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Purification and gas chromatographic–mass spectrometric characterization of non-methylene interrupted fatty acid incorporated in rat liver

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Abstract

A C20 non-methylene interrupted trienoic acid detected in the liver of rat fed with a pine (*Pinus koraiensis*) seed oil diet was purified by two-step argentation thin-layer chromatography (AgTLC) and characterized by gas chromatography–mass spectrometry (GC–MS). First, a C20 methyl trienoate fraction was obtained from fatty acid methyl esters prepared from rat liver by 5% AgTLC developed with petroleum ether–diethyl ether–acetic acid (70:20:2, v/v) as a solvent system. The fraction was then subjected to AgTLC developed with benzene–acetone–diethyl ether–acetic acid (65:15:15:5, v/v) which could separate non-methylene interrupted fatty acids (NMIFA) from usual MIFAs. The purified C20 NMIFA was partially hydrogenated, and the resulting three kinds of the C20 monoenoate were analyzed by GC–MS after conversion to their dimethyl disulfide (DMDS) adducts. The results revealed that the original C20 non-methylene interrupted trienoic acid detected in the liver of rats fed with a pine seed oil diet was Δ-5,11,14/20:3, a minor component of pine seed oil. © 1997 Elsevier Science B.V.

Keywords: Pine seed oil; Fatty acids, non-methylene interrupted; Non-methylene interrupted fatty acids

1. Introduction

Some plants contain non-methylene interrupted fatty acids (NMIFAs) [1–3]. Several investigators have reported the effects of dietary NMIFAs or NMIFAs-containing plant oil on the lipid metabolism of experimental animals [1,4,5]. Pine seed oil which contains pinolenic acid (all-*cis*-Δ-5,9,12/18:3) has been shown to attenuate the elevation of blood pressure of spontaneously hypertensive rats [4]. Dietary columbinic acid (*trans,cis,cis*-Δ-5,9,12/18:3) improves the symptoms of essential fatty acid-deficiency in the rat [1]. Berger and German have demonstrated that the dietary podocarpic acid (all-*cis*-Δ-5,11,14/20:3) can replace arachidonic acid in the hepatic phosphatidylinositol (PI) of rats [5].

To understand the mechanisms of the biomedically beneficial and unique property of NMIFAs, it is necessary to clarify the metabolic fate of NMIFA in cells with in vivo and in vitro experiments. To this end, a separation of NMIFAs from usual methylene interrupted fatty acids (MIFA) in tissues is an important problem, especially in the case of structur-

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al elucidation by gas chromatography–mass spectrometry (GC–MS).

Argentation thin-layer chromatography (AgTLC) has been used to separate fatty acid methyl esters according to their degree of unsaturation. It has been reported that AgTLC and silver ion high-performance liquid chromatography techniques are applicable to the resolution of positional and geometrical isomers of unsaturated fatty acids [6,7].

In this study, we report on methods for purification of a NMIFA incorporated into animal tissues by two-step AgTLC. We also characterize the purified NMIFA by GC–MS.

2. Experimental

2.1. Materials

Pine (*Pinus koraiensis*) seed oil was a gift from Sansho Oil Company (Nagoya, Japan). The columbine (*Aquilegia hybrida*) seed and *Sciadopitys verticillata* seed were purchased from a nursery. Precoated thin-layer plates (Silica gel 60) were from Merck (Darmstadt, Germany). Silic AR (100–200 mesh) was from Mallinckrodt (Paris, KY, USA). Methanolic HCl (10%, w/v), dimethyl disulfide and hydrazine monohydrate were from Tokyo Kasei (Tokyo, Japan).

