Gas chromatographic–mass spectrometric identification of the fatty acids in borage oil using the picolinyl ester derivatives

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ABSTRACT

When subjected to capillary gas chromatography, picolinyl ester derivatives of fatty acids were shown to have as good chromatographic properties as the corresponding methyl esters. By using a capillary column of medium polarity (Supelcowax 10), excellent resolution with respect to chain length, degree of unsaturation and positional isomers was obtained without any serious problems with a disturbed background due to column bleed in the mass spectrometric interpretation of diagnostic ions. These features permitted a simple one-step procedure to be carried out for the characterization of fatty acids with respect to molecular weight, number of double bonds and positional isomers. The usefulness of the technique for the identification of the fatty acids in borage seed oil (*Borago officinalis*) was demonstrated. In addition to γ-linolenic acid, the most important fatty acid in borage seed oil from a commercial standpoint, a further fourteen different fatty acids could be positively identified.

INTRODUCTION

Gas chromatography (GC) of methyl ester derivatives on fused-silica capillary columns of medium polarity is generally considered as the method of choice for the quantitative analysis of complex fatty acid mixtures of natural origin. However, although having excellent GC properties, the methyl esters are less suitable as derivatives for structural determination by mass spectrometry (MS), mainly because isomerization of the unsaturated fatty acids occurs during ionization. Several different methods to overcome the problem of double bond migration have been extensively reviewed [1,2].

One commonly used approach involves the fixation of the double bond by chemical means such as hydroxylation followed by trimethylsilylation. However, although this is a powerful technique for the identification of pure fatty acids, the large increase in molecular weight and retention times makes the procedure less suitable for the analysis of natural fatty acid mixtures containing polyenoic acids. Another

approach to the problem is the preparation of pyrollidide [3,4], picolinyl ester [5–8] or oxazoline [9,10] derivatives, which stabilize the double bonds during ionization in the mass spectrometer, resulting in well recognizable diagnostic ions. However, in these procedures packed columns or capillary columns coated with non-polar stationary phases have generally been used, which might cause problems with respect to low resolution and increased risk of peak overlapping.

One approach to overcome this problem has been fractionation of the methyl esters with respect to unsaturation by silver ion high-performance liquid chromatography before GC–MS [8]. In this study the usefulness of capillary GC of picolinyl ester derivatives on a medium-polarity column for the mass spectrometric identification of the fatty acids in borage oil is demonstrated. The results obtained confirm previous identifications of γ-linolenic acid based on other techniques such as equivalent chain length values [11] and permanganate–periodate oxidation [12]. Further, most of the other fatty acids in borage oil were identified by using this technique.

EXPERIMENTAL

Materials

Refined borage seed oil (*Borago officinalis*) was obtained from Kabi Nutrition, (Stockholm, Sweden). 3-(Hydroxymethyl)pyridine, dimethylaminopyridine and trifluoroacetic anhydride were purchased from Sigma (Poole, U.K.), potassium hydroxide from EKA (Bohus, Sweden), ethanol from Kemetyl (Stockholm, Sweden) and all other reagents and solvents from Merck (Darmstadt, F.R.G.). Sepralyte (Bond-Elut) NH_2 (aminopropyl-bonded silica) sorbent was purchased from Analytichem International (Atlanta, GA, U.S.A.).

Preparation of picolinyl esters

After saponification of the oil (100 mg), the free fatty acids were converted into their picolinyl derivatives essentially as described by Christie and Stefanov [13]. In order to obtain a higher yield of free fatty acids in the extraction step, the hydrochloric acid concentration was increased to 6 M. For the removal of unreacted free fatty acids after the derivatization step, Bond-Elut NH_2 was used instead of purification by Florisil column chromatography. To the derivatives dissolved in diethyl ether a few milligrams of Bond-Elut NH_2 were added. After 10 min the mixture was vortex mixed and centrifuged. The isolated supernatant was evaporated and the dried residue dissolved in hexane.

Preparation of fatty acid methyl esters

The oil (100 mg) was transesterified using 0.5 M methanolic sodium methoxide and the methyl esters were extracted into hexane.

