solution of N,N′-carbonyldiimidazole in methylene chloride was added, followed, after 5 min, by 5 μl of 3-picolinyl alcohol. After 15 min at 40°C, the solvent was removed in a stream of dry nitrogen, and the residue was dissolved in 20 μl of BSTFA. An aliquot of 1–2 μl of the solution was injected into the GC–MS instrument.

GC–MS analysis

GC–MS analysis and low-energy CID MS–MS were performed on a Finnigan TSQ70 triple quadrupole mass spectrometer directly interfaced to a Varian 3400 capillary gas chromatograph. GC separation was obtained on two different columns: (A) 30 m × 0.32 mm I.D. BPFS-type with a 0.25 μm dimethylsilicone chemically bonded stationary phase (Supelco, Bellefonte, PA, USA), and (B) 25 m × 0.32 mm I.D. BPFS-type with a 0.25 μm SE-52 stationary phase (J&W, Folsom, CA, USA). The carrier gas was helium at 69 kPa head pressure. The injector (splitless mode) and transfer line temperatures were set at 300°C, and the temperature of the ion source was 180°C.

Temperature programming for column A was between 200 and 300°C at 5°C/min with hold times of 3 min at the beginning and at the end of the gradient, and for column B between 220 and 320°C at 5°C/min, with an initial hold time of 3 min and a final one of 12 min.

Chemical ionization was accomplished with isobutane gas (200 or 460 Pa, as specified; 70 eV; source temperature, 120°C). Collisional activation was performed with argon (30–60 mPa or 80–110 mPa, as specified) in the rf-only quadrupole.

The scan time in the normal and daughter ion

mode was 1 s, and 0.5 s in the alternated parent ion scan, so that chromatographic integrity was retained.

RESULTS AND DISCUSSION

Derivatization and gas chromatographic separation

No previous report of the derivatization of a mixture of acids and alcohols was found in the literature, and only few examples of GC separation of either standard mixtures or real-life samples [1,14].

When the acid chloride derivatization methods employed by Harvey [2,15,16] were applied in sequence to a standard mixture, only very poor yields of pyridine-containing derivatives were obtained, and most of the products in the chromatogram were silylated acids and alcohols and waxy esters. The derivatization procedure that employs N,N′-carbonyldiimidazole as the carboxyl-activating reagent and methylene chloride as the solvent has recently been applied to convert acid-labile epoxides of polyunsaturated acids into their 3-picolinyl esters [12]. The analogous reaction of nicotinoyl imidazolide with long-chain alcohols has not, to our knowledge, been applied before.

Both reagent solutions appear to be very stable when stored in stoppered containers at 4°C, and can be prepared in a few minutes from the solid reagents. Moreover, the condensing agent is considerably safer than most of the previously employed reagents (thionyl chloride [1], trifluoroacetic anhydride [14], dicyclohexylcarbodiimide [17]), so the reaction can be performed with little or no potential health hazard. Another advantage of the modified derivatization procedure is that all reactions are performed at much lower temperatures and evaporation of noxious solvents and reagents can be kept to a minimum.

Although a considerable amount of 3-picolinyl nicotinate is formed as a byproduct, it elutes earlier than the C_{12} derivatives. Separation of the straight-chain acids and alcohols with the same number of carbon atoms is poor on the dimethylsilicone-type stationary phase, but on the more polar SE-52 type they can be baseline separated,

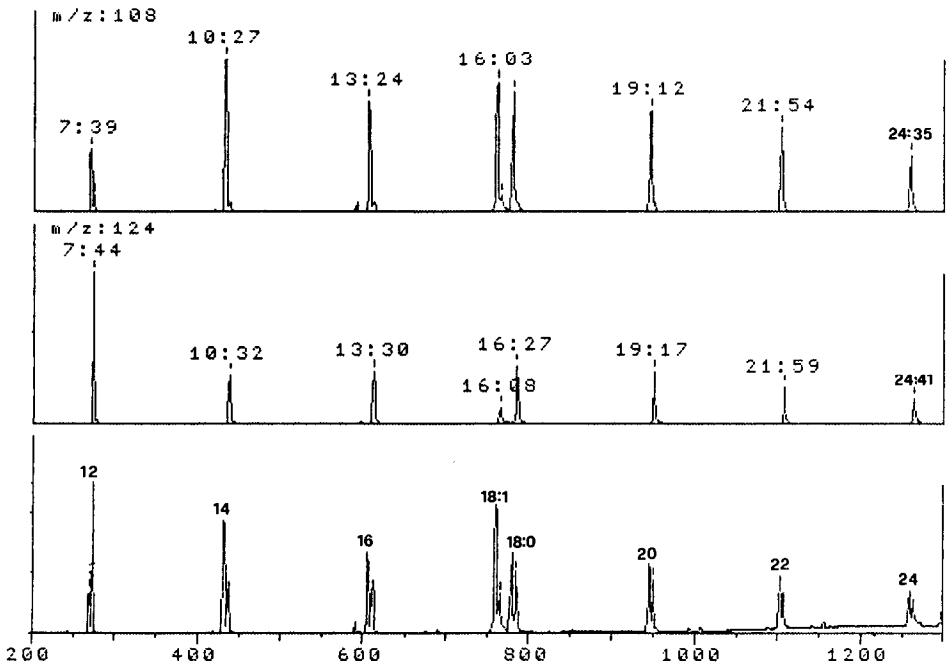

Fig. 2. GC–MS separation of a standard mixture of derivatized C_{12}–C_{24} fatty acids and alcohols on column B (see text). The number on top of the peaks of lower trace (total ion current) indicates the length of the chain.