2.2. Isolation of NMIFAs from several plant seeds

The columbine and *Sciadopitys verticillata* seeds (5 g each) were milled and ground in a mortar with pestle in 50 ml of methanol. The lipids of the seeds were extracted from the homogenate by the method of Folch [8]. The lipid extracts of these seeds and the pine seed oil were subjected to methanolysis by refluxing with 5% methanolic HCl for 60 min [9]. The resulting fatty acid methyl esters were purified by TLC using the solvent system petroleum ether–diethyl ether–acetic acid (80:30:1, v/v) to remove unsaponifiable materials. Detection was done with 0.01% primuline (in acetone–H_2O, 4:1, v/v) under ultraviolet light. The purified fatty acid methyl esters were fractionated by AgTLC using the solvent system petroleum ether–diethyl ether–acetic acid (70:20:2, v/v) (solvent system I). The AgTLC plates were prepared by immersing TLC plates (Silica gel 60) with 5% silver nitrate (in acetonitril) for 30 min in the dark. The plates were activated at 110°C for 30 min just before use. Detection was done with 0.2% 2,7-dichlorofluorescein (in ethanol) under ultraviolet light. The zone of silica gel corresponding to NMIFA methyl esters was scraped, and fatty acid methyl esters were extracted from the silica gel by the method of Bligh and Dyer [10]. To remove the remaining 2,7-dichlorofluorescein, the fatty acid methyl esters were dissolved in *n*-hexane and washed with an equal volume of water until the fluorescence yellowish color in the water phase disappeared. The purities of isolated NMIFAs were checked by gas chromatography (GC), and structural confirmations were done by GC–MS as described below.

2.3. Animals and diets

Three-week-old male Sprague–Dawley (S.D.) rats were fed pellets of standard rat chow (Oriental Yeast, Japan) or pine seed oil diet. The pine seed oil diet was a mixture of a fat-free diet (Crea, Japan) and pine seed oil (9:1, w/w). The fatty acid composition of pine seed oil is given in Table 1. Diets and water were supplied ad libitum. In another experiment, 5-week-old male rats which were maintained on standard rat chow were fasted for 48 h. They were

Table 1
Fatty acid composition of plant seed lipids used in this study

Fatty acid	Pine	Columbine %	*Sciadopitys verticillata*
14:0	–	–	–
16:0	5.0	7.9	3.3
18:0	2.0	2.5	2.2
18:1 (Δ-9)	27.6	8.7	24.5
18:2 (Δ-9,12)	46.6	27.4	45.0
18:2 (Δ-5,9)	2.0	–	–
18:3 (Δ-5t,9,12)[a]	–	49.2	–
18:3 (Δ-5,9,12)	14.6	–	–
18:3 (Δ-9,12,15)	–	–	1.1
20:0	–	–	0.2
20:1 (Δ-11)	–	–	1.2
20:2 (Δ-5,11)	–	–	0.8
20:2 (Δ-11,14)	–	-	4.8
20:3 (Δ-5,11,14)	0.7	-	15.2
20:3 (Δ-11,14,17)	–	–	0.2
20:4 (Δ-5,11,14,17)	–	–	1.1

[a] *trans,cis,cis*-Δ-5,9,12/18:3.

then fed with a fat-free diet for 24 h following the feeding of the pine seed oil diet for 24 h.

2.4. Purification of NMIFA from rat liver

After the feeding period, rats were lightly anaesthetized with diethyl ether and killed. The liver (1 g) was homogenized by ultra disperser in a mixture of 0.8 ml of water and 6 ml of chloroform–methanol mixture (1:2, v/v). The lipids were extracted from the homogenate according to the method of Bligh and Dyer [10]. The neutral lipid fraction and the phospholipid fraction were prepared from the lipid extracts using silic AR column chromatography [11]. From the phospholipid fraction, the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions were prepared by preparative TLC using the solvent system chloroform–methanol–H_2O (65:35:6, v/v). The phosphatidylinositol (PI) fraction was also prepared by TLC with chloroform–methanol–28% aqueous ammonia (65:35:5, v/v). Detection was done with 0.01% primuline (in acetone–H_2O, 4:1, v/v) under ultraviolet light. The fatty acid composition of each lipid fraction was analyzed by GC.

For isolation of NMIFA incorporated into the rat liver, the fatty acid methyl esters prepared from the phospholipid fraction were fractionated by 5% AgTLC using solvent system I. The isolated C20 methyl trienoate fraction was subjected to 5% AgTLC developed with solvent system benzene–acetone–diethyl ether–acetic acid (65:15:15:5, v/v) (solvent system II). The zone of silica gel corresponding to NMIFA methyl ester was scraped off the plate, and the fatty acid methyl esters were recovered from the silica gel by the method of Bligh and Dyer [10].

