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Identification of Fatty Acids in the Oils of Pine Nuts by GC-MS of Their Picolinyl Esters and 4,4-dimethyloxazoline Derivatives in Combination with Silver-Ion Chromatography

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Abstract: A mixture of methyl ester derivatives of fatty acids from the oils of pine nuts was well resolved to five fractions differing by degree of unsaturation by silver ion solid-phase extraction column chromatography (Ag^+-SEC). Polyunsaturated fatty acid with non-methylene interrupted conjugated double bond (NMiDB) radical held more strongly to silver ions in the column than methylene interrupted conjugated double bond (MiDB) one when they had the same number of double bonds. Although both the picolinyl ester and DMOX derivative provided clear mass ion species powerful enough to elucidate the structure of the polyunsaturated fatty acid (PUFA) with NMiDB and/or methylene interrupted conjugated double bond (MiDB) radical in the oils, the picolinyl ester of PUFA with NMiDB radical did not provide a cluster of mass ions neighboring diagnostic mass ions induced by the double bond in the proximal to the carboxyl group. However, the DMOX derivative of PUFA with NMiDB group as well as MiDB showed abundant mass ion species differing by gaps of 12 amu, which made it possible with greater ease to locate the double bonds in the molecule. The oil contained $C_{18:2\omega6}$ (46.2 %) and $C_{18:1\omega9}$ (25.4 %) as main components, and considerable amounts of PUFAs with NMiDB radical such as $\Delta^{5,9,12}$-$C_{18:3}$ (16.0 %), $\Delta^{5,9}$-$C_{18:2}$ (2.3 %) and $\Delta^{5,11,14}$-$C_{20:3}$ (0.8 %).

Keywords ; Ag^+-chromatography, picolinyl ester, pine nuts oil, 2, 2,-dimethyloxazoline (DMOX) derivative, non-methylene conjugated double bond (NMiDB).

1. INTRODUCTION

Pyrrolidide derivative (1-3) and 2-alkylbenzoxazole derivative (2, 4) have been studied most often to determine the structures of both model fatty acids and natural samples by gas-liquid chromatography-mass spectrometry (GC-MS). These derivatives, however, show lower volatility and worse resolution on GC in comparison with the methyl esters of fatty acids, and the interpretation of spectra for highly unsaturated fatty acids (greater than tetraenes) becomes substantially more difficult although distinctive modes of fragmentation can be obtained for less unsaturated fatty acids.

In recent years, Harvey (5-6) reported that

picolinyl esters were more useful than pyrrolidide esters for locating of fatty acids with double bond(s) or other functional group(s); these derivatives generally show good separation in the non-polar phase of GC with a little tailing although their volatile temperatures are about 50℃ higher than those used for the methyl esters, and they provide more abundant diagnostic ions by GC-MS analysis. Furthermore, they can be separated by high-performance liquid chromatography (HPLC) in the reverse-phase mode (7). Much research on the structural interpretation of unsaturated fatty acids and branched fatty acids has been carried out by Christie (8-10) via derivatization to picolinyl esters.

Recently, a new type of nitrogen-containing derivative, 2-alkenyl-4, 4-dimethyl-oxazoline (DMOX), was applied to the GC-MS analysis of fatty acids by Zhang (11-13). These derivatives are easily prepared in good yield by condensation of free fatty acids (or the methyl esters) with an excess of 2-amino-2-methyl-propanol. Their chromatographic behaviours on GC are comparable to those of the methyl esters and their EI mass spectra are more structurally specific than those of other nitrogen-containing derivatives. Therefore, DMOX derivatives have been widely used for locating individual double bonds in multiple double bond structural fatty acids, alkyl branching, hydroxy and oxo radicals and the presence and position of cyclopropane and cyclopropene groups (12).

However, actually all the previous studies using compounds of picolinyl esters and DMOX derivatives have been with model fatty acids (11-13), although there have been a few applications of GC-MS to both derivatives of complex fatty acids in nature (14-19).

Lipids existing in nature frequently contain fatty acids having unusual structures and/or minor compositions, so it is often difficult to identify their individual components by GC or GC-MS because some identifications with similar chemical features are not well resolved on the columns. For better resolution preliminary fractionation procedures are required to classify complicated fatty acids and to concentrate their minor components. Silver ion (Ag^+)-chromatography is one of the techniques suitable for this purpose (20, 21).

Ag^+-chromatography is based on the complexity of π-electrons localized in the double bonds of unsaturated organic molecules with silver ions, and has been widely used to simplify natural and modified lipids of different origins or even to clarify their structures. It has two operation modes, thin-layer and column chromatography including normal pressure and high-performance liquid chromatography (20).

In recent years, as one type of silver ion-normal pressure liquid chromatography, disposable solid-phase extraction columns packed with various bonded phases and the loaded with silver ion (Ag^+) are finding many applications in chromatography of lipid analysis. Small columns packed with a bonded benzene-sulfonate (or propylsulfonate) medium can be loaded with silver ions and used to achieve excellent separations of methyl ester derivatives of fatty acids according to the number of double bonds (22-24). The silver ion solid-phase extraction column chromatography (Ag^+-SEC) (22) is much easier to use than silver ion thin-layer chromatography (Ag^+-TLC) (25, 26) and also gives clean fractions without any stain of the silver ions and artefacts frequently encountered in Ag^+-TLC. In addition, this approach does not require the costly and sophiscated apparatus that a silver ion-HPLC (Ag^+-HPLC) system does (8, 27-30). This technique has, in particular, immediate practical value for preparative purification of lipid samples; for example, simpler molecular fractions prepared from complicated natural

lipid samples by Ag^+-SEC have been shown to be more easily identifiable by GC-MS than are the unfractionated materials.

Polyunsaturated fatty acids having a non-methylene interrupted conjugated double bond (NMiDB) (*e. g.*, -CH=CH-$(CH_2)_2$-CH=CH-, -CH=CH-$(CH_2)_3$-CH=CH-) such as $\Delta^{5, 9}$-$C_{18:2}$, $\Delta^{5, 9, 12}$-$C_{18:3}$ and $\Delta^{5, 11, 14}$-$C_{20:3}$ have been found in the oils of nuts of *Ginkgo biloba* (30) and of seeds of some plants belonging to the Pinaceae family (31-34) and in the oil of marine mussels (35). However, only limited research has been reported on mass spectrometric analysis of polyunsaturated fatty acids with NMiDB radical via their picolinyl esters and/or DMOX derivatives. In this study, both the picolinyl esters and DMOX derivatives were applied to the structural analyses of unsaturated fatty acids with NMiDB radical in natural oils by GC-MS. For this purpose we chose the oil of nuts of a pine tree (*Pinus koraiensis*) because it contained a considerable amount of polyunsaturated fatty acids having NMiDB radical (31-34) as well as a large proportion of unsaturated fatty acids with MiDB radical. Methyl esters of fatty acids from the sample oil were preliminarily resolved into diffrerent fractions by degree of unsaturation prior to analysis by GC-MS.

2. EXPERIMENTAL

Collection of the samples. Pine nuts were collected on October, 1998, from pine trees (*Pinus koraiensis*) growing in the area of Hongchun County in Kangwon Province, Korea.

Chemicals. Fatty acid methyl ester (FAME) standards were purchased from Nu-Chek-Prep Inc (Elysian, MN, USA). All solvents were of Analar grade and were purchased from J. T. Baker Inc (Phillipsburg, NJ, USA), and chemicals were provided by Aldrich Chemical Company Inc (Milwaukee, WI, USA). Varian Bond Elut^{R}SCX solid-phase extraction columns were purchased from Varian Inc. (Hansen, Palo Alto, CA, USA); the adsorbent (1 mL, *p*-propylbenzene sulfonic acid) was packed in a polypropylene column (0.8 cm × 6.5 cm) and then enclosed with porous frits.

Extraction of lipids from samples. The pine nuts were powdered with a mortar and then homogenized with a Waring Blendor. Total oils were extracted from the prepared samples according to the method of Bligh and Dyer (36). Chloroform-methanol extracts combined were transferred to a 250 mL volumetric separating funnel and allowed to settle overnight in a refrigerater to separate the organic and aqueous layers completely. The chloroform layer containing the lipids was concentrated in a rotary vacuum evaporator. The resulting lipids were transferred to an amber-colored vial and were stored at -30℃.

Fatty acid methyl ester (FAME) preparation (37). Total lipids (2~50 mg) were dissolved in sodium-dried diethyl ether (0.5 mL), and methyl acetate (20 μL), and 0.5 M sodium methoxide in dry methanol (20 μL) were added. The mixture was thoroughly vortexed and left at room temperature for 5 min, and the reaction was stopped by addition of acetic acid (2 μL). The solvent was evaporated carefully in a gentle stream of nitrogen at 30℃ after the internal standard solution of methyl nonadecanoate (methyl ester of $C_{19:0}$, 25 μL, 5.8 mg/25 mL hexane) along with BHT (1 mL, 50 mg/L hexane) was added. The mixture was centrifuged to consolidate the precipitate of sodium acetate at about 1,500 *g* for 2 minutes. The supernatant layer was decanted into a sample tube, and the excess solvent was evaporated to dryness in a stream of nitrogen. The sample was dissolved in 0.1 mL of hexane and a small portion (1 μL) was injected into GC.

Silver-ion chromatography using solid-

phase extraction column (22). Silver nitrate (30 mg) was dissolved in 0.5 mL acetonitrile (ACN)-water (1 : 1, v/v) and then the solution was allowed to percolate through a Varian Bond Elut™ SCX solid-phase extraction column (Varian Inc., Hansen, Palo Alto, CA) wrapped with aluminium foil at the level of the top of the adsorbent bed. The column was flushed with ACN (5 mL), acetone (5 mL), dichloromethane (DCM, 10 mL) by applying slight pressure from a pipette bulb. The column was then ready for use. Portions of FAMEs (<0.5 mg) dissolved in a small volume of DCM were loaded onto the column and eluted with 100% DCM (5 mL), followed by 90% DCM-acetone (5 mL), 100% acetone (5 mL), 97% acetone-ACN (10 mL) and 94% acetone-ACN (10 mL), for separation of saturated, monoene, diene, diene plus triene, and triene FAME fractions. Each fraction was concentrated under nitrogen and FAMEs in the fractions were dissolved in a small volume of hexane, respectively, for GC analysis.