TABLE I

RELATIVE INTENSITIES OF THE MAIN PEAKS IN THE SPECTRA OF THE 3-PICOLINYL ESTERS OF THE ANALYSED LONG-CHAIN ACIDS

	92	108	151	164	178	192	206	220	234	248	262	276	290
12:0	73.6	59.8	50.2	100.0	14.2	3.7	13.2	7.3	2.7	14.1	15.6	5.3	3.3
14:0	72.3	56.4	55.7	100.0	18.5	9.3	14.8	11.2	13.5	11.5	8.8	8.5	5.8
16:0	74.3	63.9	61.1	100.0	18.1	7.6	15.1	11.7	13.1	10.9	8.5	7.4	6.4
16Δ9[c]	100.0	67.2	20.6	84.5	14.9	3.3	7.3	5.7	[a]	[a]	7.5[b]	31.6	35.2
18:0	75.1	74.6	61.2	100.0	18.4	6.5	16.3	11.1	13.1	10.8	8.7	7.2	6.1
18Δ9	99.7	66.0	26.4	100.0	17.3	3.9	9.6	8.5	3.7	4.3	12.8	40.4	46.1
18Δ11[c]	100.0	61.5	26.6	58.5	4.1	4.5	[a]	[a]	[a]	[a]	[a]	3.0	7.6
20:0	76.4	76.9	62.9	100.0	18.8	6.7	17.4	11.9	13.1	10.3	8.3	7.9	6.1
22:0	81.6	86.4	62.2	100.0	18.9	8.5	20.6	12.2	12.7	9.5	8.9	7.8	5.8
24:0	83.5	93.5	66.2	100.0	25.4	7.1	24.7	13.2	12.4	10.3	9.5	8.7	7.0
26Δ19[c]	100.0	59.2	18.9	32.7	2.4	[a]	3.3	[a]	2.6	2.6	2.1	3.2	4.0

[a] Intensity below 2%.
[b] Mass number + 2 units.
[c] Spectrum from biological sample.

although the final eluting temperature (320°C) is very close to the maximum allowed, thus causing severe bleed (Fig. 2). The peak of the acid precedes that of the alcohol derivative, and the unsaturated compound (oleic acid and alcohol) is eluted before its saturated counterpart.

This procedure was applied to the determination of lipids possibly released by neuromelanin extracted from human *substantia nigra* [18] after alkaline degradation. In such a sample, C_{16}, C_{18} and C_{26} fatty acids were observed at very low concentrations (a few nanograms per microgram of analysed sample). In particular, baseline separation of oleic and vaccenic acid derivatives was accomplished (Fig. 3) and the EI spectra of palmitoleic, vaccenic and $^{\Delta 19}C_{26}$ acid reported in Table I (see below) are those recorded from these samples.

TABLE II

RELATIVE INTENSITIES OF THE MAIN PEAKS IN THE SPECTRA OF THE NICOTINATE ESTERS OF THE ANALYSED LONG-CHAIN ALCOHOLS

	106	107	124	151	164	178	192	206	220	234	248	262	276
12:0	46.2	55.2	100	6.0	7.5	15.2	10.8	8.9	5.1	6.9	12.0	11.7	4.1
14:0	55.8	57.3	100	8.3	12.6	25.1	21.3	14.2	11.5	12.2	11.3	8.2	6.6
16:0	58.7	55.2	100	7.8	7.6	23.6	20.7	13.7	10.6	11.9	10.6	7.9	7.9
18:0	42.8	52.6	100	6.6	7.2	22.1	15.6	13.6	9.2	10.9	8.9	7.7	7.6
18Δ9	39.9	35.1	100	10.9	14.6	20.8	8.8	12.6	4.4	4.1	6.7	6.3	23.6[b]
20:0	40.7	50.7	100	6.2	5.7	20.3	14.7	13.5	9.0	9.8	8.2	7.5	7.8
22:0	34.4	43.1	100	5.3	5.8	14.2	11.8	12.6	7.3	8.4	7.9	6.5	6.7
24:0	30.2	39.3	100	4.0	6.4	15.1	14.7	28.0	6.5	7.5	6.6	5.5	6.2

[a] Intensity below 2%.
[b] Mass number + 2 units.

304	318	332	346	360	374	388	402	416	430	444	458	M^+	
												291.0	7.1
[a]												319.0	3.4
5.1	3.9	2.1	2.9									347.0	7.4
7.5	6.5[b]	[a]	3.8[b]									345.0	17.5
5.2	4.2	6.5	6.6	3.3	6.9							375.0	17.5
10.9	12.1	8.7	4.8	[a]								373.0	13.4
21.9	21.2	6.3[b]										373.0	2.7
5.4	5.0	6.6	7.2	8.8	10.4	4.5	10.0					403.0	25.9
5.7	4.9	8.1	8.2	10.5	13.4	15.5	14.7	5.2	20.8			431.0	54.9
6.5	5.4	7.8	9.4	11.2	16.5	17.0	17.1	16.2	16.9	7.2	24.6	459.0	54.9
2.6	4.1	3.7	2.1	3.0	[a]	[a]	[a]	8.4[b]	6.6[b]	2.2[b]	[a]	485.0	7.5

Electron ionization studies

The relative intensities of the main peaks in the 70 eV source spectra of the derivatives of acids and alcohols are summarized in Tables I and II, respectively. The two derivatives (*i.e.* the nicotinate ester of the alcohol and the picolinyl ester of the acid) have the same relative molecular mass, and their spectra differ only in the mass of the low-mass ions related to the derivatizing head and in the relative abundance of the molecular and backbone ions, which is higher for the acid derivative.

