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Location of double bonds in polyunsaturated fatty acids by gas chromatography–mass spectrometry after 4,4-dimethyloxazoline derivatization

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ABSTRACT

The location of double bonds in polyunsaturated fatty acids is determined by gas chromatography–mass spectrometry after 4,4-dimethyloxazoline (DMOX) derivatization. A procedure for DMOX preparation, starting from fatty acid methyl esters (FAMEs) is proposed. The most interesting properties of these derivatives are presented especially for the analysis of long-chain polyenoic fatty acids. The use of DMOX derivatives in combination with FAMEs can be very useful for the identification of fatty acids in unknown samples.

INTRODUCTION

A major problem in the analysis of unsaturated fatty acids is the determination of the position of the double bond in the alkyl chain. In the past, a number of different mass spectrometric methods for the structure elucidation of fatty acids have been proposed and excellent reviews [1,2] summarize all the different aspects.

In contrast to the methods of analysing underivatised fatty acids by tandem mass spectrometry [3,4], derivatization methods which introduce a group of low ionization potential (fragmentation-directing functionality) are attractive because they can be performed on a simple gas chromatographic-mass spectrometric (GC–MS) system working in the electron impact (EI) ionization mode. Following the latter approach, many different derivatives of the carboxylic group have been investigated: pyrrolidides [5,6], picolinyl [7–12], piperidyl and morpholinyl [13] esters, triazolopyridines [14] and 2-alkenylbenzoxazoles [15]. Using these derivatives, the determination of the position of functionalities such as double and triple bonds, branches or cyclopropane rings has been described.

However, these derivatives have low volatility, especially with long-chain polyunsaturated fatty acids, and their chromatographic behaviour is not good enough to permit the separation of all the acid derivatives which are often present in complex mixtures. In addition, their mass spectra sometimes leave doubt about the sites of unsaturation.

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Recently, Zhang *et al.* [16] described the use of 2-alkenyl-4,4-dimethyloxazolines (DMOX) for the location of double bonds in long-chain olefinic acids. These derivatives have a high volatility for chromatographic analysis and their mass spectra show easily recognizable diagnostic peaks for the determination of the position of unsaturation. The same derivatives have also been used for the determination of methyl branching [17] and cyclopentenyl and triple bond location [18,19]. However, the described reaction between free fatty acids and 2-amino-2-methylpropanol was not quantitative and residual material could not be removed.

This paper describes another way of obtaining DMOX derivatives, starting from FAMEs routinely used for the analysis of lipids by GC. The methyl esters, obtained by transesterification of the triglycerides, form the corresponding DMOX derivatives by reaction with 2-amino-2-methylpropanol (Scheme 1). The products can easily be extracted from the reaction mixture.

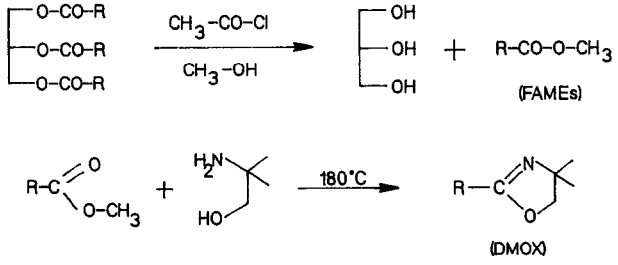
EXPERIMENTAL

Materials

All fatty acid standards were purchased from Supelco (Gland, Switzerland). Acetyl chloride and 2-amino-2-methylpropanol were obtained from Fluka (Buchs, Switzerland) and were used without further purification. All solvents were of analytical-reagent grade.

Derivatization

The direct transesterification method of Lepage and Roy [20] was slightly modified (hexane replacing benzene [21]). In a Reacti-vial (Pierce, Socochim, Pully, Switzerland), 10 μl of fat sample were dissolved in 1 ml of methanol–hexane (4:1) and 100 μl of acetyl chloride were slowly added. The vials were tightly closed and heated at 100°C for 1 h. After cooling, 1 ml of 6% K_2CO_3 and 1 ml of hexane were added and the mixture was gently shaken. The hexane phase was dried (Na_2SO_4) and evaporated under a stream of nitrogen at room temperature. The DMOX derivatization was carried out by adding 500 μl of 2-amino-2-methylpropanol, heated overnight at 180°C. After cooling, the reaction mixture was dissolved in 5 ml of dichloromethane and washed twice with 2 ml of distilled water. The dichloromethane solution was dried (Na_2SO_4) and evaporated under a stream of nitrogen at room temperature. The residue was dissolved in hexane and was ready for injection.