Fatty acid picolinyl derivative preparation. Free fatty acids released from total lipids by alkaline hydrolysis and acidification were converted into their picolinyl esters according to the method recommended by Balazy and Nies (38); *i. e.*, a solution of 1, 1'-carbonyl diimidazole in DCM, freshly prepared (100 μL, 100 mg/mL) was added to the free fatty acids (1~5 mg) dissolved in DCM (100 μL) and the mixture was left 1 min at room temperature. Picolinyl reagent (100 μL), prepared by dissolving 3-(hydroxymethyl) pyridine (300 μL) and 4-pyrrolidinopyridine (60 mg) in DCM (3 mL), and triethylamine (100 μL) was added to the reaction mixture, which was then agitated and kept in a water bath at 37℃ for 10 min before the excess solvent was blown off in a block heater at 30℃ in a gentle stream of nitrogen. Hexane (5 mL) and distilled water (2 mL) were added to the product mixture which was then agitated and centrifuged (at 4,400 rpm) for 5 min. The upper hexane layer recovered was percolated through a short column of anhydrous sodium sulphate for removal of water traces. Combined hexane eluants were reduced to 1~2 mL under nitrogen and loaded into a pasteur-pipette Florisil™column. The column was washed with 5 mL of hexane/acetone (99 : 1, v/v) to remove contaminants and was then eluted with 8 mL of hexane/acetone (80 : 20, v/v) to isolate the picolinyl derivatives. The latter eluant was evaporated to dryness for GC and GC-mass spectrometry.

Fatty acid DMOX derivative preparation (15, 19). A portion (100 mg) of the total lipids was transferred to a screw-cap vial (1 mL) and 2-amino-2-methyl propanol (0.25 mL) was added. The mixture in the vial was reacted at 180℃ in a constant-temperature oil bath for 6 hr after being flushed with a stream of nitrogen and sealed. The reaction solution was added with DCM (2 mL) and water (2 mL) and was then agitated by vortexing. The organic (DCM) layer was transferred to another glass tube and was removed completely under a nitrogen atmosphere. Hexane (2 mL) was added to the residue and the mixture was agitated vigorously by vortexing. The hexane layer recovered was dried by passing it through a Pasteur pipette filled with anhydrous sodium sulfate and reduced to dryness under nitrogen. The fatty acid DMOX derivatives thus obtained were finally dissolved in chloroform for GC and GC-MS analysis.

FAME analysis by GC (30). Sample (1 μL) was injected into a fused silica capillary column (Carbowax 20M, 2.5 m × 0.22 mm, i. d., film thickness; 0.25 μm, Hewlett-Packard) in an HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector (FID) and eluted with H_2 (split ratio 1 : 50). The column was temperature-programmed from 165℃ (held 3 min) to 205℃ at 3℃/min and then held a further 5 min at this final

temperature. The injection and detector temperature were 250℃ and components of fatty acids were identified by GC-mass spectrometry.

GC-MS analysis of picolinyl and DMOX derivatives of fatty acids (8). The derivatives were submitted to GC-mass spectrometry with an HP 5890 gas chromatograph (Hewlett-Packard) coupled with an HP model 5989 quadrupole mass-selective detector. The mass selective detector was used in the electron impact mode at 70eV with a source temperature of 250℃. The picolinyl esters were injected into a capillary column of fused-silica coated with DB5-MS™ (30 m × 0.25 mm i.d., film thickness ; 0.25 μm, J & W Scientific, Folsom, CA, USA) by cold on-column injection at 80℃ (held for 3 min). The column was temperature-programmed at 20℃/min to 160℃, then at 4℃/min to 350℃, where it was held for 20 min. Helium was the carrier gas. On the other hand, DMOX derivatives were separated in a polar capillary column, BPX 70 (50 m × 0.22 mm, 0.25 μm film thickness) coated with 70% cyanopropyl polysilphenylenesiloxane (SGE, Austin, TX, USA).

3. RESULTS AND DISCUSSION

3.1. Fractionation of fatty acid by degree of unsaturation.

The oils of pine nuts were transesterified in anhydrous mehtanol in the presence of sodium methoxide and the methyl esters of fatty acids (FAME) produced were recovered with hexane. A small portion of the complex FAME derivatives was introduced onto GC and 16 distinct peaks were all well resolved as represented in Fig. 1-A. The seed oil of *Pinus koraiensis* fatty acid contained three unsaturated fatty acids with NMiDB group such as $\Delta^{5,\ 9,\ 12}$-$C_{18:3}$ (16.0 %), $\Delta^{5,\ 9}$-$C_{18:2}$ (2.3 %) and $\Delta^{5,\ 11,\ 14}$-$C_{20:3}$ (0.8 %), in addition to conventional saturated fatty acids of $C_{16:0}$ (4.8 %) and $C_{18:0}$ (2.0 %), monoenoic acids of $C_{18:1\omega9}$ (25.4 %) and $C_{20:1\omega9}$ (0.9 %), and dienoic acids of $C_{18:2\omega6}$ (46.2 %), as listed in Table 1.

In order to enrich minor components and obtain simultaneously better resolved mass spectra of them by GC-MS, the FAME derivatives remaining were classified into five fractions with simpler components according to degree of unsaturation by Ag^{+}-SEC (no

Table 1. Solvent Elution Scheme for the Resolution of FAME Derivatives Derived from Pine Nut Oils, According to the Double Bond Number, by Using a Bond Elut^{R}SCX Solid-Phase Extraction Column in the Silver Ion Mode

Fraction number	Solvent				Fatty acid fraction
	DCM	acetone (%)**	ACN*	volume (mL)	
1	100	—	—	5	saturated
2	90	10	—	5	monenes
3	—	100	—	5	dienes
4	—	97	3	10	dienes, trienes
5	—	94	6	10	trienes

*DCM; dichloromethane, ACN; acetonitrile, (%)**; % by volume

chromatogram shown), using stepwise elution of mobile-phases with increases of the ratio of acetone to DCM and then with ones of ACN into acetone (Table 2). All the fractions eluted were essentially pure, but the component of $\Delta^{5,9}$-$C_{18:2}$ was eluted much later than the usual dienoic acids with the MiDB structure such as $\Delta^{9,12}$-$C_{18:2}$ and $\Delta^{9,12}$-$C_{20:2}$, although they have the same number of double bonds as exhibited in Fig. 1-E; unusual NMiDB unsaturated fatty acids were held more strongly than their positional isomers with usual MiDB unsaturated fatty acids, in which an interaction between π-electron of double bond and silver ion was much stronger in the NMiDB unsaturated fatty acids than in the MiDB ones. Similar results have been reported by Niko-lova-Damyanova *et al.* (27). According to them the fatty acid isomers with two methylene groups between two double bonds were held most strongly of all the isomers, followed by those with three and then those with five.

3.2. Identification of the components in each fraction by GC-MS

A portion of the FAME derivatives in each of the fractions resolved by Ag^+-SEC was analyzed by GC for quantification of the fatty acid components (Fig. 1-A~F). The remainder was hydrolyzed into free fatty acids which were then derivatized into their corresponding picolinyl esters and DMOX derivatives prior to analysis by GC-MS.

Each of the picolinyl esters showed mass ion species at *m/z* 92 (base peak), 93 and 108 formed by cleavages of carbon-carbon bonds in the proximity of the pyridine ring of the molecule, as well as the mass ion species of *m/z* 151 produced by induction of a McLafferty rearrangement (39) as depicted in Fig. 2-A and that of *m/z* 164, which could be rationalized by formation of a conjugated species as illustrated in Fig. 2-B. There was a series of even-numbered mass ion species differing by regular gaps of 14 atomic mass unit (amu) from *m/z* 178 to $[M-15]^+$ ($[M]^+$; molecular mass ion) derived by radical-induced cleavage at each methylene group. While the DMOX derivatives of fatty acids had no abundant mass ion fragments below the base peak at *m/z* 113 which was formed by a McLafferty rearrangement (12), they all also showed odd-numbered molecular mass ion and a series of even-numbered mass ion species with an interval of 14 amu from *m/z* 126 to $[M-15]^+$, as was the case with the picolinyl esters.

Table 2. Fatty Acid Composition of the Total Oils of Pine Nuts

Fatty acid	weight %
$C_{14:0}$	trace*
$C_{16:0}$	4.8
$C_{16:1\omega7}$	0.2
$C_{17:0}$	0.1
$C_{18:0}$	2.0
$C_{18:1\omega9}$	25.4
$\Delta^{5,9}$-$C_{18:2}$	2.3
$C_{18:2\omega6}$	46.2
$\Delta^{5,9,12}$-$C_{18:3}$	16.0
$C_{18:3\omega3}$	0.2
$C_{20:0}$	0.3
$C_{20:1\omega9}$	0.9
$C_{20:1\omega7}$	0.2
$C_{20:2\omega6}$	0.5
$\Delta^{5,11,14}$-$C_{20:3}$	0.8
$C_{22:0}$	0.1

trace*; below 0.1%

The first fraction (Fr. 1) separated by elution of 100% DCM (5 mL) contained six saturated components. The first component in the fraction exhibited a molecular mass ion at