Although this does not impair characterization of the standard mixture of acids and alcohols, if it is analysed on the SE-54 column, which affords the best chromatographic resolution, it would be advantageous to detect each class of compounds selectively even in the case of a less efficient chro-

290	304	318	332	346	360	374	388	402	416	430	444	458	M^+	
3.2													291.0	[a]
6.2	[a]	[a]											319.0	[a]
6.6	4.6	5.1	[a]	2.9									347.0	2.3
6.7	6.2	5.0	7.6	6.3	2.7	6.1							375.0	5.3
44.1	23.4	17.2	10.2	6.2	[a]								373.0	21.0
6.6	6.7	5.3	8.3	7.1	11.5	12.2	3.6	9.2					403.0	9.1
6.2	6.5	5.1	7.1	7.6	10.1	11.9	16.5	11.8	[a]	12.7			431.0	13.8
5.8	5.5	4.6	6.5	6.8	8.6	11.6	14.9	13.6	20.1	11.1	3.1	13.1	459.0	16.1

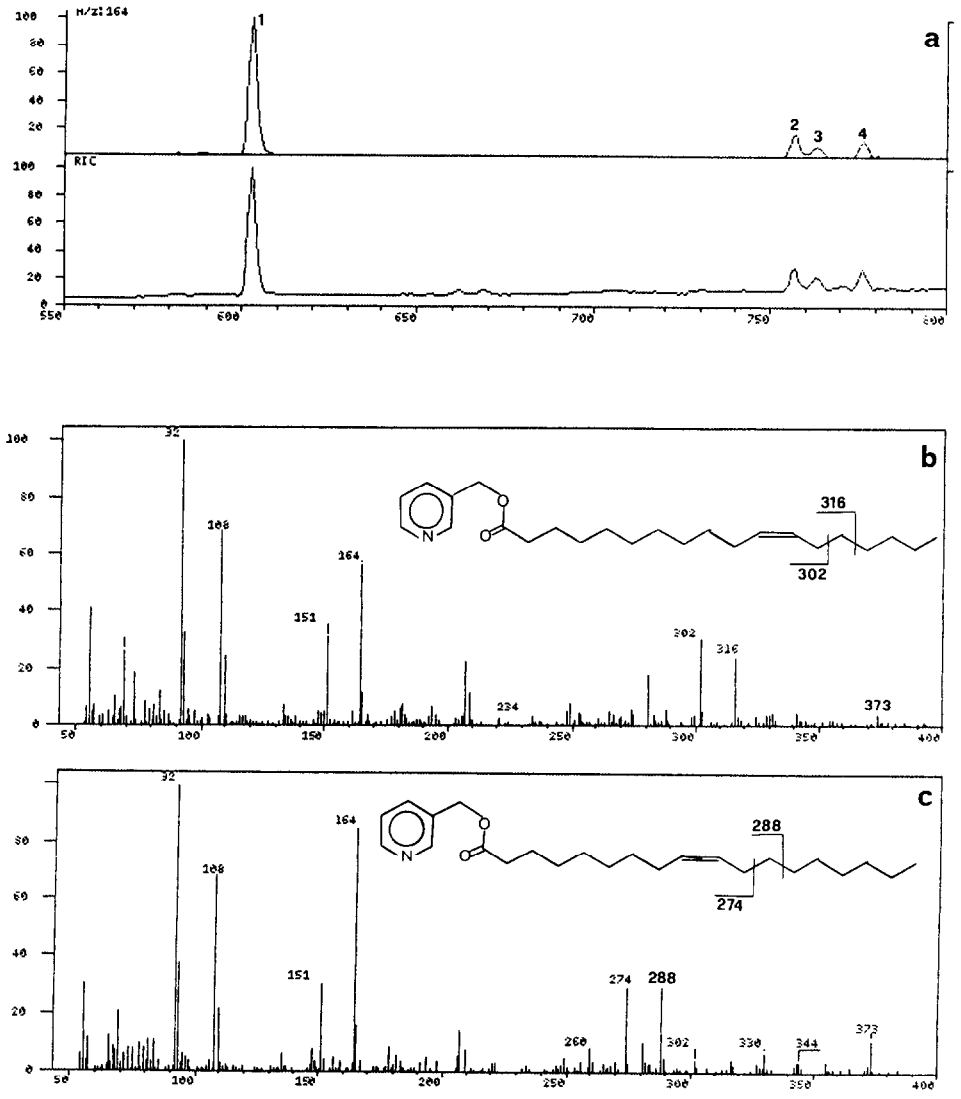

Fig. 3. (a) Partial GC–MS trace from the analysis of a derivatized lipid sample from human *substantia nigra* neuromelanin (see text). Chromatographic conditions as in Fig. 1. The labelled peaks are those of palmitoleic (1),vaccenic (2), oleic (3) and stearic (4) acids. The reported spectra are those of peaks 2 (b) and 3 (c).

matographic separation, such as that obtained on the dimethylsilicone stationary phase, which allows elution at a lower temperature.