Scheme 1.

Gas chromatography–mass spectrometry

The equipment used was a Hewlett-Packard 5880 gas chromatograph, a Kratos MS-30 mass spectrometer and a DS55 data system (Kratos, Manchester, U.K.). The GC–MS conditions were as follows: fused-silica column (30 m × 0.32 mm I.D.) DB-WAX; carrier gas (helium) pressure, 10 p.s.i.; on-column injection at 60°C; oven temperature programme, 60°C (1 min), increased at 30°C/min to 200°C and then at 3°C/min to 240°C (5 min); ion source temperature, 240°C; EI ionization at 70 eV.

An HP-5971A GC–MS system with an HP-G1030A MS Chemstation (Hewlett-Packard, Geneva, Switzerland) was also used. The oven temperature programme was 50°C (1 min), increased at 30°C/min to 150°C and then at 4°C/min to 250°C (10 min).

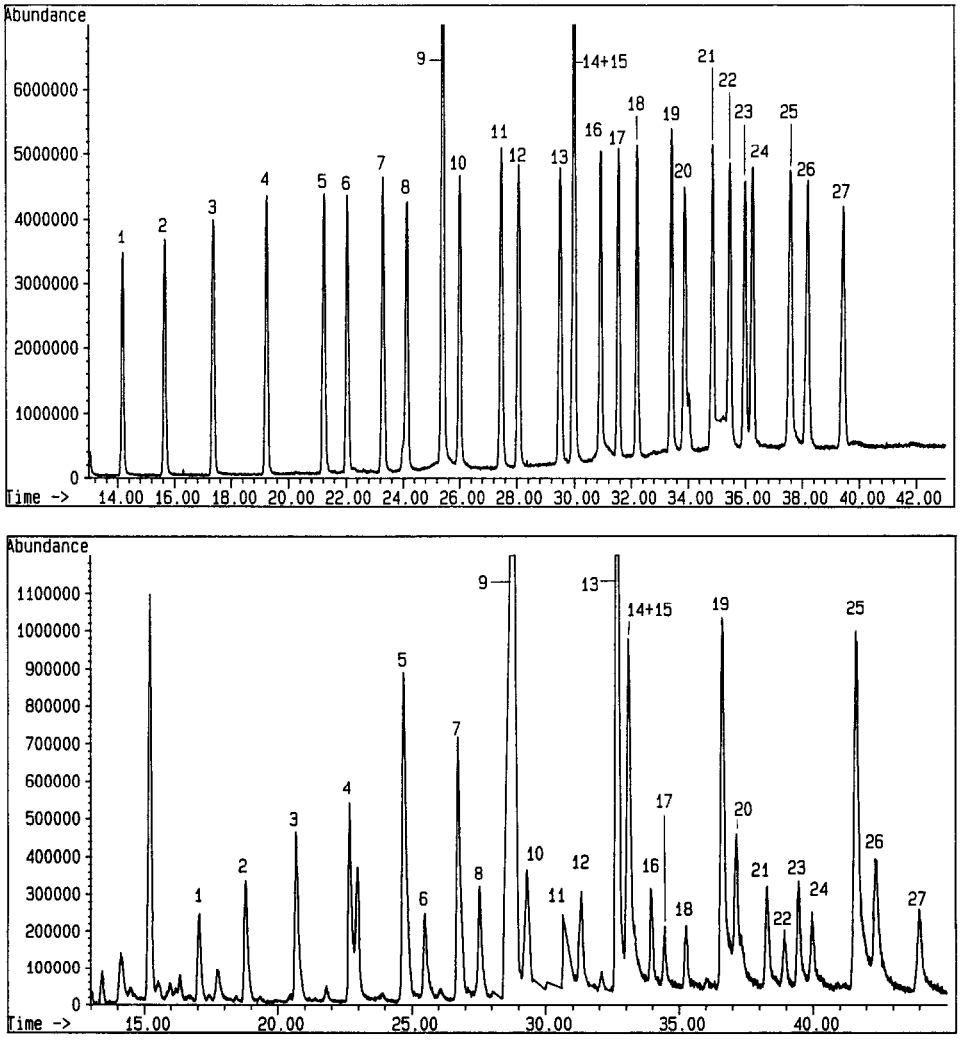


Fig. 1. Separation of a FAME standard mixture (top) and the corresponding DMOX derivatives (bottom) on a DB-WAX capillary column. See Experimental for the chromatographic conditions.