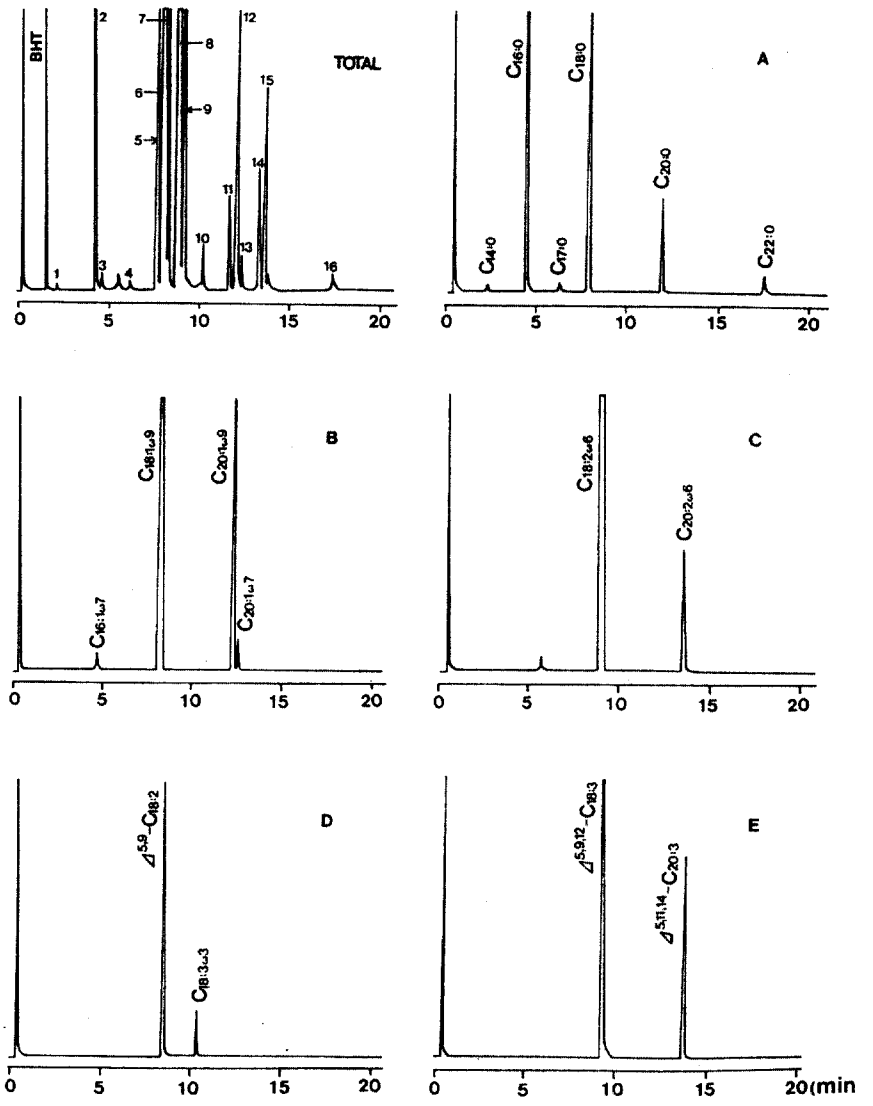

Fig. 1. GC analysis of fatty acid components in the fatty acid methyl ester fractions obtained by Ag^{+}-SEC from the seed oil of pine nuts.

A; 100% DCM fr., B; 90% DCM-10% Acetone fr., C; 100% Acetone,
D; 97% Acetone-3% ACN fr., E; 94% Acetone-6% ACN fr.

1. $C_{14:0}$, 2. $C_{16:0}$, 3. $C_{16:1\omega7}$, 4. $C_{17:0}$, 5. $C_{18:0}$, 6. $C_{18:1\omega9}$, 7. $\Delta^{5,9}$- $C_{18:2}$,
8. $C_{18:2\omega6}$, 9. $\Delta^{5,9,12}$-$C_{18:3}$, 10. $C_{18:3\omega3}$, 11. $C_{20:0}$, 12. $C_{20:1\omega9}$, 13. $C_{20:1\omega7}$,
14. $C_{20:2\omega6}$, 15. $\Delta^{5,11,14}$-$C_{20:3}$, 16. $C_{22:0}$

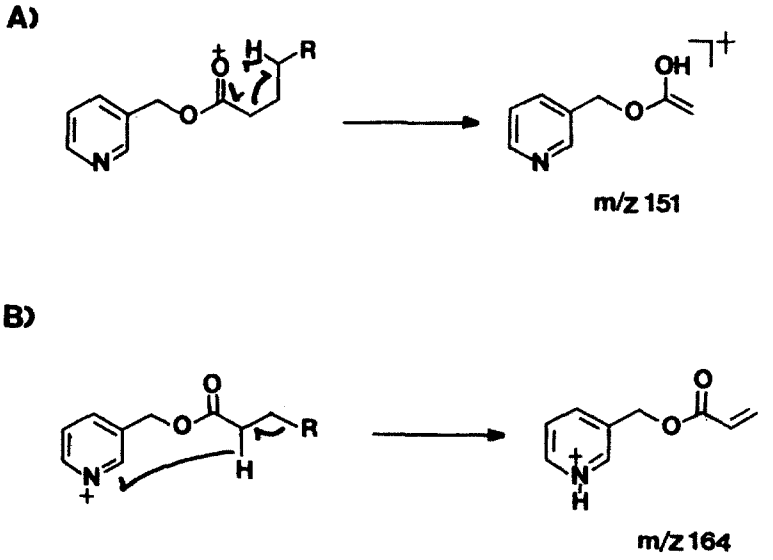

Fig. 2. Mechanism for the formation of the mass ion at *m/z* 151 by a McLafferty rearrangement (A) and the mass ion at *m/z* 164 (B) in the mass spectra of picolinyl esters

1*m/z* 319 (tetradecanoic acid, $C_{14:0}$), the mass ion species of *m/z* 92, 108 and 151 specific to picolinyl ester, and abundant mass ion species such as *m/z* 164, 178, 192, 206, 220, 234, 248, 262, 276, 290 and 304, which were separated by 14 amu from *m/z* 164 to *m/z* 304 $[M-15]^+$ in the picolinyl ester, although some of them were of low abundance. Whereas the DMOX derivative of this component had a molecular ion at *m/z* 281 (tetradecanoic acid, $C_{14:0}$) and the most intense mass ion species at *m/z* 113 as base peak. There were also prominent mass ion species of *m/z* 140, 154, 168, 182, 196, 210, 224, 238, 252 and 266 $[M-15]^+$, each of which increased by an interval of 14 amu. From these mass spectrometric results, this fatty acid was confirmed as myristic acid (*n*-tetradecanoic acid, $C_{14:0}$).

The picolinyl ester of the second component in Fr. 1 showed predominantly a molecular mass ion at *m/z* 347 (hexadecanoate, $C_{16:0}$) and a series of abundant mass ion species differing from each other by gaps of 14 amu from *m/z* 164 to 332 $[M-15]^+$; *m/z* 164, 178, 192, 206, 220, 234, 248, 262, 276, 290, 304, 318 and 332, as well as the mass ion species of *m/z* 92, 108 and 151 typical of the picolinyl esters of fatty acids. In addition, the DMOX derivative revealed a molecular mass ion at *m/z* 309 (hexadecanoate, $C_{16:0}$) and abundant mass ions such as *m/z* 140, 154, 168, 182, 196, 210, 224, 238, 252, 266, 280 and 294 with a difference of regular gaps of 14 amu from *m/z* 140 to 294 $[M-15]^+$. Thus, this fatty acid was easily confirmed as palmitic acid (*n*-hexadecanoic acid, $C_{16:0}$).

The third component in Fr. 1 presented a molecular mass ion at *m/z* 361 (heptadecanoate, $C_{17:0}$) and prominent mass ion species at *m/z* 164, 178, 192, 206, 220, 234, 248, 262, 276, 290, 304, 318, 332 and 346 $[M-15]^+$. While the DMOX derivative of the component also revealed a molecular mass ion at *m/z* 323 (heptadecanoate, $C_{17:0}$) and other prominent mass ion species at *m/z* 126, 140, 154, 168, 182, 196, 210, 224, 238, 252, 266, 280, 294 and 308 $[M-15]^+$. There were regular intervals of 14 amu from *m/z* 164 to 346 for

the picolinyl ester and those from *m/z* 126 to *m/z* 308 for the DMOX derivative. In this way, this fatty acid could be assigned to margaric acid (*n*-heptadecanoate, $C_{17:0}$).

The fourth component in Fr. 1 gave a molecular mass ion at *m/z* 375 (octadecanoate, $C_{18:0}$) and strong mass ion species such as *m/z* 164, 178, 192, 206, 220, 234, 248, 262, 276, 290, 304, 318, 332, 346 and 360 $[M-15]^+$ in the picolinyl ester as shown in Fig. 3-A, while the DMOX derivative showed a molecular mass ion at *m/z* 337 and many abundant mass ion species such as *m/z* 140, 154, 168, 182, 196, 210, 224, 238, 252, 266, 280, 294, 308 and 322 $[M-15]^+$ as illustrated in Fig. 3-B. From these results this component proved to be stearic acid (*n*-octadecanoic acid, $C_{18:0}$).

The fifth component in Fr. 1 showed a molecular mass ion at *m/z* 403 (eicosanoate, $C_{20:0}$) and a series of intense mass ion species separated by a series of gaps of 14 amu from *m/z* 164 to 388 $[M-15]^+$ in the picolinyl ester. And the DMOX derivative of the component also exhibited a molecular mass ion at *m/z* 365 (eicosanoate, $C_{20:0}$) and abundant mass ion species with intervals of 14 amu from *m/z* 140 to *m/z* 350 $[M-15]^+$ as experienced in the saturated fatty acids described above. While the sixth component was a small one and had a molecular mass ion species at *m/z* 431 (docosanoate, $C_{22:0}$) and abundant mass ion species spaced out at gaps of 14 amu from *m/z* 164 to *m/z* 416 $[M-15]^+$ in the picolinyl ester. The DMOX derivative revealed a molecular mass ion at *m/z* 393 (docosanoate, $C_{22:0}$) and a series of abundant mass ion species separated by 14 amu from *m/z* 140 to *m/z* 378 $[M-15]^+$. Thus, the components of the fifth and sixth were identified as arachidic acid (*n*-eicosanoic acid, $C_{20:0}$) and behenic acid (*n*-docasanoic acid, $C_{22:0}$), respectively.

Both the picolinyl esters and the DMOX derivatives of saturated fatty acids provided diagnostic mass ion species, but it should be noted that the mass ion of $[M-15]^+$ stood in a striking contrast in its abundance to the picolinyl ester and the DMOX derivative; the mass ion $[M-15]^+$ was less abundant than its neighboring mass ion species in the picolinyl ester (*e. g.*, *m/z* 360 for $C_{18:0}$ in Fig. 3-A), but it was much more abundant than other ones including molecular mass ions $[M]^+$ in the DMOX derivative (*e. g.*, *m/z* 322 for $C_{18:0}$ in Fig. 3-B).