This problem was also addressed by Harvey [1], during his studies on the characterization of waxy secretions of animals and humans. To recognize the acidic constituents of wax esters, he cleaved the ester bond by lithium aluminum deuteride reduction. The acidic component was thus converted into an alcohol that incorporated two deuterium atoms in the 1,1′ position. All diagnostic ions in the spectrum of the nicotinate ester were shifted by two mass units and could therefore be recognized.

An alternative approach involving the use of low-energy CID MS–MS can be designed. A desirable feature of the method, besides detection of the molecular peak of each eluting compound, would be the acquisition of ions produced by fission of the hydrocarbon backbone. This can be

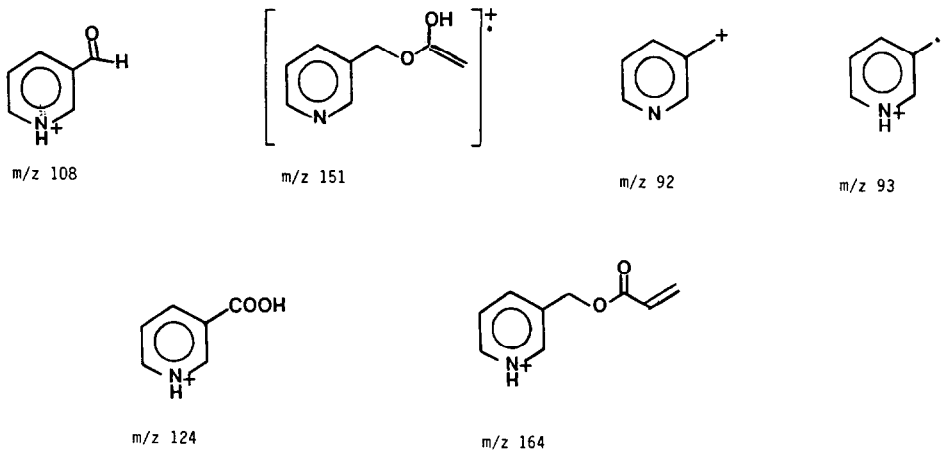

Fig. 4. Low-mass ions in the EI spectra of the picolinyl and nicotinate ester derivatives.

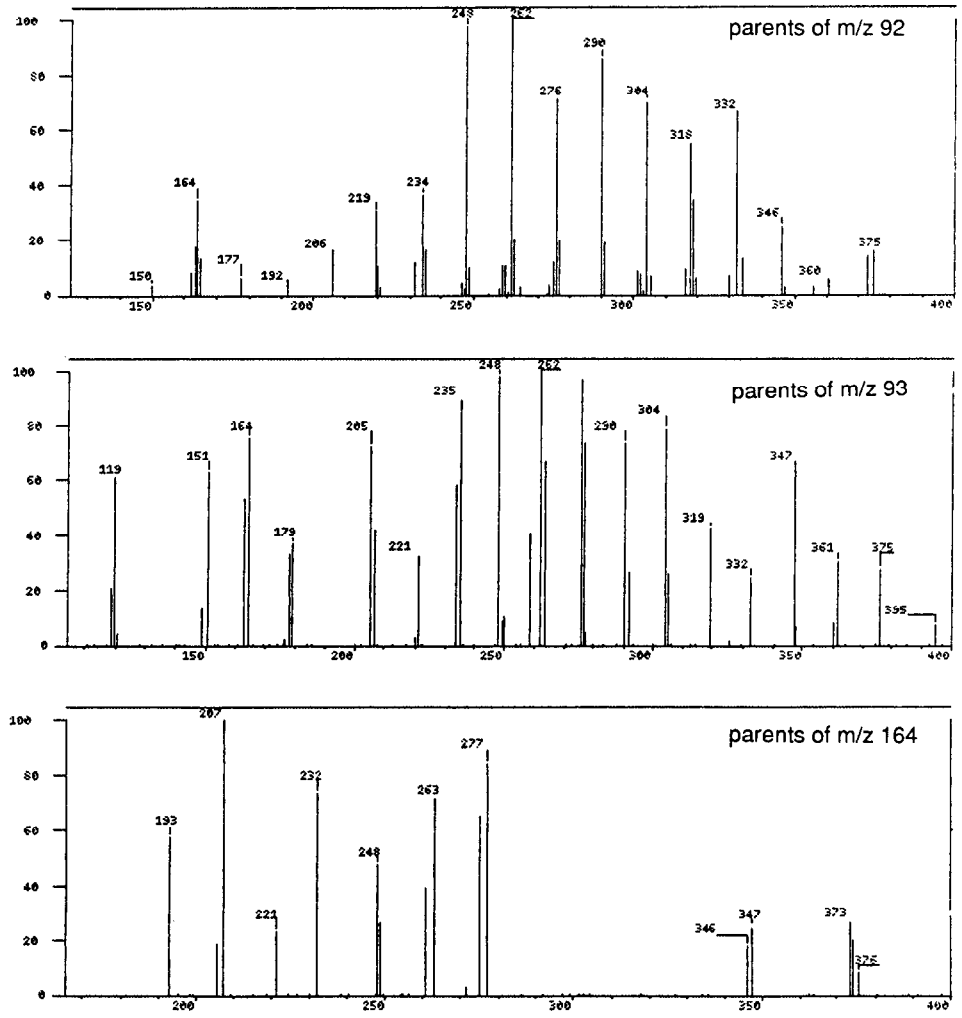

Fig. 5. MS–MS parent ion spectra of some low-mass fragments of the picolinyl ester of stearic acid. Collision pressure 30–60 m Pa; collision energy, 30 eV.