2.5. Determination of double bond position of NMIFA

The determination of double bond positions of purified NMIFA from plant seeds or rat liver was conducted by the method essentially reported by Yamamoto et al. [12]. The NMIFA methyl ester (0.1–0.5 mg) dissolved in 1 ml of methanol was mixed with 0.1 ml of hydrazine monohydrate and 0.1 ml of 30% hydrogen peroxide. After heating at 50°C for 60 min, 1 ml of 6 *M* HCl, 6 ml of *n*-hexane and 2 ml of H_2O were added. The *n*-hexane layer was repeatedly washed with H_2O. The resulting mixture of hydrogenated fatty acid methyl esters was recovered from the *n*-hexane layer, and methyl monoenoates were isolated by AgTLC using solvent system I. The methyl monoenoates were dissolved in 0.5 ml of dimethyl disulfide (DMDS) containing 6.5 mg of I_2 and heated for 30 min at 35°C. After adding 2 ml of *n*-hexane–diethyl ether (1:1, v/v) mixture, 10% (w/v) aqueous $Na_2S_2O_3$ solution was added to the reaction mixture until the color of I_2 disappeared. The DMDS adducts of methyl monoenoate were recovered from the upper phase and fractionated by normal TLC using the solvent system *n*-hexane–diethyl ether–acetic acid (80:20:1, v/v). The DMDS adducts recovered from the TLC plate were dissolved in a small amount of *n*-hexane and analyzed by the GC–MS system.

2.6. GC and GC–MS

Fatty acid methyl esters were analyzed by GC (Shimadzu GC-14A, Kyoto, Japan) equipped with a capillary column coated with 0.25 mm film of polar CBP 20 (0.22 mm×30 m; Shimadzu) as previously described [9]. The analyses of DMDS adducts of methyl monoenoate were carried out on a Shimadzu QP-2000 quadrapole mass spectrometer equipped with an interface for capillary GC (column coated with a 0.25-μm film of nonpolar CBJ1, 0.25 mm×30 m, Shimadzu). The column temperature was raised from 215 to 265°C (for analysis of DMDS adducts of C20 methyl monoenoates) or 200 to 250°C (for analysis of DMDS adducts of C18 methyl monoenoates) at a rate of 5°C/min, and the temperatures of the injection port, ion source and interface were set at 250°C. The sample was injected in the split mode and helium was used as the carrier gas. The ionization energy was 70 eV.

3. Results and discussion

3.1. Isolation of NMIFAs from plant seeds

The major NMIFA in pine seed oil was pinolenic acid (all-*cis*-Δ-5,9,12/18:3). Pine seed oil also contained all-*cis*-Δ-5,9/18:2 and all-*cis*-Δ-5,11,14/20:3 as minor NMIFAs (Table 1). The lipid extract of

columbine seeds abundantly contained *trans,cis,cis*-Δ-5,9,12-18:3, named columbinic acid, a geometrical isomer of pinolenic acid (Table 1). The major NMIFA in the lipid extract of *Sciadopitys verticillata* seeds was all-*cis*-Δ-5,11,14/20:3, named podocarpic acid (Table 1). These results were essentially comparable to those reported by Wolff and Bayard [2], Houtsmuller [1] and Takagi and Itabashi [3]. We purified pinolenic acid, columbinic acid and podocarpic acid by AgTLC with solvent system I from pine seed oil, the lipid extract of columbine seeds and *Sciadopitys verticillata* seeds, respectively. As shown in Table 2, AgTLC with solvent system I could separate C18 (except for columbinic acid) and C20 methyl trienoates. Both methyl pinolenate and methyl columbinate were isolated by single AgTLC with solvent system I, with a purity over 99%. However, repeated chromatography was necessary to obtain highly purified podocarpic acid from *Sciadopitys verticillata* seeds. The first isolated C20 methyl trienoates fraction by AgTLC with solvent system I contained methyl podocarpate (93%) and methyl α-linolenate (7%) checked by GC. We could obtain highly purified methyl podocarpate (99%) from this fraction again by AgTLC. The methyl podocarpate was recovered from the upper band on the TLC plate.