The second fraction (Fr. 2) eluted by DCM-acetone (90 : 10, v/v, 5 mL) was a major one and showed three monoenoic acids as represented in Fig. 1-C. From their elution times on the Ag^+-column (no data shown) they seemed to be in the *cis*-configurational form; if monoenoic acids have *trans*-configurational double bond systems, they have to be eluted in the saturated fatty acid fraction (8, 27, 28).

The picolinyl ester of the first component in Fr. 2 (Fig. 1-B) provided a prominent molecular ion at *m/z* 345 (hexadecamonoenoic acid, $C_{16:1}$) and mass ion species such as *m/z* 206, 220, 234, 260, 274, 288, 302, 316 and 330 separated by gaps of 14 amu from *m/z* 260 to $[M-15]^+$ (*m/z* 330). There was a diagnostic gap of 40 amu (as opposed to a gap of 42 amu for saturated acids) between *m/z* 220 (C-7) and 260 (C-10). While the DMOX derivative also showed a molecular mass ion at *m/z* 307 $[M^+]$ (hexadecamonoenoic acid, $C_{16:1}$) and abundant mass ion species such as *m/z* 182, 196, 208, 222, 236 250, 278 and 292, there was a gap of 12 amu between *m/z* 196 (C-8) and 208 (C-9) as well as a gap of 40 amu between *m/z* 182 (C-7) and 222 (C-10) (or 26 amu between *m/z* 196 and 222). These results indicated the presence of a double bond between C-9 and C-10 in the molecule. Thus, this fatty acid was palmitoleic acid (*cis*-Δ^9-hexadecamonoenoic acid, *cis*-$C_{16:1\omega7}$).

The second component in Fr. 2 upon electron impact gave a prominent molecular ion at *m/z* 373 (octadecamonoenoic acid, $C_{18:1}$)

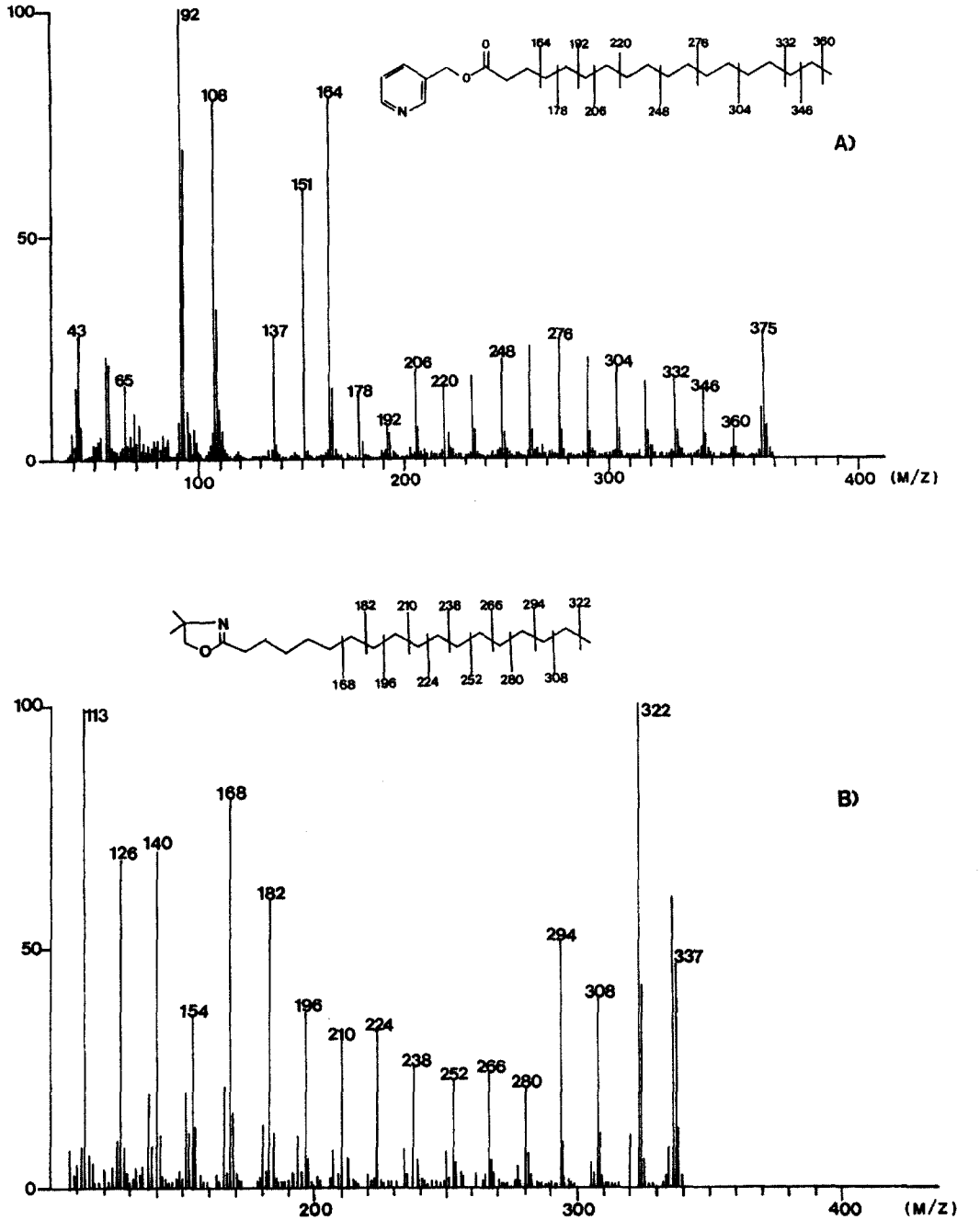

Fig. 3. Mass spectrum of the picolinyl ster (A) and the DMOX derivative (B) of $C_{18:0}$ (stearic acid)

and mass ion species at *m/z* 260, 274, 288, 302, 316, 330 and 358 differing by gaps of 14 amu from *m/z* 260 to $[M-15]^+$ (*m/z* 358), which indicated the absence of double bonds at the sites of C-10 to C-17 in the chain. In addition, a gap of 40 amu (as opposed to a gap of 42 amu for saturated acids) between *m/z* 220 (C-7) and 260 (C-10) was diagnostic for locating a double bond between C-9 and C-10, although a gap of 26 amu between *m/z* 234 and 260 instead of 40 amu was more instructive for this purpose, as shown in Fig. 4-A. Picolinyl esters of monoenoic acids produced more abundant molecular mass ions $[M^+]$ (*e. g., m/z* 373 for $C_{18:1}$, in Fig. 4-A) than those of saturated fatty acids (*e. g., m/z* 375 for $C_{18:0}$, in Fig. 3-A). In general, picolinyl esters of unsaturated fatty acids have been known to have distinct cleavages at either side of a given double bond; for example, when a double bond occurs between the '**n**'th and '**n+1**'th carbon in the molecule of a fatty acid, diagnostic mass ion species formed by cleavages between the '**n-2**'th and '**n-1**'th carbon, and the '**n-1**'th and '**n**'th carbon, and the '**n-1**'th and '**n+1**'th carbon can be predominantly produced (2, 7).

While the DMOX derivative of this component showed a molecular mass ion at *m/z* 335 $[M^+]$ (octadecamonoenoic acid, $C_{18:1}$) and other intense mass ion species such as *m/z* 182, 196, 208, 222, 236 250, 278, 292 and 320, there were diagnostic gaps of 12 amu between *m/z* 196 and 208, and 40 amu between *m/z* 182 and 222 (or 26 amu between *m/z* 196 and 222) as represented in Fig. 4-B. In particular, a gap of 12 amu between *m/z* 196 (C-8) and 208 (C-9) provided more decisive evidence of the presence of a double bond at the site between C-9 and C-10 because DMOX derivatives of unsaturated fatty acids predominantly revealed a gap of 12 amu induced by splitting between the '**n-1**'th and '**n**'th carbon when a double bond is positioned at the '**n**'th and '**n+1**'th carbon in the chain (19). Although this diagnostic gap does not systematically apply to the mass spectra of picolinyl esters, it provides more accurate information on the location of a double bond than does a gap of 26 amu or 40 amu in the spectra of DMOX derivatives of unsaturated fatty acids (11, 12, 19). Thus, this fatty acid was oleic acid (*cis*-Δ^9-octadecamonoenoic acid, *cis*-$C_{18:1\omega9}$).

The third and fourth components in Fr. 2 both had the same molecular mass ion, but they gave a different splitting feature from each other in the mass spectra of picolinyl esters as well as DMOX derivatives; the third component showed a molecular mass ion at *m/z* 401 (eicosamonoenoic acid, $C_{20:1}$) and abundant diagnostic mass ion species such as *m/z* 206, 220, 234, 248, 262, 288, 302, 316 and 358 in the picolinyl ester. There was a distinct gap of 40 amu between *m/z* 248 (C-9) and 288 (C-12) [or 26 amu between *m/z* 262 (C-10) and 288 (C-12)] in the chain. While the DMOX derivative gave a molecular ion at *m/z* 363 and abundant mass ion species at *m/z* 210, 224, 236, 250, 264, 278, 292 and 348, a diagnostic gap of 12 amu between *m/z* 224 (C-10) and 236 (C-11) indicated the presence of a double bond between C-11 and C-12, which was supported by an interval of 40 amu between *m/z* 210 (C-9) and 250 (C-12) [or 26 amu between *m/z* 224 (C-10) and 250 (C-12)], Thus, this component was confirmed as *cis*-Δ^{11}-$C_{20:1}$ ($C_{20:1\omega9}$).