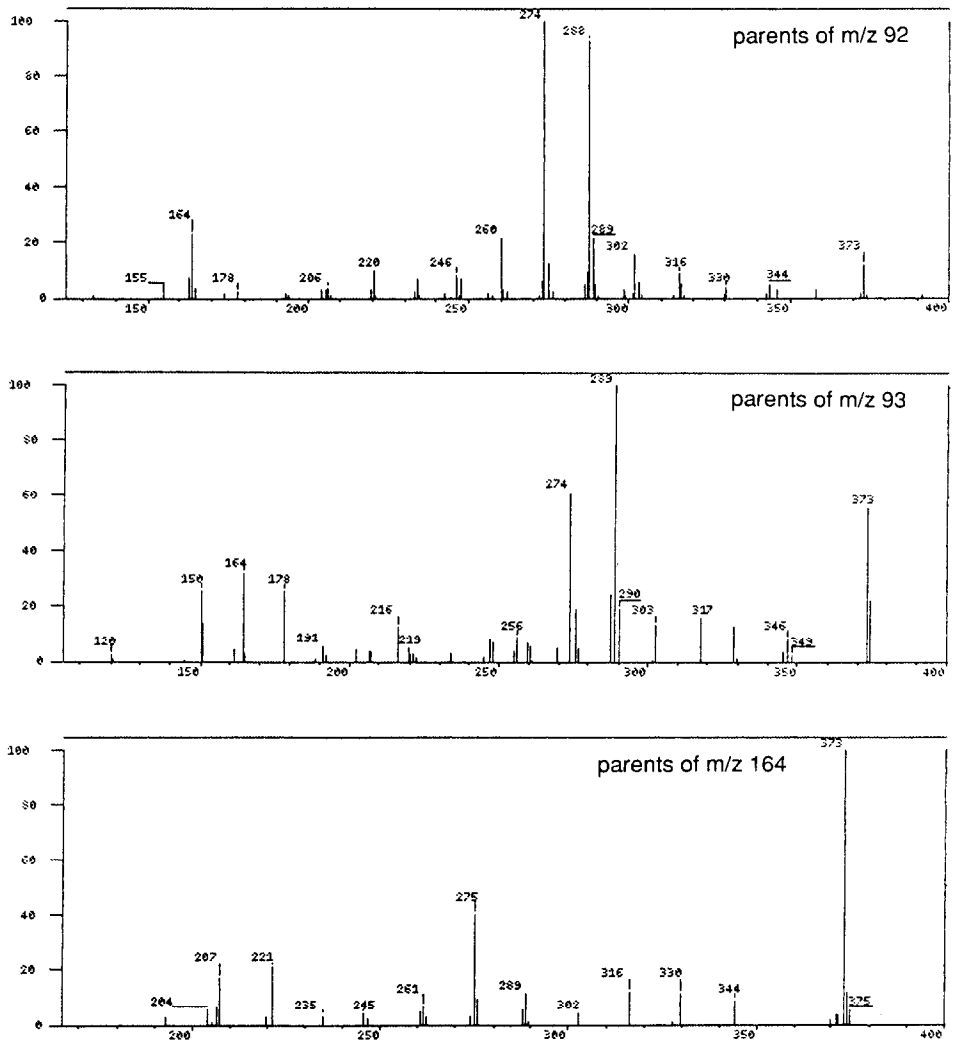

Fig. 6. MS–MS parent ion spectra of some low-mass fragments of the picolinyl ester of oleic acid. Collision pressure, 30–60 mPa; collision energy, 30 eV.

accomplished by performing a "parent scan" of an appropriate low-mass fragment ion related to each derivatizing function (Fig. 4). For practical use in the characterization of real samples, optimization of collision energy and pressure is important to obtain both intense and informative spectra [19].

Among the daughter ions tested were *m/z* 92, 93, 108, 151 and 164 for the acid derivatives and *m/z* 106, 107 and 124 for the alcohols. The parent spectra of *m/z* 108 afforded only *m/z* 151 and the molecular peak, and those of *m/z* 151 were of very weak intensity, thus being of limited, if any, analytical utility.

Figs. 5–8 show the parent ion spectra (collision energy −30 eV and pressure 30–60 mPa) obtained for selected fragments from stearic acid and oleic acid, and from the corresponding alcohols. The parent scan of *m/z* 92 yields the whole series of fragments expected from straight-chain saturated and monounsaturated acid derivatives, and those of *m/z* 93 and 164 are either too weak or leave gaps in the array. The parent scan of *m/z* 124 affords intense readily interpretable spectra