Gas chromatography of fatty acid methyl esters

A Hewlett-Packard 5880 gas chromatograph equipped with flame ionization detector, a split injection system and a Supelcowax 10 fused-silica capillary column, (30 m × 0.25 mm I.D.) was used. The carrier gas (helium) flow-rate was 0.9 ml/min and the splitting ratio was 1:100. The oven temperature was held at 180°C for 8 min

and then programmed at 10°C/min to 225°C, at which it was held for 23 min. The injection and detection temperatures were 250°C.

Gas chromatography of picolinyl ester derivatives

The same equipment and analytical conditions were used as in the analysis of fatty acid methyl esters except for the temperature settings. The oven temperature was held isothermally at 260°C throughout and the injection and detection temperatures were 280°C.

Gas chromatography–mass spectrometry of picolinyl ester derivatives

A Hewlett-Packard Model 5890 gas chromatograph equipped with a split/splitless injection system in combination with a Hewlett-Packard 5970 mass-selective detector was used. The column was the same as for the GC analysis. The helium flow-rate was set at 1 ml/min and the oven temperature was 260°C. The injection and ion source temperatures were 280 and 250°C, respectively. The spectra were recorded at an ionization energy of 70 eV.

RESULTS AND DISCUSSION

A comparison of the gas chromatograms obtained for methyl esters and picolinyl derivatives of the fatty acids in borage oil is show in Fig. 1. In order to elute the picolinyl derivatives within a reasonable period of time, a higher column temperature was needed. A temperature of 260°C, 20°C below the recommended maximum temperature of the column, was chosen as a satisfactory compromise with respect to column bleeding and analysis time. Despite the differences in retention times, the elution patterns are very similar and the picolinyl derivatives seem to have as good GC properties as the methyl esters. The small peak appearing on the shoulder of peak 8 in the chromatogram of the picolinyl esters might be an artefact introduced during the preparation of the picolinyl esters.

The fatty acid composition of borage oil as obtained by analysis of the methyl esters is shown in Table I. The distribution is similar to previously published results [11]. In an attempt to use the peak-area distribution of the picolinyl derivatives instead of the methyl esters as a measure of the fatty acid composition, it was observed that polyunsaturated and late-eluting fatty acids were discriminated against to some extent (cf., Table I).

The mass spectra of the fatty acid picolinyl ester derivatives of borage oil contained good molecular ions and distinct diagnostic ions, which facilitated identification of the fatty acid moieties. As expected, a certain degree of bleeding from the medium-polarity column could be observed. As a consequence, disturbing background peaks interfered to some extent in the interpretation of the mass spectra of minor constituents. By increasing the sample size and by using background subtraction, these problems were essentially eliminated as could be demonstrated by the very informative mass spectrum of 18:4 ($n-3$), a minor fatty acid in borage oil comprising only 0.2% (Fig. 4). The negative intensities of some peaks in Figs. 2, 3 and 4 are due to background subtraction.