On the other hand, the fourth component revealed a molecular mass ion at *m/z* 401 (eicosamonoenoic acid, $C_{20:1}$) and diagnostic mass ion species in the picolinyl ester: *m/z* 206, 220, 234, 260, 274, 290, 302, 316, 330 and 344. There was a prominent gap of 40 amu between *m/z* 276 (C-11) and 316 (C-14) [or 26 amu between *m/z* 290 (C-12) and 316 (C-14)], suggesting the position of a double bond between C-13 and C-14. Furthermore, the DMOX derivative gave a molecular mass ion at *m/z* 363 (eicosamonoenoic acid, $C_{20:1}$)

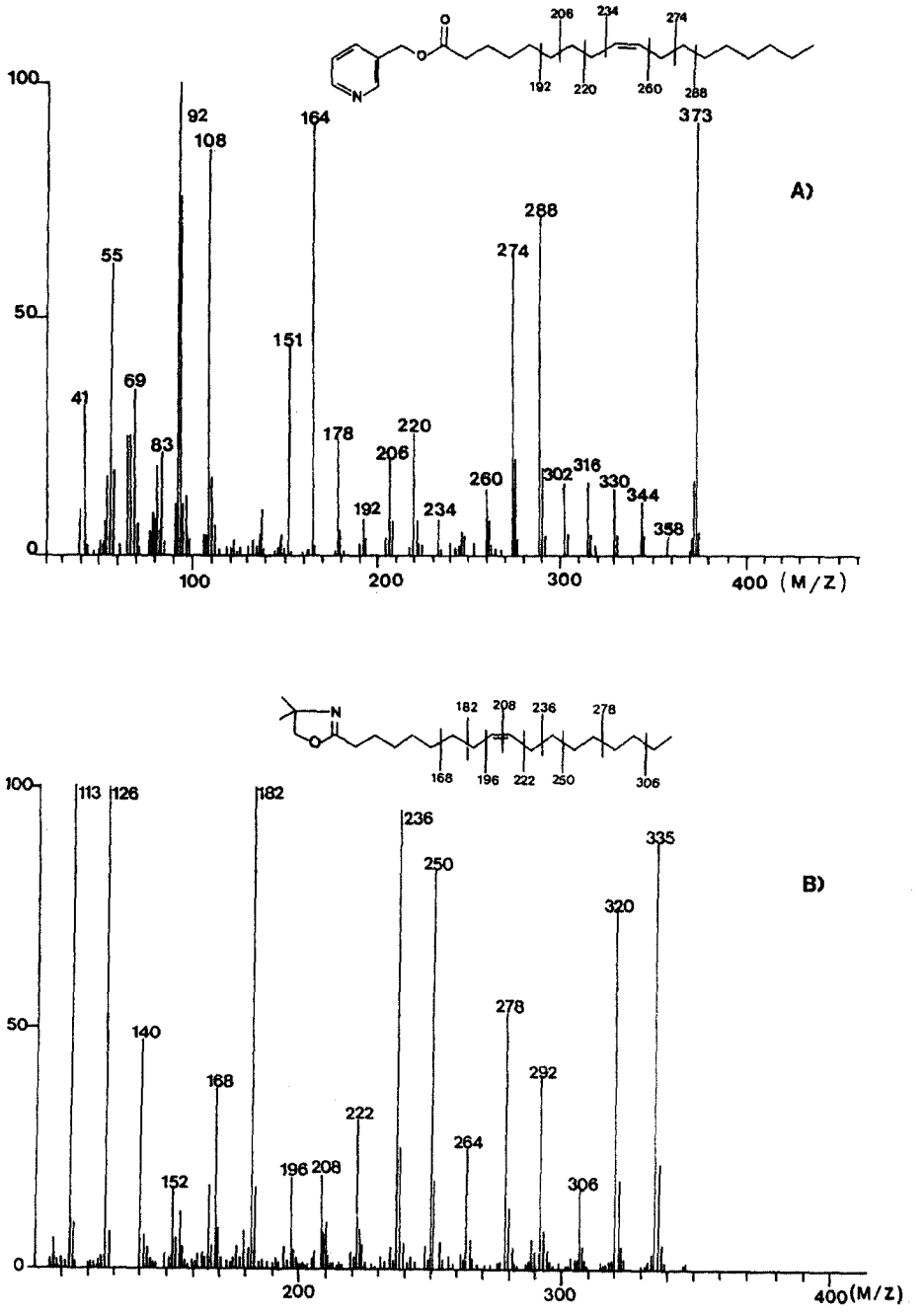

Fig. 4. Mass spectrum of the picolinyl ester (A) and the DMOX derivative (B) of Δ^9-$C_{18:1}$ ($C_{18:1}$ ω9, oleic acid)

as well and diagnostic mass ion species such as *m/z* 238, 252, 278, 264, 292, 306 and 348. A gap of 12 amu between *m/z* 252 (C-12) and 264 (C-13) served a guide to finding the position of a double bond between C-13 and C-14 in the molecule, which was again manisfested by a gap of 40 amu between *m/z* 238 (C-11) and 278 (C-14) [or 26 amu between *m/z* 252 (C-12) and 278 (C-14)]. Therefore, this fatty acid could be confirmed as *cis*-Δ^{13}-$C_{20:1}$ ($C_{20:1\omega7}$).

The third fraction (Fr. 3) separated with 100% acetone (5 mL) was a large one and contained three components (Fig. 1-C). The first component in this fraction exhibited a molecular ion at *m/z* 371 $[M]^+$ (octadecadienoic acid, $C_{18:2}$) and other intense mass ion species such as *m/z* 206, 220, 234, 260, 274, 300, 314 and 328 in the picolinyl ester as depicted in Fig. 5-A. There were regular gaps of 40 amu between *m/z* 220 (C-7) and 260 (C-10), and *m/z* 260 (C-10) and 300 (C-13) [or gaps of 26 amu between *m/z* 234 (C-8) and 260 (C-10), and *m/z* 274 (C-11) and 300 (C-13)]. Additionally, the DMOX derivative represented a molecular mass ion at *m/z* 333 $[M]^+$ (octadecadiencic acid, $C_{18:2}$) and abundant mass ion species as follows: *m/z* 196, 208, 222, 236, 248, 262, 276 and 290 as shown in Fig. 5-B. By checking the gaps of 12 amu between *m/z* 196 (C-8) and 208 (C-9), and *m/z* 236 (C-11) and 248 (C-12), the positions of two double bonds were easily determined at the sites between C-9 and C-10, and C-12 and C-13, respectively. In this way, this fatty acid was identified as $\Delta^{9,\ 12}$-$C_{18:2}$ ($C_{18:2\omega6}$).

While the second component in Fr. 3 had a molecular ion at *m/z* 399 $[M]^+$ (eicosadienoic acid, $C_{20:2}$) and diagnostic mass ion species such as *m/z* 234, 248, 262, 288, 302, 328 and 342 in the picolinyl ester, there were a series of regular gaps of 40 amu between *m/z* 248 (C-9) and 288 (C-12) [or 26 amu between *m/z* 262 (C-10) and 288 (C-12)], and between *m/z* 288 (C-12) and 328 (C-15) [or 26 amu between *m/z* 302 (C-13) and 328 (C-15)]. The DMOX derivative again showed a molecular ion species at *m/z* 361 $[M]^+$ (eicosadienoic acid, $C_{20:2}$) and intense mass ion species of *m/z* 210, 224, 236, 250, 264, 276, 290, 304, 318 and 346. A series of diagnostic gaps of 12 amu between *m/z* 224 (C-10) and 236 (C-11), and *m/z* 264 (C-13) and 276 (C-14) were easily checked, along with gaps of 40 amu between *m/z* 210 (C-9) and 250 (C-12), and *m/z* 250 (C-12) and 290 (C-15). These mass spectrometric results proved the component to be *cis*-11, 14-$C_{20:2}$ ($C_{20:2\omega6}$).

The fourth fraction (Fr. 4) was isolated by elution of a mixture of acetoneACN (97 : 3, v/v, 10 mL) and was again a minor one and contained two components (Fig. 1-D). They seemed to be in *cis*-cofigurational form from their elution patterns on Ag^+-SEC. The first component in Fr. 4 had a molecular mass ion at *m/z* 371 $[M^+]$ (octadecadienoic acid, $C_{18:2}$) and a series of prominent diagnostic mass ion species such as *m/z* 164, 178, 219 and 272 in the picolinyl ester, as seen in Fig. 6-A. An odd-numbered mass ion species of *m/z* 219 formed by cleavage at the center site of two methylene groups located between two double bonds is specific to $\Delta^{5,\ 9}$-unsaturated fatty acids and rarely found in the picolinyl esters of the usual fatty acids with MiDB structure (30). Although some (*e. g.*, *m/z* 204 and 258) of diagnostic mass ion species produced by splitting at either side of the double bonds were of very low intensity, intervals of 40 amu between *m/z* 164 and 204 (very small) and between *m/z* 219 ($218+H^+$) and 258 (very small) were be recognizable and then made it possible to assign two double bonds to the sites between C-5 and C-6, and C-9 and C-10. On the other hand, the DMOX derivative revealed a molecular mass ion at *m/z* 333 $[M^+]$ (octadecadienoic acid, $C_{18:2}$) and diagnostic mass ion species such as *m/z* 140, 153, 166, 180, 194, 206, 220, 234 and 318, as well as a mass ion species of *m/z* 153