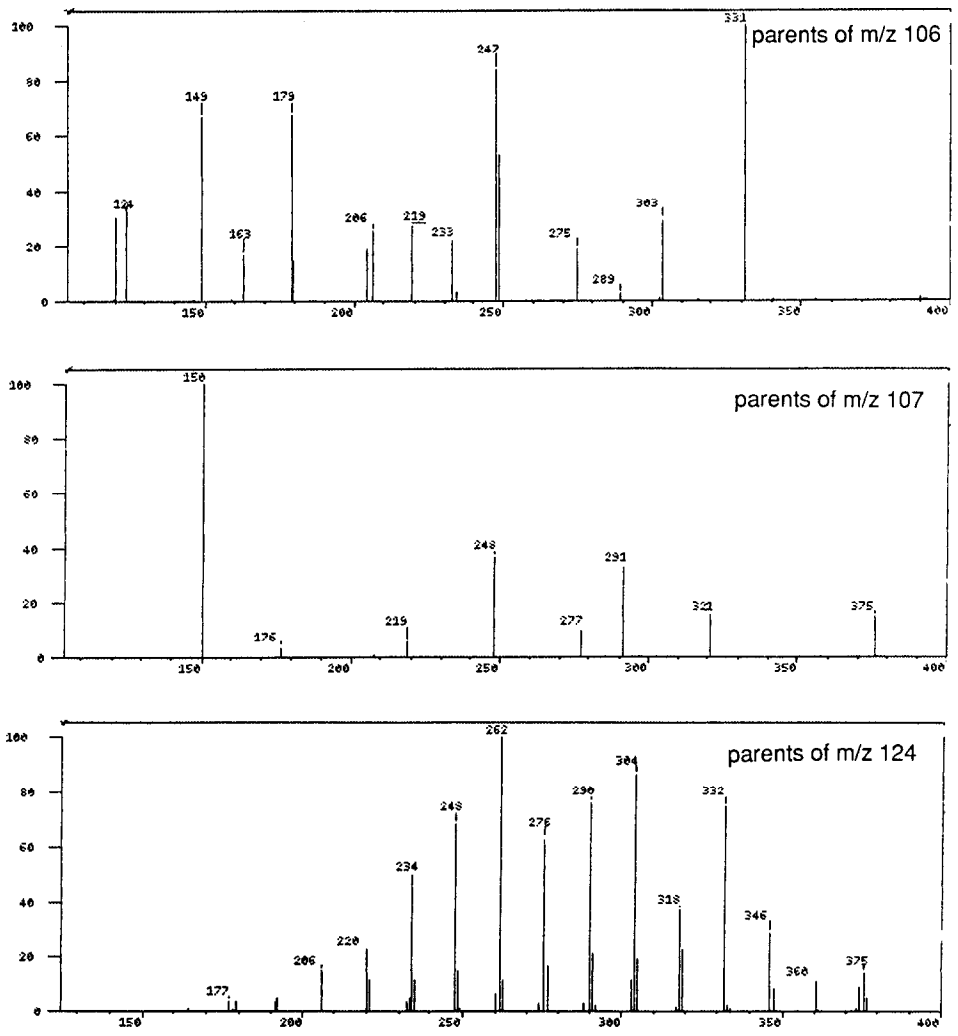

Fig. 7. MS–MS parent ion spectra of some low-mass fragments of the nicotinyl ester of stearyl alcohol. Collision pressure, 30–60 mPa; collision energy, 30 eV.

for both saturated and unsaturated alcohol derivatives, but those of m/z 106 and 107 do not contain the same amount of information. In particular, the parent spectra of m/z 92 and 124 of the derivatives of oleic acid and oleyl alcohol, respectively, also feature the fragment ions from which the position of the double bond can be determined according to the equation:

$$\Delta\text{position from C-1} = \frac{(\text{highest-mass } \Delta\text{fragment} + 2) - 164}{14}$$

By alternating the two scans (parents of m/z 92 and 124) over the GC elution time, the total ion chromatogram of Fig. 9a is obtained from the standard mixture. Computer reconstruction of the ion current pertinent to each experiment permits acquisition of the separate GC–MS traces for the acids and the alcohols shown in Fig. 9b and c, respectively, even if the chromatographic separation is poor.

Chemical ionization studies

The presence of a basic site in the analysed derivatives allows formation of an MH^+ species