Table 2
Argentation TLC of C18 and C20 trienoic acid methyl esters

Fatty acid	R_F in solvent system	
	Solvent I[a]	Solvent II[b]
C18		
18:3 (Δ-6,9,12) (γ-linolenic acid)	0.20	0.51
18:3 (Δ-9,12,15) (α-linolenic acid)	0.20	0.50
18:3 (Δ-5,9,12) (pinolenic acid)	0.17	0.45
18:3 (Δ-5t,9,12)[c] (columbinic acid)	0.21	0.51
C20		
20:3 (Δ-8,11,14) (dihomo-γ-linolenic acid)	0.24	0.57
20:3 (Δ-5,11,14) (podocarpic acid)	0.22	0.52

[a] Petroleum ether–diethyl ether–acetic acid (70:20:2, v/v).
[b] Benzene–acetone–diethyl ether–acetic acid (65:15:15:5, v/v).
[c] *trans,cis,cis*-Δ-5,9,12/18:3.

AgTLC with solvent system II is effective for the separation of all- *cis*-NMIFA from all-*cis*-MIFA with the same chain length and the same degree of unsaturation (Table 2). It is known that fatty acids with double bonds separated by more than one methylene groups are strongly retained on silver-ion high-performance liquid chromatography, because they form stable three-center complexes with silver ions [7]. As C18 MIFA and methyl podocarpate overlapped in the AgTLC with solvent system II (Table 2), AgTLC with solvent system I was better for separation of these fatty acids. The combination of these two AgTLC developing systems is effective for the isolation of NMIFAs incorporated in animal tissues as described below.

3.2. Purification of C20 NMIFA detected in the phospholipid fraction of rat liver

An unknown peak was detected between the peaks of Δ-5,8,11/20:3 and Δ-8,11,14/20:3 in the gas chromatogram of fatty acid methyl esters derived from the PC of rat liver (Fig. 1). This unknown fatty acid was detected only in the liver of the rat fed with a pine seed oil (Po) diet for 24 h after starved–refed treatment (Stv-Rf-Po), but not in the rat fed with control chow nor in the rat continuously fed the Po diet. The unknown fatty acid was contained in the PC, PE and PI fraction of the liver of Stv-Rf-Po rat for 1.5%, 1.0% and 1.5%, respectively; however, TG did not contain it. The retention time of this fatty acid on GC was coincident with standard podocarpic acid methyl ester. At this level of information, the two possible structures for this fatty acid were podocarpic acid (Δ-5,11,14/20:3) and Δ-7,11,14/20:3. The former is the minor NMIFA contained in pine seed oil. Because the starved–refed with fat-free diet treatment is considered to cause temporary essential fatty acid deficiency in the rat, the Stv-Rf-Po rat would have extensively incorporated podocarpic acid from dietary pine seed oil to accumulate it in the phospholipid fraction of the liver. The latter candidate is the C2-elongated metabolite of pinolenic acid (Δ-5,9,12/18:3), a major NMIFA in pine seed oil. Because that starved–refed with fat free diet

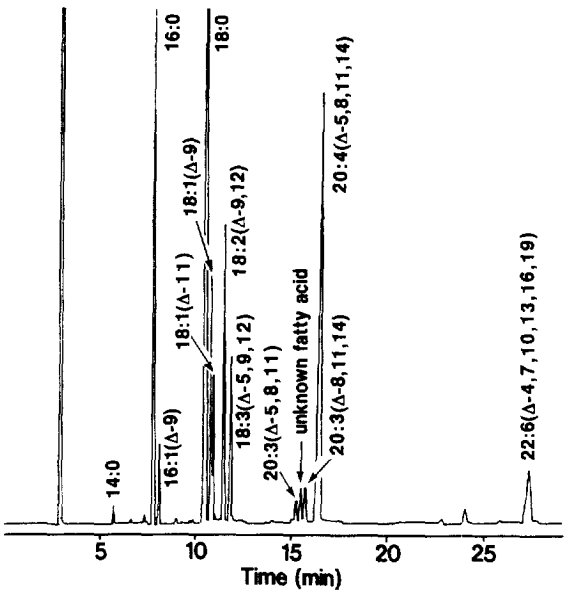

Fig. 1. Typical GC of fatty acid methyl esters prepared from the PC fraction of the liver of rat fed with a pine seed oil diet after the starved–refed treatment. The rats which had been maintained on standard chow were fasted for 48 h. They were then fed with a fat-free diet for 24 h following the feeding of pine seed oil diet for 24 h.