RESULTS AND DISCUSSION

In our first experiments, the DMOX derivatization reaction of free fatty acids according to Zhang *et al.* [16] was not complete and the gas chromatograms showed residual materials interfering with DMOX derivatives. Therefore, we developed a new DMOX derivatization procedure starting from FAMEs. This method allows the qualitative and quantitative analysis of fatty acids in an unknown sample in two steps. First, all the common fatty acids are easily determined by analysis of the FAME derivatives obtained after the direct transesterification of triglycerides. In a second step, if there is doubt about the structure of unsaturated acids, the FAMEs can be derivatized directly to the corresponding DMOX derivatives, which are then analysed by GC–MS.

Fig. 1 shows the chromatograms of a standard mixture of 27 FAMEs and of the DMOX derivatives obtained with the same column under the same conditions. The elution orders of DMOX and FAME derivatives are identical. The chromatographic properties of DMOX derivatives allow their resolution on a polar column without applying high temperature or long isothermal periods. Typical elution temperatures of DMOX are about 10–15°C higher than those required by the corresponding FAMEs.

The proposed reaction of FAMEs with 2-amino-2-methylpropanol is carried out overnight at 180°C. The procedure yields a quantitative derivatization and after extraction with dichloromethane no residual FAMEs interfere with the DMOX derivatives (Fig. 2).

These drastic conditions are required in order to obtain a complete reaction between FAMEs and 2-amino-2-methylpropanol and quantitative formation of oxazoline derivatives. If the reaction is performed at lower temperatures residual FAMEs are still present and produce interferences with DMOX derivatives. Under the chosen conditions, no discrimination effect and no decomposition products of fatty

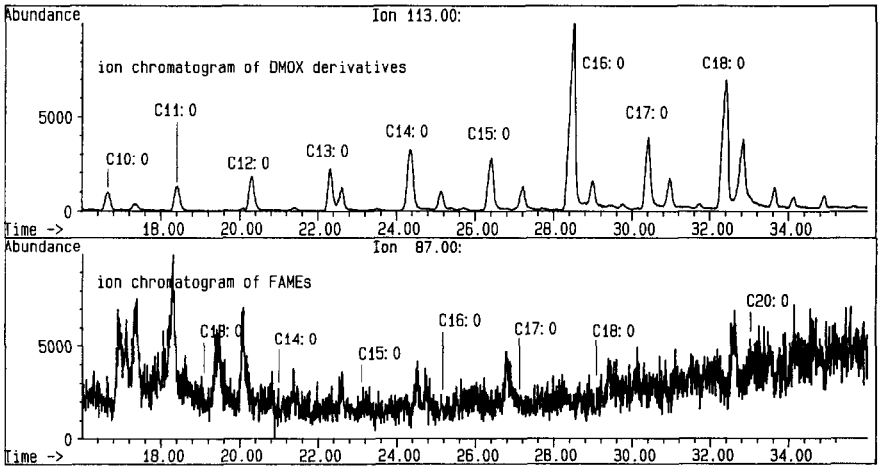


Fig. 2. Mass chromatograms of DMOX derivatives (m/z 113) and FAMEs (m/z 87) in a standard mixture after DMOX derivatization carried out overnight at 180°C. No residual FAMEs can be detected in the sample.

acids have been detected. The derivatives obtained are stable for at least 1 week at room temperature.

The mass spectra of DMOX derivatives clearly show the positions of the double bonds in mono- or polyunsaturated fatty acids. An unsaturation is located by an interruption of the regular pattern produced by successive chain cleavages of methylene units. The double bond position can be determined using the rule formulated by Andersson and co-workers [22,23] for pyrrolidide derivatives: if an interval of 12 atomic mass units (a.m.u.), instead of the regular 14 a.m.u., is observed between the most intense peaks clusters of fragments containing n and $n - 1$ carbon atoms in the acid moiety, a double bond occurs between carbons n and $n + 1$ in the molecule.

Fig. 3 shows the mass spectrum of C22:1 (13), which contains two peaks at m/z 252 and 264 locating a C-13 double bond. The base peak at m/z 113 and the intense ion at m/z 126 are produced by McLafferty rearrangement and a cyclization reaction, respectively [16].