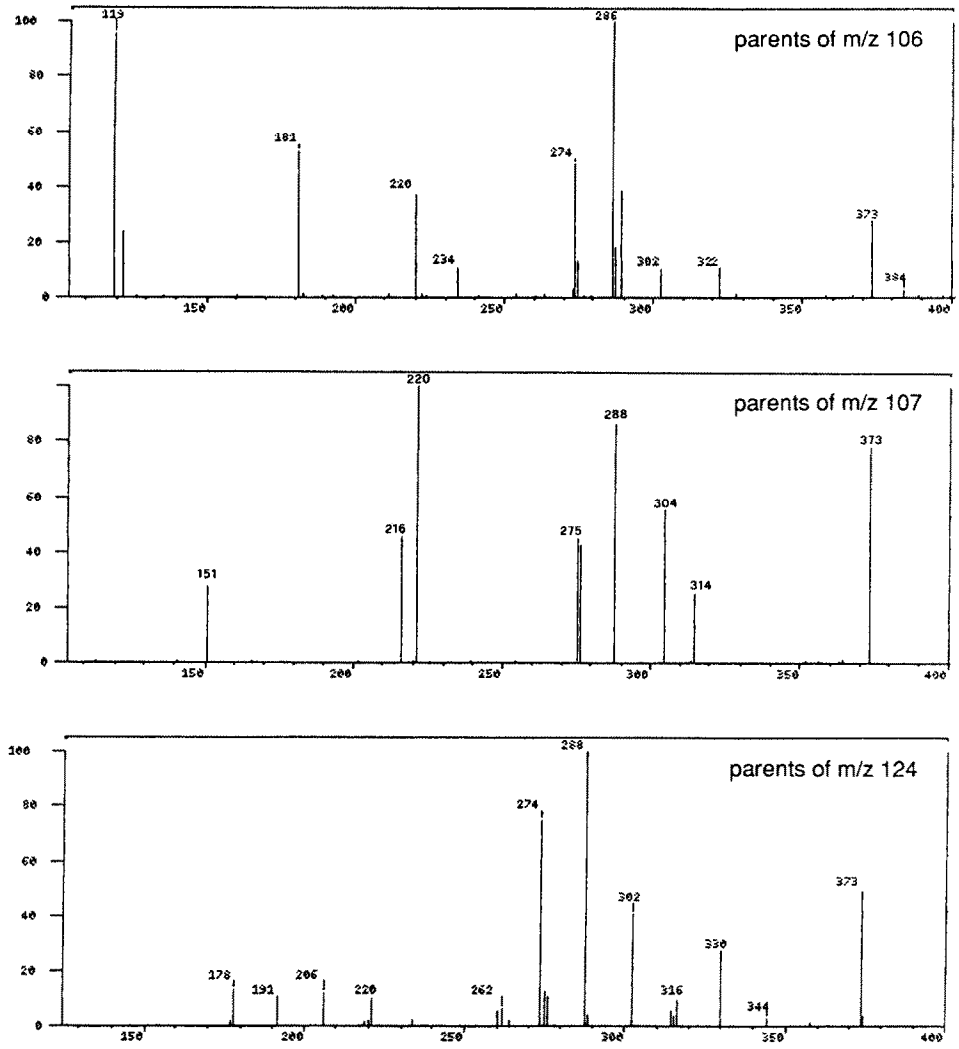

Fig. 8. MS–MS parent ion spectra of some low-mass fragments of the nicotinyl ester of oleyl alcohol. Collision pressure, 30–60 mPa; collision energy, 30 eV.

under isobutane chemical ionization. At a lower pressure of the reagent gas (200 Pa), a mixed EI–CI spectrum is obtained (Fig. 10a and b), which features the protonated molecule as the base peak, accompanied by a complete EI spectrum and by fragments corresponding to $RCOO^+$ (acid derivatives) and RCH_2^+ (alcohol derivatives). Under these conditions, however, the sensitivity is somewhat lower than at higher pressure (460 Pa), where this behaviour is suppressed, and the spectrum only shows the MH^+ signal.

CID MS–MS of the protonated molecule at low collision energy (30–150 eV) did not prove a useful way to map the hydrocarbon chain, because mainly charge-triggered decompositions are activated, which lead to the characteristic ions of the pyridine head (*m/z* 108 for the acid derivative and *m/z* 124 for that of the alcohol). Even at the highest collision energy tested, the abundance of the expected charge-remote fragments is negligible. This result, although discouraging, is by no means unexpected, since it is

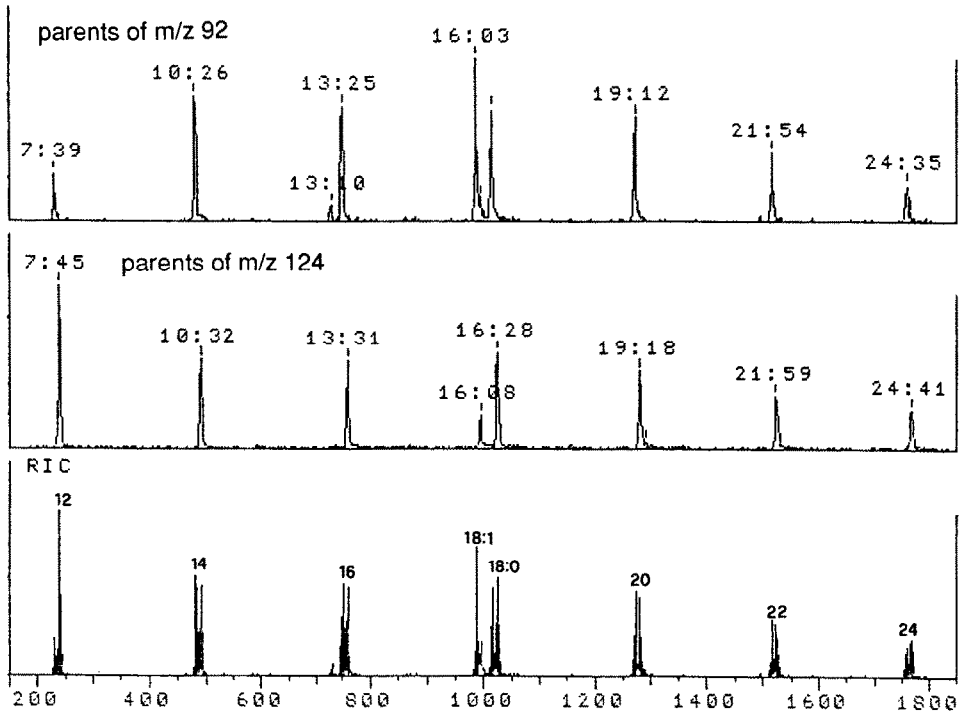

Fig. 9. GC–MS–MS trace of the standard mixture of derivatized acids and alcohols (chromatographic conditions as in Fig. 1). The two upper traces are relative to the selective scans for acids (parents of m/z 92) and for alcohols (parents of m/z 124). Collision pressure and energy as Figs. 5–8.