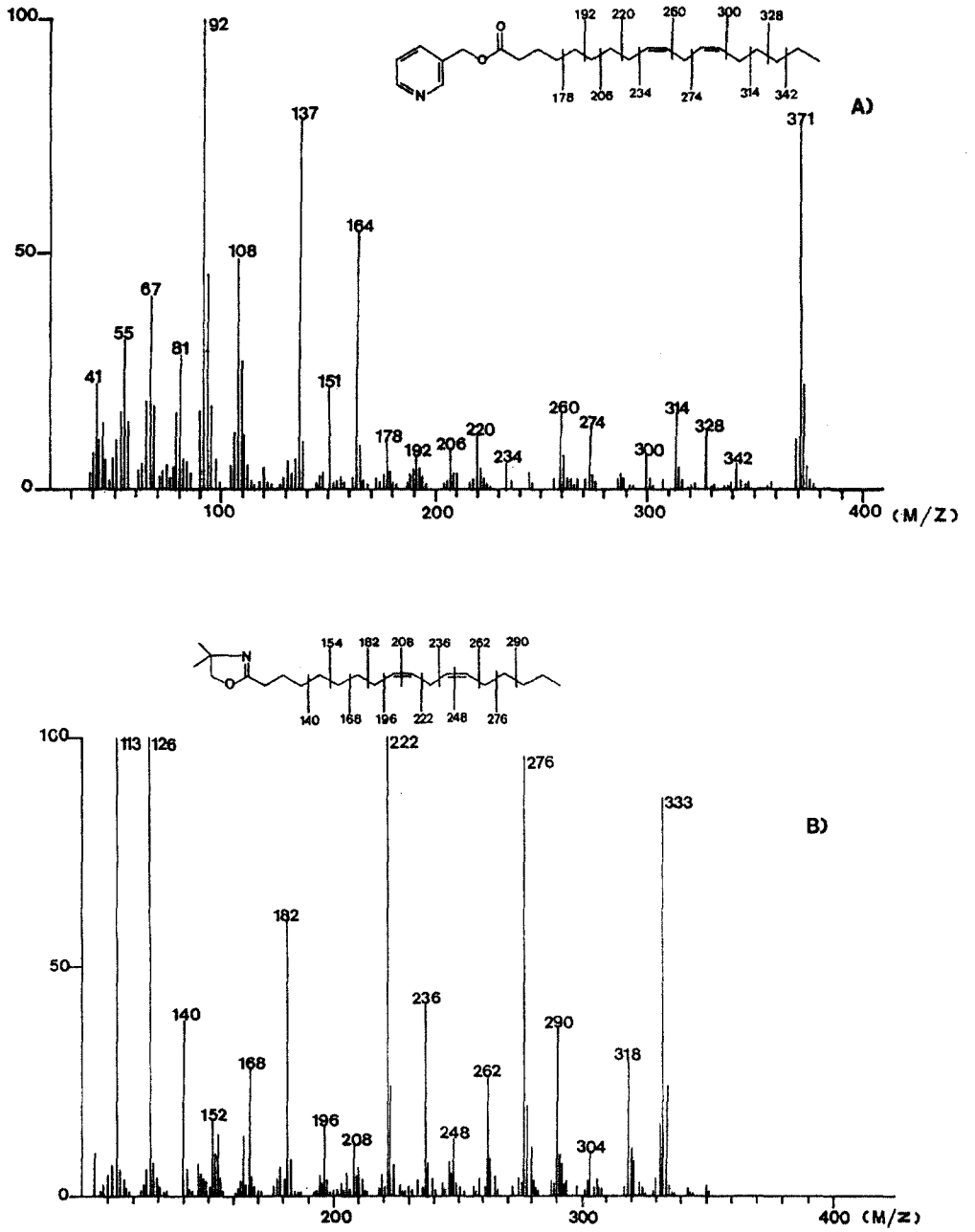

Fig. 5. Mass spectrum of the picolinyl ester (A) and the DMOX derivative (B) of $\Delta^{9,12}$-$C_{18:2}$ ($C_{18:2\omega6}$, linoleic acid)

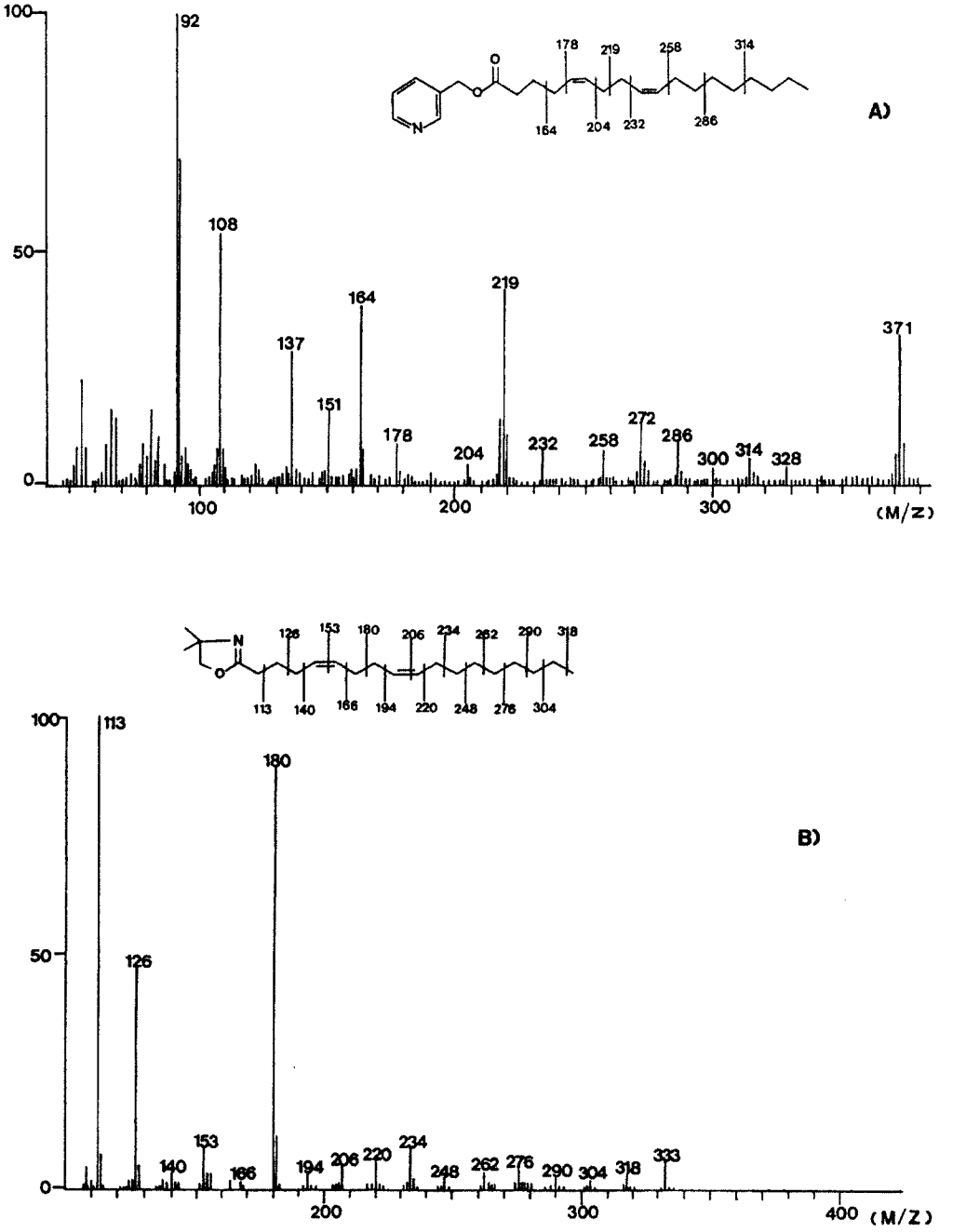

Fig. 6. Mass spectrum of the picolinyl ester (A) and the DMOX derivative (B) of $\Delta^{5,9}$-$C_{18:2}$ (taxoleic acid)

specific to $\Delta^{5,9}$-unsaturated fatty acid (37, 40), as illustrated in Fig. 6-B. Gaps of 12 amu between *m/z* 140 (C-4) and 153 (152+H$^+$) (C-5) and *m/z* 194 (C-8) and 206 (C-9) indicated the presence of two double bonds between C-5 and C-6, and C-9 and C-10 in the chain. This assignment was also supported by gaps of 40 amu between *m/z* 126 (C-3) and 166 (C-6) and between *m/z* 180 (C-7) and 220 (C-10) (or 26 amu between *m/z* 140 and 166, and *m/z* 194 and 220). Thus, this fatty acid was confirmed as $\Delta^{5,9}$-$C_{18:2}$ (taxoleic acid).

While the second component in Fr. 4 showed a molecular mass ion at *m/z* 369 (octadecatrienoic acid) and abundant mass ion species: *m/z* 164, 178, 206, 220, 260, 274, 300, 314 and 340 as shown in Fig. 7-A. There was a series of gaps differing by 40 amu between *m/z* 220 and 260, *m/z* 260 and 300, and *m/z* 300 and 340, which suggested the presence of three double bonds between C-9 and C-10, C-12 and C-13, and C-15 and C-16. Furthermore, the DMOX derivative also gave a molecular ion at *m/z* 331 [M$^+$] corresponding to octadecatrienoic acid ($C_{18:3}$) and intense mass ions such as *m/z* 196, 208, 222, 236, 248, 262, 276, 288, 302 and 316 as represented in Fig. 7-B. By detecting regular gaps of 12 amu between *m/z* 196 (C-8) and 208 (C-9), *m/z* 236 (C-11) and 248 (C-12), and *m/z* 276 (C-13) and 288 (C-14), the positions of three double bonds were easily determined between C-9 and C-10, C-12 and C-13, and C-15 and C-16, respectively. From the results, this component was confirmed as $\Delta^{9,12,15}$-$C_{18:3}$ ($C_{18:3\omega3}$).

The final fraction (Fr. 5) showed two unknown polyunsaturated fatty acids on the GC profile as shown in Fig. 1-E. The first component revealed a strong molecular mass ion species at *m/z* 369 [M$^+$] (octadecatrienoic acid, $C_{18:3}$) and abundant mass ion species such as *m/z* 164, 178, 232, 258, 272, 298, 312 and 326 including a diagnostic mass ion of *m/z* 219 specific to $\Delta^{5,9}$-double bond system (30) in the picolinyl ester (Fig. 8-A). There was a prominent gap of 40 amu between *m/z* 219 [218+H] and 258, and between *m/z* 258 and 298, indicating the presence of two double bonds at the sites between C-9 and C-10, and C-12 and C-13, respectively. However, since there was a cluster of neighboring mass ion species of similar intensity around the diagnostic ones of *m/z* 204, it was difficult to select correctly a gap of 40 amu among mass ion species formed by cleavages of carbon-carbon bonds in the proximal double bond. We might mistakenly choose the gap between *m/z* 178 (C-4) and 219 (218+H$^+$) (C-7) instead of that between *m/z* 164 (C-3) and 204 (C-6), which was hardly recognizable owing to its low abundance, whereas the DMOX derivative showed a molecular mass ion species at *m/z* 331 [M$^+$] (octadecatrienoic acid, $C_{18:3}$) which was of low abundance compared to that of the picolinyl ester, and other diagnostic ions at *m/z* 140, 153, 194, 206, 234 and 246 (Fig. 8-B). Positions of double bonds were easily found by checking gaps of 12 amu and/or 26 amu, although the diagnostic ions (*m/z* 140) formed by splitting of the double bond closer to the carboxyl radical were low in abundance. From the results of both spectra, this fatty acid could be assigned to $\Delta^{5,9,12}$-$C_{18:3}$ (pinolenic acid).