For all monoenoic acids studied, the double bond located between carbons n and $n + 1$ also gives abundant ions with $n + 2$, $n - 2$ [16] and $n + 3$ carbon atoms. In this case, the small ions locating the double bond are always surrounded by three ions with more abundant intensities. For example, the spectrum in Fig. 3 presents such a pattern with prominent peaks at m/z 238, 292 and 306.

DMOX derivatives are very attractive for the analysis of long-chain polyenoic fatty acids. Unlike pyrrolidide, picolinyl or triazolopyridine derivatives, DMOX of polyunsaturated fatty acids are well eluted on polar GC columns and easily transferred into the MS source. After EI ionization, they produce mass spectra which clearly show the molecular ion and the positions of unsaturation.

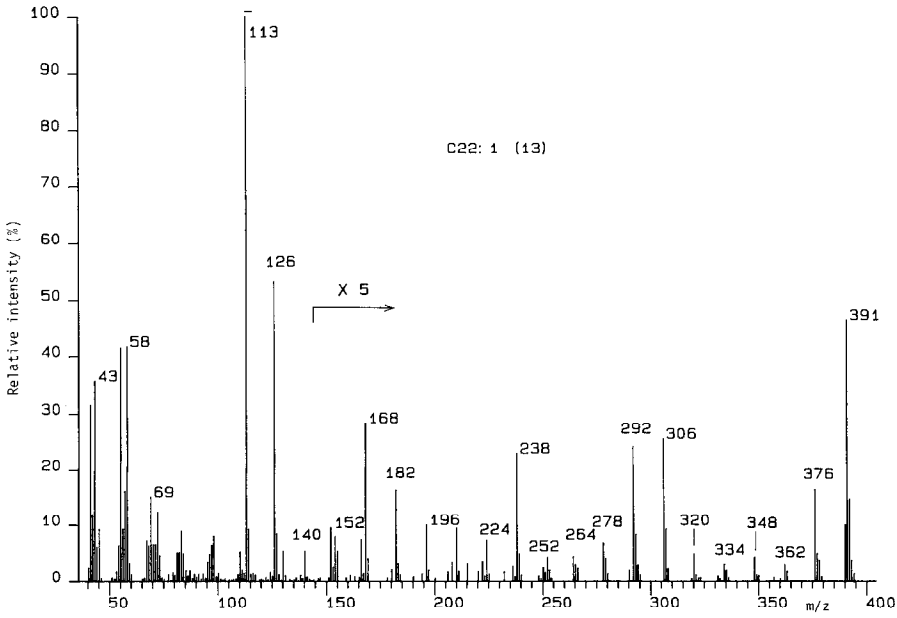


Fig. 3. Mass spectrum of the DMOX derivative of C22:1 (13).

Table I summarizes the results obtained for 22 unsaturated fatty acids after DMOX derivatization. Only the diagnostic fragments, locating the double bond positions, are presented.

Fig. 4 shows the spectrum of C22:6 (4,7,10,13,16,19). The rule of the 12 a.m.u. intervals can easily be applied and indicates double bonds at the C-7, -10, -13, -16 and -19 positions. However, the location of the C-4 unsaturation cannot be identified by the 12 mass units rule. This unsaturation is located by an intense odd-mass ion at m/z 139. The formation of this ion could be explained by ionization of the nitrogen atom followed by cyclization and cleavage of the rest of the aliphatic chain (Scheme 2). For C-5 and C-6 unsaturations, corresponding ions were observed at m/z 153 and 167.

The use of ions at m/z 139, 153 and 167 allows the unambiguous location of C-4, -5 and -6 double bonds even for fatty acids with a non-regular distribution of the unsaturations. As an illustration, Fig. 5 presents the mass spectrum of the C18:3 (5,9,12) acid. The C-9 and -12 double bonds are located by the 12 a.m.u. interval and the C-5 unsaturation by the intense peak at m/z 153.