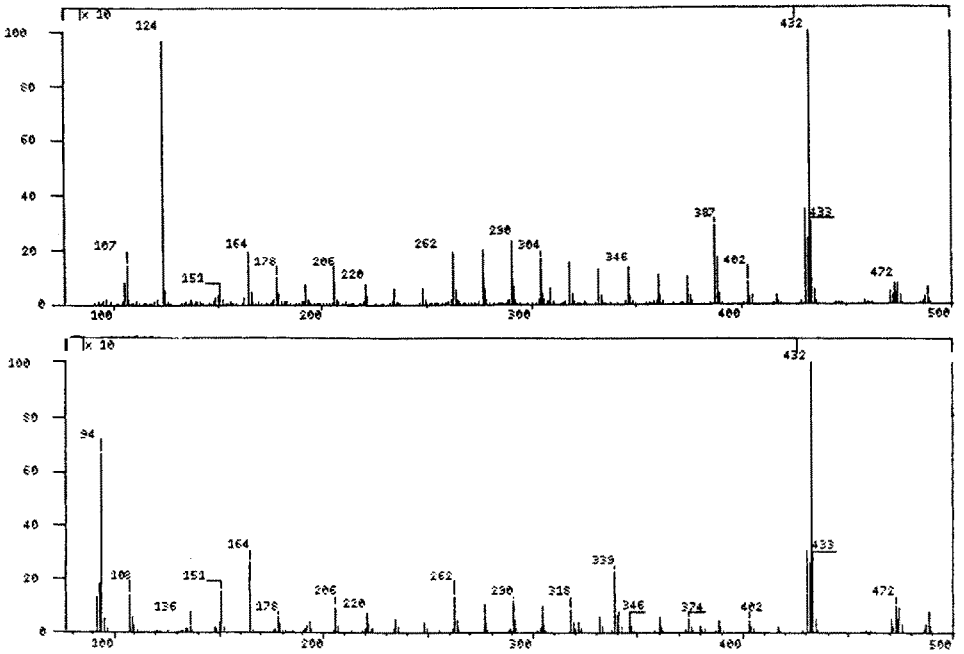

Fig. 10. Mixed EI–CI (iso-C_4H_{10}, 200 Pa 70 eV) spectra of the derivatives of behenyl alcohol and behenic acid.

known that only high-energy CID is capable of triggering charge-remote fragmentations [20].

CONCLUSIONS

Our results demonstrate the useful properties of the pyridine-containing derivatives of fatty acids and alcohols for the characterization of the hydrocarbon moiety. The parent-ion MS–MS approach under electron ionization is very convenient, since it does not require previous knowledge of the mass of the eluting compounds, as a more conventional daugther-ion experiment would. Moreover, complete deconvolution of the MS–MS spectra of coeluting acid and alcohol derivatives is possible, thus allowing separate detection of the two series, even in the case of complex mixtures.

Mixed EI–CI at low pressure of the reagent gas can also offer some advantages, owing to the much enhanced intensity of the molecular ion. The possibility of analysing the derivatives of acids and alcohols under either EI of CI may also be advantageous as a means of coupling the recently described high-performance liquid chromatographic separation method [17] to mass spectrometric detection. Although under our present conditions the use of the intense MH^+ signal as a MS–MS precursor is precluded, the use of xenon instead of argon and of a higher collision energy (*e.g.* 200–400 eV on a hybrid instrument) to raise the centre-of-mass collision energy could prove a practicable, though more expensive, alternative.

REFERENCES

1 D. J. Harvey, *Spectros Int. J.*, 8 (1990) 211–244.
2 W. Vetter and W. Meister, *Org. Mass Spectrom.*, 16 (1981) 118–122.
3 W. Vetter, W. Meister and G. Oesterhelt, *Org. Mass Spectrom.*, 23 (1988) 566–572.
4 D. J. Harvey and J. M. Tiffany, *J. Chromatogr.*, 301 (1984) 173–187.
5 D. J. Harvey, *J. Chromatogr.*, 494 (1989) 23–30.
6 D. J. Harvey, *Biomed. Environm. Mass Spectrom.*, 18 (1989) 719–723.
7 D. J. Harvey, *Biomed. Chromatogr.*, 3 (1989) 251–254.
8 D. J. Harvey, *J. Chromatogr.*, 565 (1991) 27–34.
9 D. J. Harvey, *Biol. Mass Spectrom.*, 20 (1991) 61–69.
10 D. J. Harvey, *Biomed. Chromatogr.*, 5 (1991) 143–147.
11 L. J. Deterding and M. L. Gross, *Anal. Chim. Acta*, 200 (1987) 431–445.
12 M. Balazy and A. S. Nies, *Biomed. Environm. Mass Spectrom.*, 18 (1989) 328–336.
13 W. W. Christie, E. Y. Brechany, M. S. F. Lie Ken Je and O. Bakare, *Biol. Mass Spectrom.*, 20 (1991) 629–635.
14 W. W. Christie, E. Y. Brechany, S. B. Johnson and R. T. Holman, *Lipids*, 21 (1986) 657–661.
15 D. J. Harvey, *Biomed. Mass Spectrom.*, 9 (1982) 33–38.
16 D. J. Harvey and J. M. Tiffany, *Biomed. Mass Spectrom.*, 7 (1984) 353–359.
17 W. W. Christie and K. Stepanov, *J. Chromatogr.*, 392 (1987) 259–265.
18 L. Zecca, C. Mecacci, R. Seraglia and E. Parati, *Biochim. Biophys. Acta*, 1138 (1992) 6–10.
19 H. Schweer, G. Mackert and H. W. Seyberth, *Biomed. Environm. Mass Spectrom.*, 19 (1990) 94–96.
20 A. J. Alexander, P. Thibault and R. K. Boyd, *Rap. Commun. Mass Spectrom.*, 3 (1989) 30–34.