In Table II, all the fatty acids which could be identified in the borage oil are given together with the most important diagnostic ions.
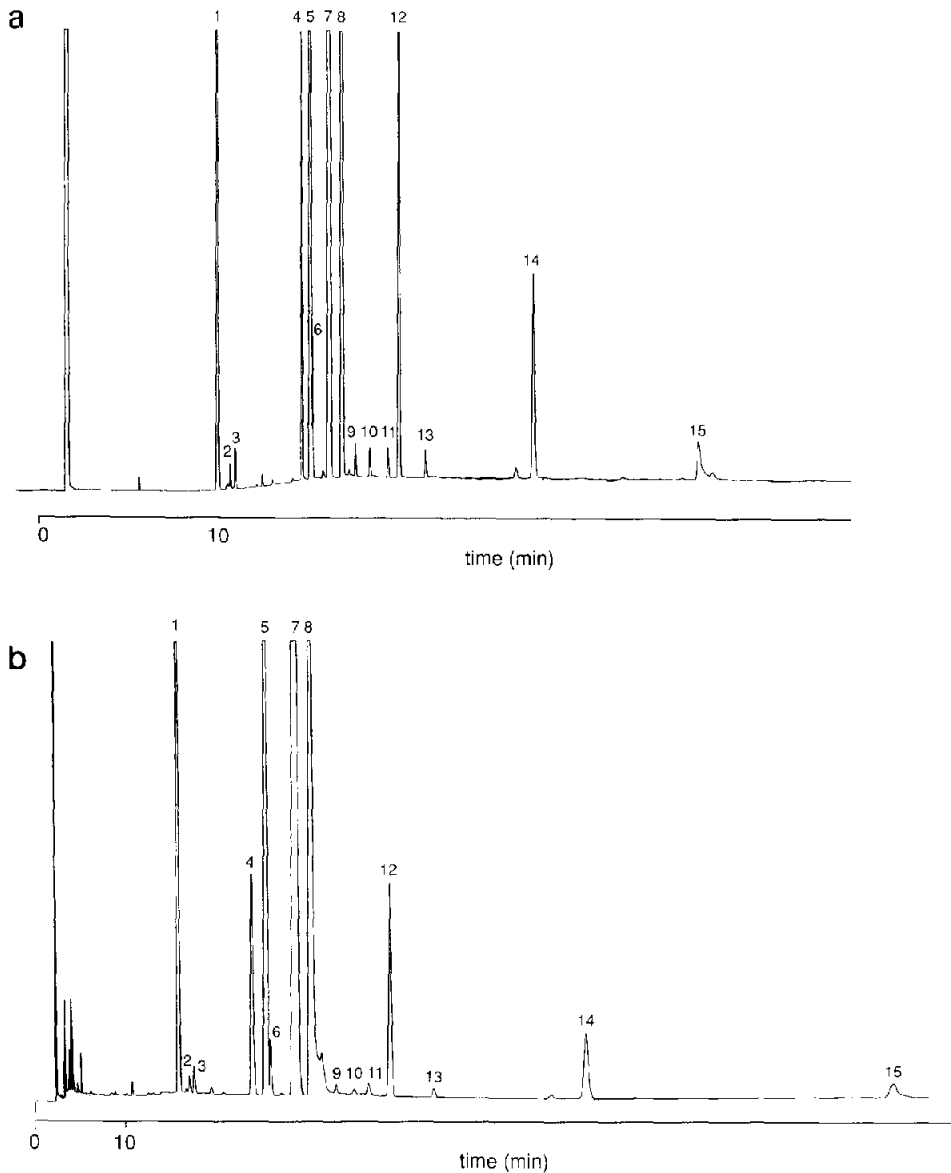
Fig. 1. Chromatograms showing separation of fatty acids in borage oil as (a) methyl esters and (b) picolinyl esters on a fused-silica capillary column coated with a polyethylene glycol phase (Supelcowax 10). For detailed analytical conditions, see Experimental. Peaks: 1 = 16:0; 2 = 16:1 (*n* − 7); 3 = 16:1 (*n* − 5); 4 = 18:0; 5 = 18:1 (*n* − 9); 6 = 18:1 (*n* − 7); 7 = 18:2 (*n* − 6); 8 = 18:3 (*n* − 6); 9 = 18:3 (*n* − 3); 10 = 18:4 (*n* − 3); 11 = 20:0; 12 = 20:1 (*n* − 9); 13 = 20:2 (*n* − 6); 14 = 22:1 (*n* − 9); 15 = 24:1 (*n* − 9).