The second component of the last fraction showed a molecular ion at m/z 397 (eicosatrienoic acid, $C_{20:3}$) and intense mass ion species such as *m/z* 164, 178, 218, 232, 246, 286, 300, 340 and 358 in the picolinyl ester (Fig. 9-A). Gaps of 40 amu between *m/z* 246 and 286 and between *m/z* 286 (C-12) and 326 (C-15) were checked with ease, so the positions of double bonds were determined between C-11 and C-12, and C-14 and C-15. However, a double bond in the proximal to the carboxyl group could be not easily located because there were no diagnostic mass ion species between *m/z* 178 and 218. On the contrary, the DMOX

derivative also produced a molecular ion (m/z 359) corresponding to eicosatrienoic acid ($C_{20:3}$) and many intense mass ion species as follows: *m/z* 140, 153, 180, 208, 222, 234, 248, 262, 274, 302, 316 and 344 (Fig. 9-B). Intervals of 12 amu between *m/z* 140 and 153 (152+H^+), *m/z* 222 and 234, and *m/z* 262 and 274 suggested the presence of a double bond at the sites between C-5 and C-6, and C-11 and C-12, and C-14 and C-15. Thus, this fatty acid could be ascribed to sciadnoic acid ($\Delta^{5,\ 11,\ 14}$-$C_{20:3}$).

Even though a fatty acid component occurs at concentration as low as 0.05% in the mixture of over 34~40 fatty acids in a single species, the mass spectra of its picolinyl ester and DMOX derivative are all powerful enough to elucidate its molecular structure with much greater ease than has been possible with pyrrolidide (1, 7) and alkylbenzoxazole derivatives (4).

However, it is allegedly often difficult to identify MiDB polyunsaturated fatty acids with more than three double bonds occurring in nature by reading the mass spectra of their picolinyl esters; *i. e.*, the diagnostic intervals of 40 amu (or 26 amu) for finding the positions of double bonds may be easily selected out because the relevant mass ion species may occur as a cluster of neighboring mass ion species of similar intensity (41). In particular, the proximal double bonds closer to the carboxyl group are difficult to locate in the molecule of a polyunsaturated fatty acid with MiDB structure present in mammalian and fish oils (41), but knowing the total number of double bonds (from the molecular ion which is always prominent) and the positions of the distal double bonds allows one to deduce the positions of unassigned double bonds.

While the picolinyl esters of polyunsaturated fatty acids with NMiDB radical also give abundant molecular mass ions and distinctive diagnostic mass ion species, some diagnostic mass fragments formed by cleavages at both sides of double bonds in the proximal and/or the distal to the carboxyl group are often hardly appreciated due to their low abundance as well. For instance, the picolinyl ester of $\Delta^{5,\ 11,\ 15}$-$C_{20:3}$ (sciadonic acid) represents diagnostic mass ions at *m/z* 164, 178, 218, 232, 246, 286, 300 and 340, as well as a molecular mass ion at *m/z* 397 (Fig. 9-A). And intense gaps of 40 amu between *m/z* 246 and 286 and between *m/z* 286 and 326 verify the positions of two double bonds between C-11 and C-12, and C-14 and C-15, but picking up a gap of 40 amu (or 26 amu) among the rest of the diagnostic mass ion species requires a closer examination; if a gap of 40 amu between *m/z* 178 and 218 instead of between *m/z* 164 and 204 (not checked) is chosen, one may made a mistake in trying to find the correct position of the rest double bond. A gap of 40 amu between the '**n-2**'th and '**n+1**'th carbon (or gaps of 26 amu between the '**n-1**'th and '**n+1**'th carbon) is thought to be a guide requisite for locating a double bond between the '**n**'th and '**n+1**'th carbon in the molecule of MiDB fatty acid (2, 7), but caution must be taken to apply this principle to finding double bonds in NMiDB fatty acids.

On the other hand, the mass spectra of DMOX derivatives of fatty acids with three or more double bonds are relatively free of the groups of mass ions which often interfere with diagnostic mass ion species and make the corresponding picolinyl esters difficult to interpret. They are usually easy to interpret by checking gaps of 12 amu for the position of double bonds (12, 14, 15, 19). Taking pinolenic acid ($\Delta^{5,\ 9,\ 12}$-$C_{18:3}$, Fig. 8-B) as an example, the molecular ion at *m/z* 331 (it has an intensity as low as its neighboring mass ion species) indicates a C_{18} fatty acid with three double bonds. The intervals of 12 amu between *m/z* 140 and 153 (*m/z* 152+H), between 194 and 206, and between 234 and 246, as well as gaps of 40 amu between *m/z* 126 and 146, between *m/z* 208 and 248, and

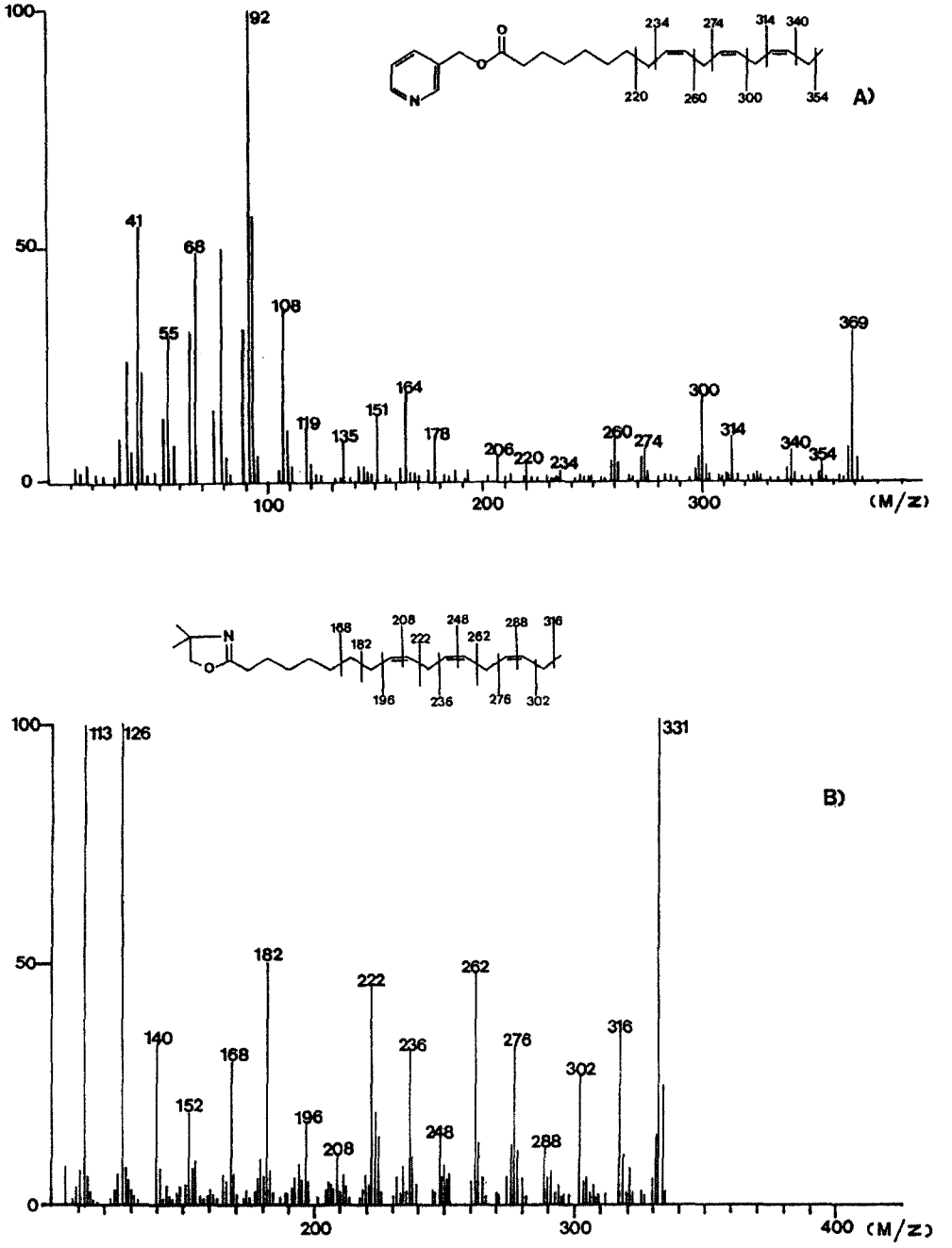

Fig. 7. Mass pectrum of the picolinyl ester (A) and the DMOX derivative (B) of $\Delta^{9, 12, 15}$-$C_{18:3}$ ($C_{18:3\omega3}$, α-linolenic acid)