The method described here has also been used successfully for fatty acids with conjugated double bonds. An example is the analysis of seed oil from *Calendula*

TABLE I

CHARACTERISTIC IONS IN EI MASS SPECTRA OF DMOX DERIVATIVES OF 22 UNSATURATED FATTY ACIDS

Fatty acid DMOX	$M^{+\cdot}$ m/z (intensity, %)	Diagnostic fragments m/z (intensity, %)
C22:6 (4,7,10,13,16,19)	381(5)	166(4), 178(3), 206(3), 218(2), 246(3), 258(2), 286(1), 298(1), 326(0.5), 338(0.1), 139(9)
C20:4 (5,8,11,14)	357(9)	180(3), 192(2), 220(3), 232(2), 260(1), 272(1), 153(19)
C20:5 (5,8,11,14,17)	355(5)	180(3), 192(2), 220(3), 232(2), 260(2), 272(1), 300(1), 312(1), 153(17)
C18:1 (6)	335(4)	166(13), 167(24)
C18:3 (6,9,12)	331(18)	194(13), 206(8), 234(7), 246(4), 220(27), 274(19), 166(21), 167(33)
C18:4 (6,9,12,15)	329(8)	194(6), 206(3), 234(4), 246(2), 274(2), 286(1), 220(7), 260(6), 166(9), 167(12)
C22:4 (7,10,13,16)	385(14)	168(7), 180(13), 208(7), 220(4), 248(9), 260(4), 288(3), 300(2)
C22:5 (7,10,13,16,19)	383(7)	168(6), 180(9), 208(6), 220(4), 248(6), 260(2), 288(3), 300(2), 328(2), 340(1)
C20:3 (8,11,14)	359(4)	182(2), 194(1), 222(2), 234(1), 262(1), 274(1)
C14:1 (9)	279(7)	196(2), 208(3), 182(16), 236(13), 250(9)
C16:1 (9)	307(7)	196(2), 208(2), 182(15), 236(11), 250(12)
C18:1 (9)	335(13)	196(3), 208(3), 182(16), 236(13), 250(11)
C18:2 (9,12)	333(10)	196(3), 208(2), 236(5), 248(2), 222(15), 276(11)
C18:3 (9,12,15)	331(20)	196(3), 208(2), 236(6), 248(3), 276(8), 288(2)
C15:1 (10)	293(8)	210(1), 222(2), 196(10), 250(11), 264(9)
C17:1 (10)	321(7)	210(2), 222(2), 196(9), 250(9), 264(9)
C18:1 (11)	335(10)	224(1), 236(3), 210(7), 264(9), 278(9)
C20:1 (11)	363(10)	224(1), 236(2), 210(6), 264(8), 278(8)
C20:2 (11,14)	361(15)	224(1), 236(3), 264(5), 276(2)
C20:3 (11,14,17)	359(16)	224(2), 236(1), 264(5), 276(2), 304(5), 316(2)
C22:1 (13)	391(9)	252(1), 264(1), 238(5), 292(5), 306(5)
C22:2 (13,16)	389(19)	252(1), 264(1), 292(3), 304(2)

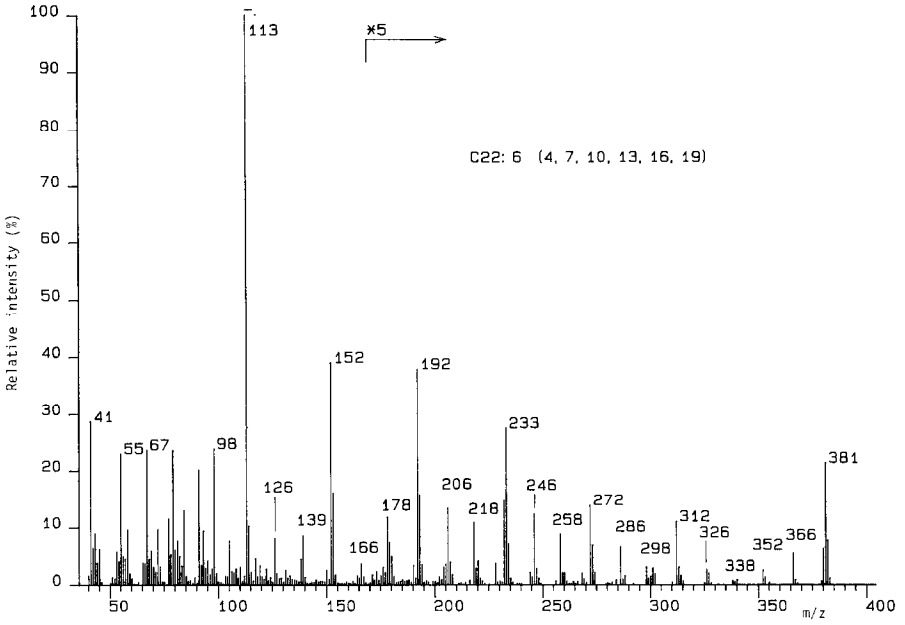
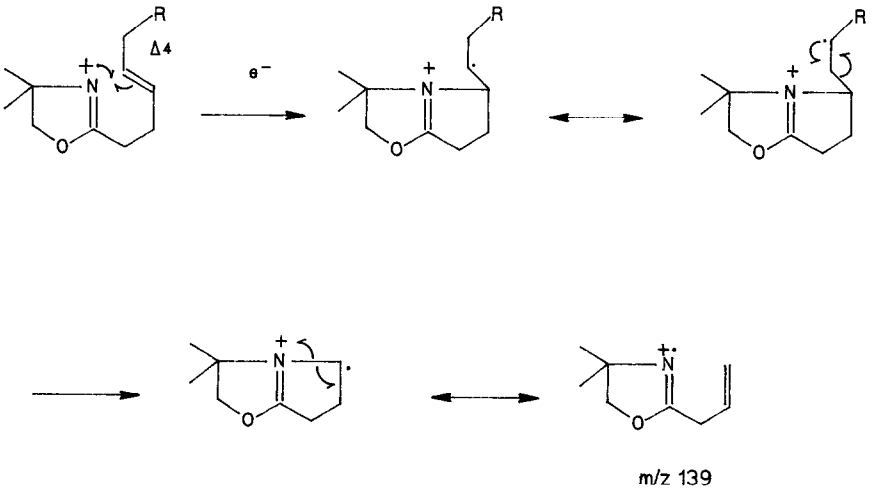