The mass spectra of the picolinyl esters of saturated fatty acids showed a regular pattern of fragments 14 a.m.u. apart, representing cleavage at each methylene group. Among the monoenoic fatty acids, two 16:1 isomers could be found, *i.e.*, the

TABLE I

THE FATTY ACID DISTRIBUTION IN BORAGE OIL DETERMINED BY GC ANALYSIS OF FATTY ACIDS AS METHYL AND PICOLINYL ESTERS

Peak No.	Component	Methyl esters (area %)	Picolinyl esters (area %)
1	16:0	9.77	12.35
2	16:1 $(n-7)$	0.14	0.16
3	16:1 $(n-5)$	0.21	0.26
4	18:0	3.44	3.92
5	18:1 $(n-9)$	14.80	16.60
6	18:1 $(n-7)$	0.52	0.63
7	18:2 $(n-6)$	37.51	38.22
8	18:3 $(n-6)$	24.58	19.18
9	18:3 $(n-3)$	0.20	0.13
10	18:4 $(n-3)$	0.18	0.07
11	20:0	0.22	0.21
12	20:1 $(n-9)$	4.18	4.12
13	20:2 $(n-6)$	0.22	0.17
14	22:1 $(n-9)$	2.51	2.03
15	24:1 $(n-9)$	1.11	0.71

$(n-7)$ and $(n-5)$ isomers, the occurrence of the latter not having been reported previously in borage seed oil. Except for the gap of 26 a.m.u., or sometimes better a gap of 40 a.m.u., being indicative of the position of the double bond, each monoenoic fatty acid as a general rule shows a doublet of peaks representing hydrogen abstraction allylic to the double bond [6]. For instance, 16:1 $(n-5)$ could be identified partly
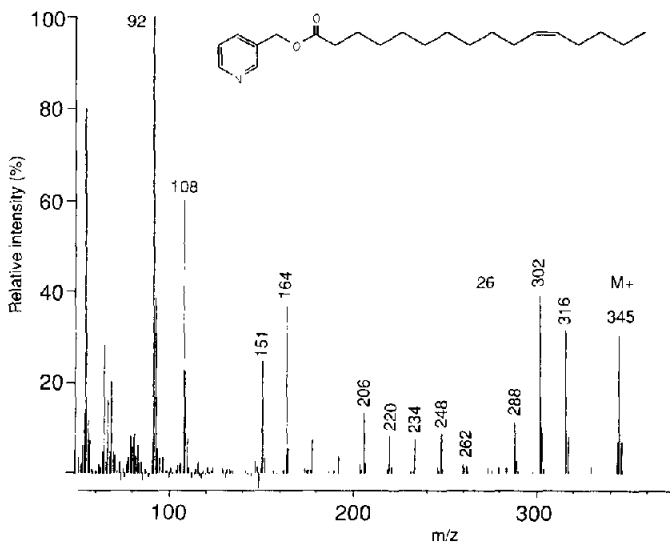

Fig. 2. Mass spectrum of 11-hexadecenoic acid picolinyl ester [16:1 $(n-5)$] in borage oil.