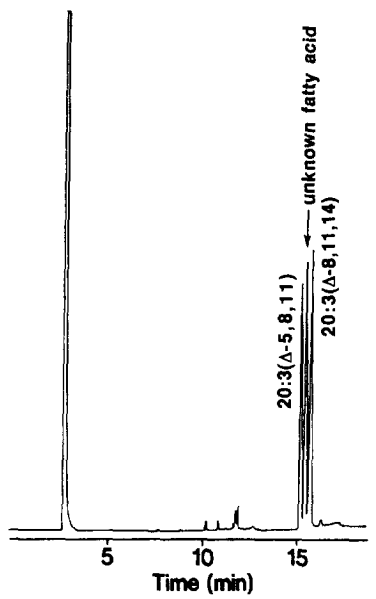

Fig. 2. GC of C20 methyl trienoate fraction obtained from the phospholipid fraction of the rat liver. The C20 methyl trienoate fraction was obtained from the fatty acid methyl esters derived from the phospholipid fraction of the rat liver by AgTLC developed with the solvent system petroleum ether–diethyl ether–acetic acid (70:20:2, v/v).

treatment has been shown to affect the activity of fatty acid chain elongation of the rat liver [13], and because pinolenic acid is structurally related to γ-linolenic acid, the latter possibility arose from the metabolism of pinolenic acid by the fatty acid chain elongation system similar to the case for the conversion of γ-linolenic acid to dihomo-γ-linolenic acid. Because the latter candidate, Δ-7,11,14/20:3 was not commercially available, we isolated the unknown fatty acid from the liver and determined its double bond positions by GC–MS as follows: first, the mixture of fatty acid methyl esters prepared from the phospholipid fraction of the liver was subjected to the AgTLC developed with solvent system I. The obtained C20 methyl trienoate fraction contained the unknown fatty acid as shown in Fig. 2. The unknown fatty acid was then purified from the C20 methyl trienoates fraction by the AgTLC developed with solvent system II (Fig. 3). The unknown fatty acid methyl ester migrated slower than the methyl esters of Δ-5,8,11/20:3 and Δ-8,11,14/20:3, suggesting the

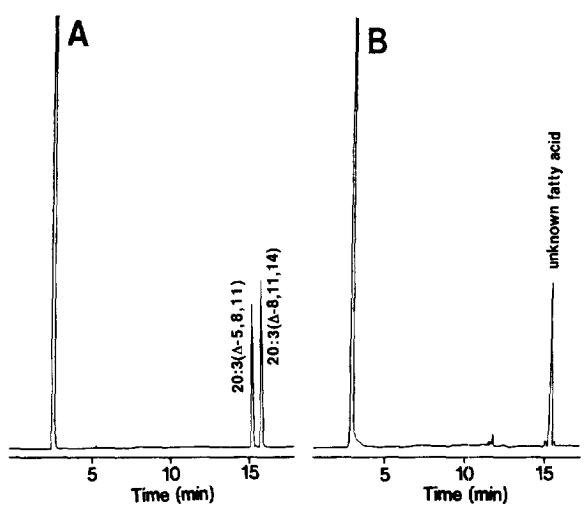

Fig. 3. GC of C20 MIFA methyl ester fraction (A) and C20 NMIFA methyl ester fraction (B) obtained from the C20 methyl trienoate fraction. The C20 MIFA methyl ester fraction and C20 NMIFA methyl ester fraction were obtained from the C20 methyl trienoate fraction by AgTLC developed with the solvent system benzene–acetone–diethyl ether–acetic acid (65:15:15:5, v/v).

existence of a *cis* non-methylene interrupted double bond in the molecule.