Fig. 4. Mass spectrum of the DMOX derivative of C22:6 (4,7,10,13,16,19).

officinalis. After transesterification, the GC–MS analysis of the FAMEs obtained shows two unknown peaks. The chromatogram of the corresponding DMOX derivatives is presented in Fig. 6 and the mass spectrum of the first unknown peak in Fig. 7. This peak 1 was identified as C18:3 (8,10,12). Its spectrum shows characteristic ions between *m/z* 182 and 260. Peak 2 has the same mass spectrum and is identified as



Scheme 2.

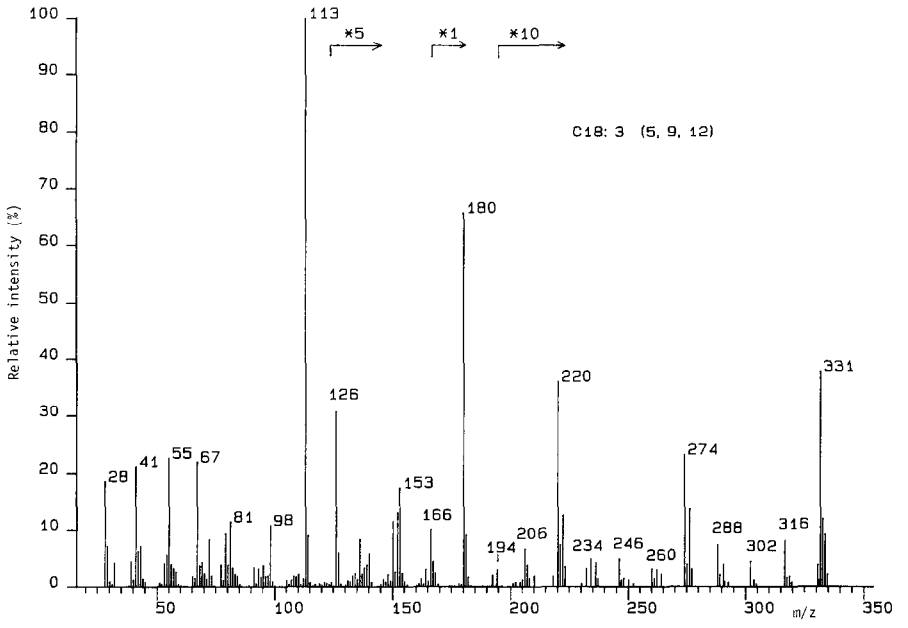


Fig. 5. Mass spectrum of the DMOX derivative of C18:3 (5,9,12).