TABLE II

GC–MS ANALYSIS OF FATTY ACID PICOLINYL ESTERS IN BORAGE OIL

| Peak No.	Component	Retention time (min)	Molecular ion m/z	Base ion m/z	Other useful or diagnostic ions m/z (%)							
1	16:0	12.35	347	92	318(13)	304(15)	290(15)	276(16)	262(17)	248(13)	234(11)	220(10)
2	16:1 (n−7)	13.35	345	92	316(8)	302(10)	288(39)	274(32)	260(6)	234(4)	220(11)	206(10)
3	16:1 (n−5)	13.61	345	92	316(31)	302(39)	288(11)	262(2)	248(9)	234(7)	220(8)	206(13)
4	18:0	19.21	375	92	346(10)	332(13)	318(14)	304(14)	290(15)	276(17)	262(16)	248(13)
5	18:1 (n−9)	20.28	373	92	316(10)	302(9)	288(38)	274(33)	260(10)	234(4)	220(12)	206(8)
6	18:1 (n−7)	21.63	373	92	330(9)	316(27)	302(35)	288(15)	262(4)	248(11)	220(10)	206(16)
7	18:2 (n−6)	22.87	371	92	328(12)	314(15)	300(7)	274(12)	260(17)	234(4)	220(8)	206(6)
8	18:3 (n−6)	24.18	369	92	312(11)	298(7)	272(9)	258(11)	232(3)	218(3)	192(2)	178(3)
9	18:3 (n−3)	27.43	369	92	340(7)	314(8)	300(26)	274(8)	260(8)	234(4)	220(9)	206(8)
10	18:4 (n−3)	28.59	367	92	338(3)	312(6)	298(7)	272(5)	258(9)	232(3)	218(1)	178(2)
11	20:0	30.63	403	92	374(9)	360(10)	346(10)	332(13)	318(16)	304(17)	290(17)	276(20)
12	20:1 (n−9)	32.12	401	92	344(7)	330(7)	316(31)	302(40)	288(11)	262(3)	248(10)	234(8)
13	20:2 (n−6)	35.67	399	92	356(9)	342(13)	328(8)	302(13)	288(30)	262(5)	248(10)	234(7)
14	22:1 (n−9)	45.98	429	92	358(7)	344(27)	330(39)	316(13)	290(4)	276(13)	262(9)	248(9)
15	24:1 (n−9)	64.36	457	92	386(11)	372(23)	358(33)	344(13)	318(4)	304(14)	290(13)	276(13)
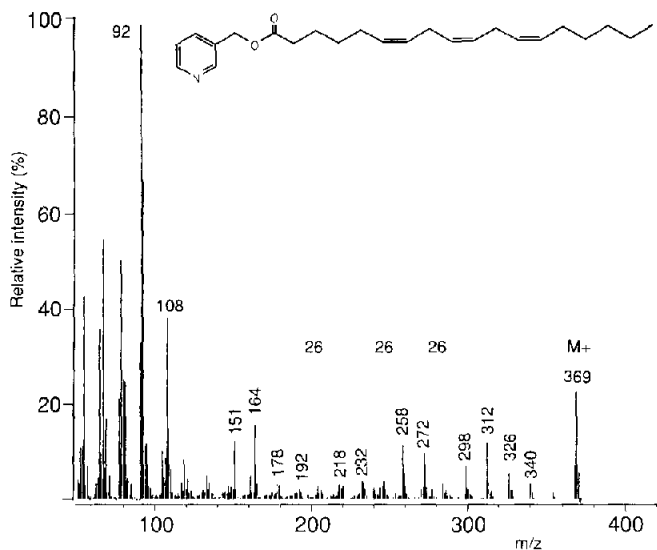
Fig. 3. Mass spectrum of 6,9,12-octadecatrienoic acid picolinyl ester [18:3 $(n-6)$] in borage oil.

because of a gap of 26 a.m.u. between the fragments 262 and 288 or of 40 a.m.u. (m/z = 248–288) and partly because of a doublet of ions at m/z 302 and 316 (Fig. 2). The spectrum of the analogous C_{18} fatty acid derivative has been published elsewhere [5].

The structures of the dienoic fatty acids could be determined in the same way as for the monoenoic compounds. For instance, in the spectrum of linoleic acid the position of the double bond could be determined by a gap of 26 a.m.u. between the
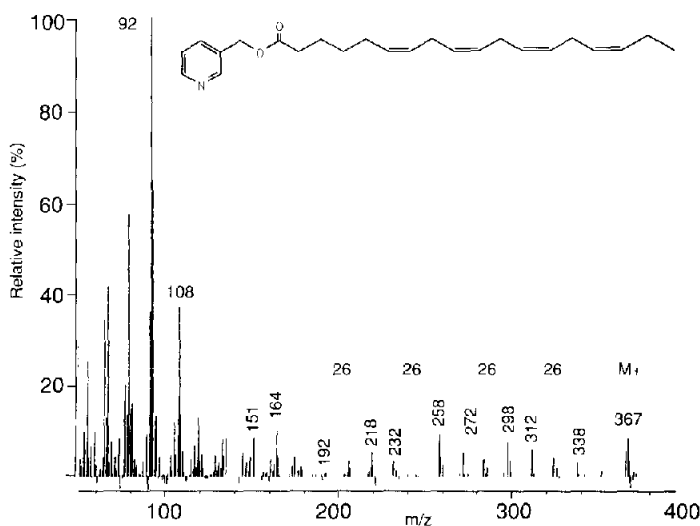

Fig. 4. Mass spectrum of 6,9,12,15-octadecatetraenoic acid picolinyl ester [18:4 $(n-3)$] in borage oil.

peaks of m/z 274 and 300. After a gap of 14 a.m.u., representing a methylene group, another gap of 26 a.m.u., representing the second double bond, appears. Three diagnostic ions representing hydrogen abstraction allylic to the double bonds could also be found at m/z 274, 314 and 328. The spectrum was similar to that obtained by Harvey [6].