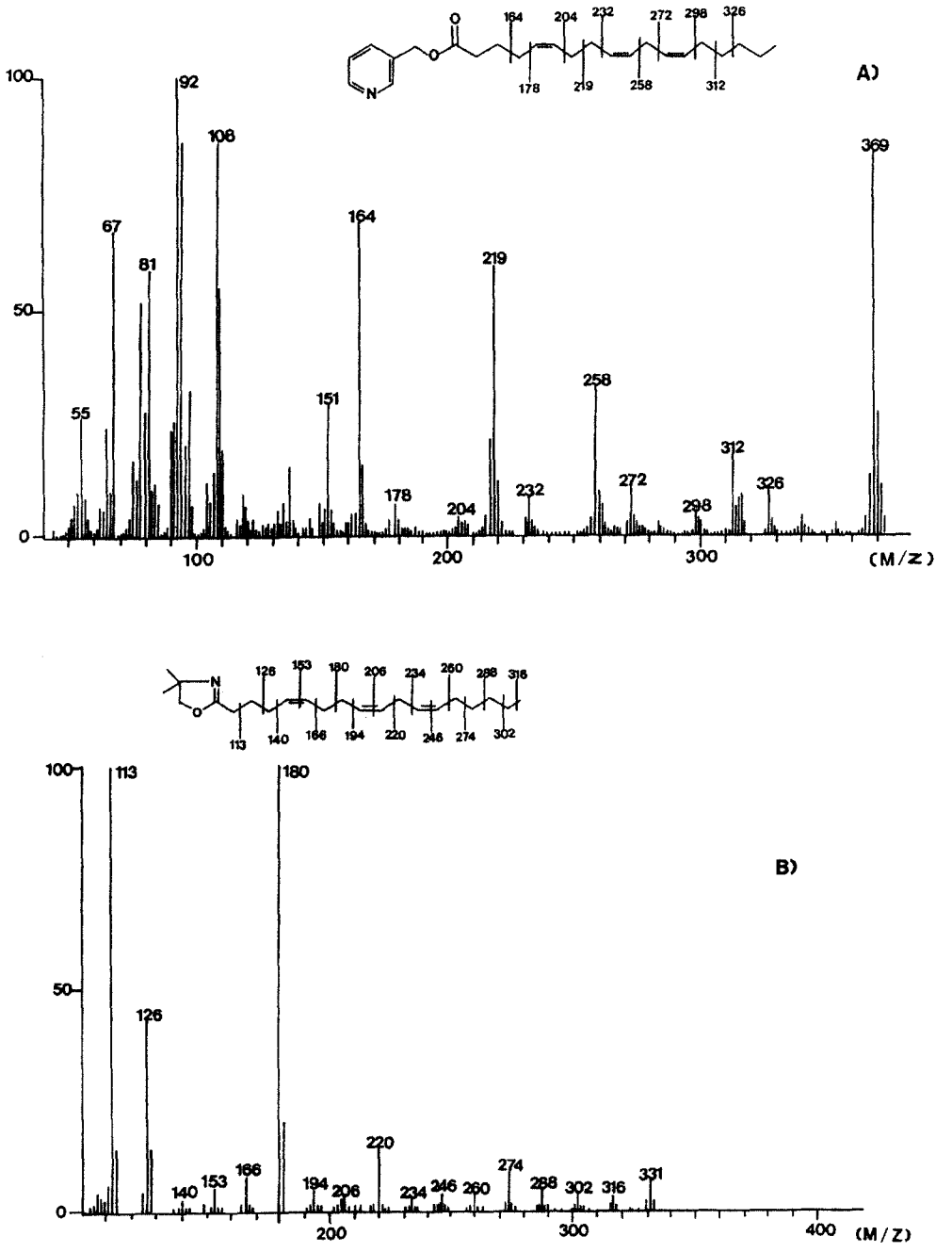

Fig. 8. Mass spectrum of the picolinyl ester (A) and the DMOX derivative (B) of $\Delta^{5,9,12}$-$C_{18:3}$ (pinolenic acid)

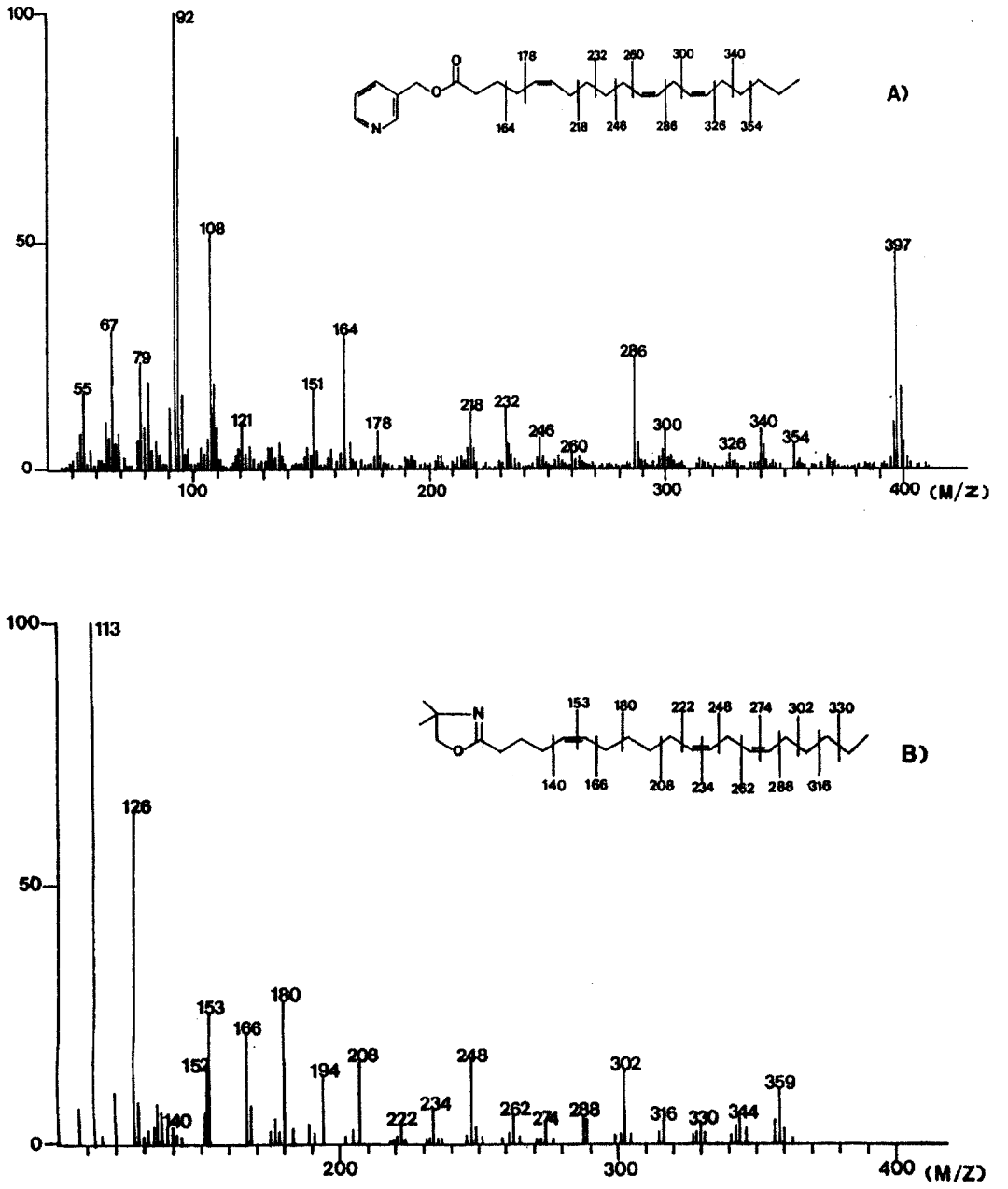

Fig. 9. Mass spectrum of the picolinyl ester (A) and the DMOX derivative (B) of $\Delta^{5,\ 11,\ 14}$-$C_{20:3}$ (sciadonic acid)

between *m/z* 248 and 288, are easily checked, although the gap found in the proximity of the carboxyl group is slightly confusing owing to the low abundance of the mass ion speceis of *m/z* 140 and the appearance of an oddnumbered mass ion species at *m/z* 153.

Both picolinyl esters and DMOX derivatives were complementary to each other and enabled to acquire useful information on locating double bonds of polyunsaturated fatty acids with MiDB and NMiDB groups by GC-MS. Virtually, their mass spectra represent equally distinctive and easily interpretable diagnostic mass ion species for many types of fatty acids and bear great resemblance to each other in some respects. However, DMOX derivatives seem to provide more informative mass spectra for locating double bonds in polyunsaturated fatty acids having NMiDB structure, although picolinyl esters of saturated to trienoic fatty acids with MiDB radical give also prominent diagnostic mass spectra, as indicated in the fatty acids with conjugated double bonds (42), cyclopropyl (2, 6) and branched methyl radicals (43).

DMOX derivatives can be obtained by a much simpler process, but require very rigorous conditions (6 hr at 180℃) than picolinyl esters (10 min at 37℃) in which the high temperature may cause the components of interest, especially polyunsaturated fatty acids, to decrease in stability or lose their molecular structures. However, DMOX derivatives volatilize at just 10℃ higher temperature than methyl esters and then show chromatographic feature comparable to that of methyl esters in less polar columns (11). Picolinyl ester generally require a boiling temperature of about 50℃ higher than methyl esters (2), so they are not suitable for analyzing polyunsaturated fatty acids with longer chains by GC. This is one of the reasons why DMOX derivatives are more popular with lipid analysts.

4. CONCLUSIONS

Methyl ester derivatives of fatty acids from the oils of pine nuts were well resolved to saturated, monoenes, dienes, trienes with MiDB group, and trienes with NMiDB group by silver ion solid-phase extraction column chromatography (Ag^+-SEC). NMiDB structural polyunsaturated fatty acid held more strongly to silver ions in the column than MiDB one when they had the same number of double bond. A technique of GC-MS combined with Ag^+-SEC made it possible to identify minor fatty acid components with very low abundance below 0.1 % to the total composition. Although both the picolinyl ester and DMOX derivative provided mass spectra powerful enough to elucidate the structure of parent polyunsaturated fatty acid (PUFA) with NMiDB and/or MiDB radical in the oils, the picolinyl ester of PUFA with NMiDB radical did not provide clearly diagnostic mass ion species induced by the double bond in the proximal to the carboxyl group. Whereas the DMOX derivative of PUFA with NMiDB group including MiDB showed abundant mass ion species differing by gaps of 12 amu, which provided us with more accurate information on locating the double bonds in the molecule with greater ease. The fatty acid composition of the oil were mainly composed of $C_{18:2\omega6}$ (46.2 %), followed by $C_{18:1\omega9}$ (25.4 %) and $\Delta^{5,9,12}$-$C_{18:3}$ (16.0 %). Level of PUFAs with NMiDB radical in the oil approximately amounted to 20 % ($\Delta^{5,9,12}$-$C_{18:3}$; 16.0 %, $\Delta^{5,9}$-$C_{18:2}$; 2.3 %, $\Delta^{5,11,14}$-$C_{20:3}$; 0.8 %).

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