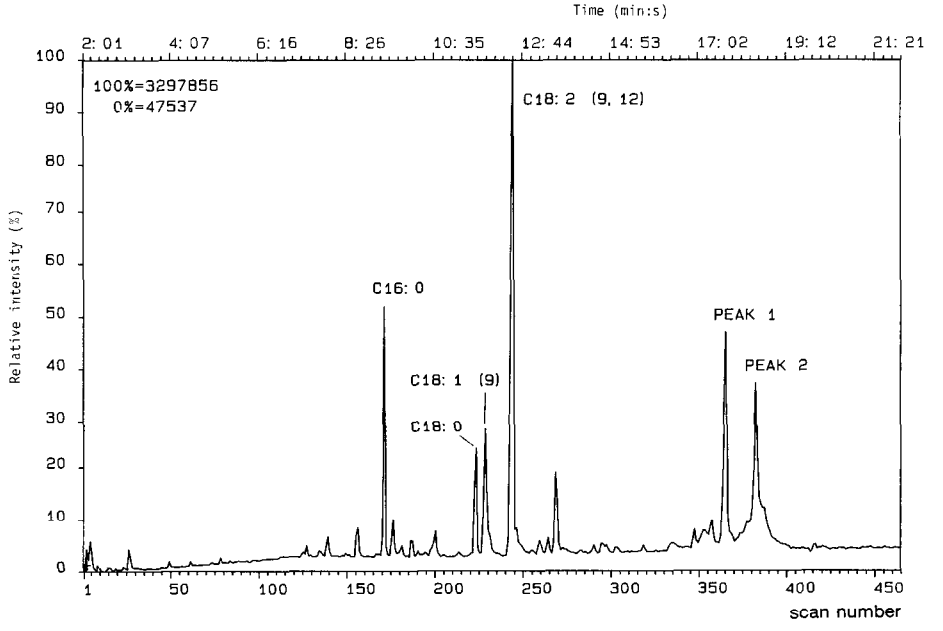


Fig. 6. Separation of the DMOX derivatives of the seed oil of *Calendula officinalis*.

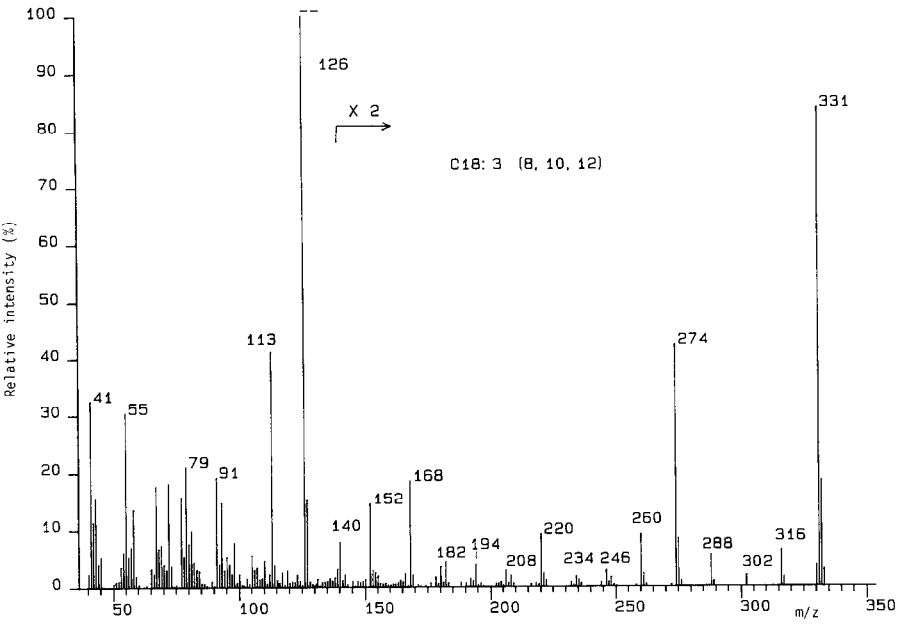


Fig. 7. Mass spectrum of the DMOX derivative of C18:3 (8,10,12) identified in the seed oil of *Calendula officinalis*.

an isomer. The difference between these two acids is probably the *cis*/*trans* configuration of the double bonds. For this oil various isomers of octadecatrienoic acids, including calendic acid (*trans*-8,*trans*-10,*cis*-12-octadecatrienoic acid), have been proposed [24].

In conclusion, DMOX derivatives of fatty acids can be obtained easily and quantitatively from the corresponding methyl esters. Mass spectra of DMOX derivatives are very useful for double bond location, especially in polyunsaturated and conjugated systems. The good chromatographic properties of DMOX derivatives allow the effective resolution of complex mixtures. The combined use of FAMEs and DMOX derivatives can be a very attractive method for fatty acid analysis.

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