All the trienoic and tetraenoic fatty acids in borage seed oil could be identified. None of the double bonds of these fatty acids were close to the carboxyl group, which could lead to difficulties in identification [7]. The pattern 26–14 a.m.u. indicating double bonds could be seen and also a strong diagnostic ion representing cleavage by loss of an allylic radical adjacent to the terminal double bond [6]. In the spectrum of the picolinyl ester of γ-linolenic acid ions 14 a.m.u. apart, counted from the methyl end, were found (Fig. 3). The first double bond is reached at the gap of 26 a.m.u. between the fragments of m/z 272 and 298. After 14 a.m.u. ($-CH_2$) another gap of 26 a.m.u. is reached, representing the second double bond. Finally, the third double bond is found between C_6 and C_7. The third gap of 26 a.m.u. was not as distinct as the others, the gap of 40 a.m.u. between the fragments of m/z 178 and 218 being more indicative of the double bond. The strong diagnostic ion of this trienoic acid is, according to the schemes of Harvey [6], at m/z 258. The fatty acid octadecatetraenoic acid or 18:4 (n 3), previously identified by Kleiman et al. [11] by means of equivalent chain-length values, was also confirmed by GC–MS. The spectrum is shown in Fig. 4. The positions of the three double bonds closest to the methyl end are determined by the pattern 26–14 a.m.u.. The fourth double bond closest to the carboxyl group does not give very distinct ions, but again a gap of 40 a.m.u. between the fragments of m/z 178 and 218 is found. The diagnostic ion representing cleavage adjacent to the terminal double bond is found at m/z 298 [6].

Picolinyl ester derivatives have been shown to be very useful for the structure determination of fatty acids by GC–MS, also reported elsewhere [5–8], because of their good mass spectrometric qualities. For the GC separation, non-polar columns have mostly been chosen because of the high molecular weight and also the polarity of picolinyl esters. However, on non-polar columns overlapping of peaks might be expected, especially with the ($n-6$) and ($n-3$) families of fatty acids. When a medium-polarity column was used, as in this instance, positional isomers could be separated easily. Linoleic acid and α-linoleic acid were also well resolved which might not be the case on non-polar columns [13]. An early attempt to use such a column was not successful [7], but there have been improvements in manufacturing techniques recently to give polar phases of great stability. Although the picolinyl esters of fatty acids were run isothermally at 260°C and the corresponding methyl esters according to a temperature programme from 80 to 225°C, their gas chromatograms were very similar (Fig. 1). This circumstance would facilitate identification. This technique, involving only one instrumental step, is very suitable for the identification of fatty acids in oils of moderate complexity. For more complex oils, such as fish oils, probably a liquid chromatographic prefractionation is required [8]. Positive identification of the fatty acids in oil of *Borago officinalis* by GC–MS was achieved previously by Craig and Bhutty [12] by means of permanganate–periodate oxidation after the isolation of each of the fatty acids. In that work, 11-hexadecenoic acid [16:1 ($n-5$)], α-linolenic acid [18:3 ($n-3$)] and 6,9,12,15-octadecatrienoic acid [18:4 ($n-3$)] were not identified. These three fatty acids were probably not detectable by the procedures available at that

time. According to the present work, 16:1 $(n-5)$, 18:3 $(n-3)$ and its corresponding tetraene, 18:4 $(n-3)$, are present but in small amounts, *viz.*, 0.21%, 0.20% and 0.18%, respectively (Table I).

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