3.3. Structural determination of C20 NMIFA purified from the phospholipid fraction of the rat liver

Several derivatives have been developed for the determination of the double bond positions in the unsaturated fatty acids by GC–MS [14]. The introduction of a charge-stabilizing group, such as dimethyloxazoline [15] and pyrrolidide [16], at the carboxyl group has been used to minimize the migration of the double bond by electron impact. However, in preliminary experiments, applications of these two derivatizations for the mass spectrometric distinction of pinolenic acid and γ-linolenic acid were unsuccessful. There are no informative fragment ions which indicate the difference in the location of the double bond between the pinolenic acid (double bond position: Δ5) and γ-linolenic acid (double bond position: Δ6) (data not shown). Therefore, we used the method reported by Yamamoto et al. [12]. The method involved the conversion of DMDS adducts of methyl monoenoates obtained by partial hydrogenation of purified polyunsaturated fatty acids.

The purified C20 methyl trienoate from the rat liver was partially hydrogenated with hydrazine monohydrate as indicated in the Section 2. By checking with GC, the presence of eight kinds of fatty acid methyl ester in the reaction mixture was confirmed (data not shown). As the treatment with hydrazine randomly hydrogenates the double bonds in the polyunsaturated fatty acid molecule and the position of the double bond unchanged by this treatment, these were considered to be one kind of methyl trienoate (20:3), three kinds of methyl dienoates (20:2), three kinds of methyl monoenoates (20:1) and saturated acid methyl ester (20:0). From the reaction mixture, methyl monoenoates were isolated by AgTLC using solvent system I. After the conversion of methyl monoenoates to their DMDS adducts, they were fractionated by normal TLC (upper and lower bands) as described in the Section 2. Two kinds of DMDS adducts of methyl monoenoate were detected (Fig. 4A) from the upper band on TLC, and one kind of DMDS adduct from the lower band (Fig. 4B). The mass spectra obtained at the top of peaks 1, 2 (upper band) and peak 3 (lower band) are shown in Fig. 5A–C, respectively. The sets of key fragment ions at *m/z* 173, 213 and 245 (peak 1), at *m/z* 131, 255 and 287 (peak 2) and at *m/z* 257, 129 and 161 (peak 3) confirmed the presence of the DMDS adducts of methyl esters of Δ-11/20:1, Δ-14/20:1 and Δ-5/20:1, respectively. The results indicated that double bond positions of the original 20:3 were Δ-5,11,14. The double bonds of the Δ-5,11,14-20:3 were considered to be all *cis* configuration because the retention time of 20:3 was coincident

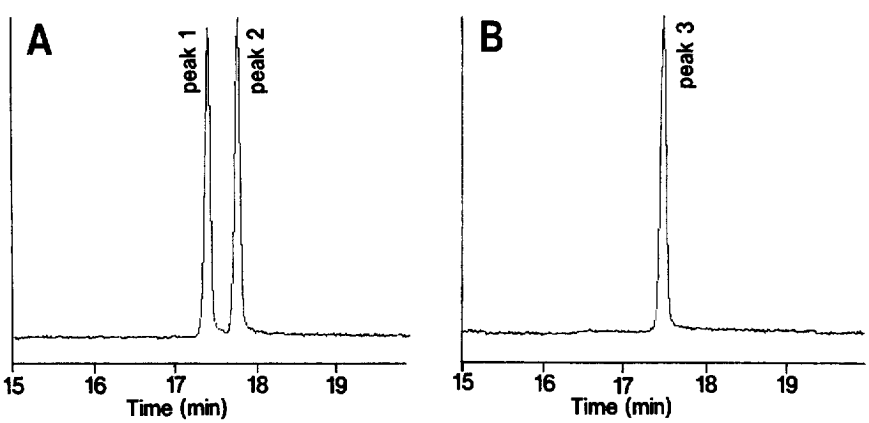

Fig. 4. Total ion chromatograms of DMDS adducts of methyl monoenoates derived from the purified C20 methyl trienoate. The C20 methyl monoenoates were obtained from purified methyl trienoate by partial hydrogenation with aqueous hydrazine. They were converted to DMDS adducts and fractionated into two fractions by TLC. The upper (A) and lower (B) bands on TLC plate were analyzed by GC–MS.

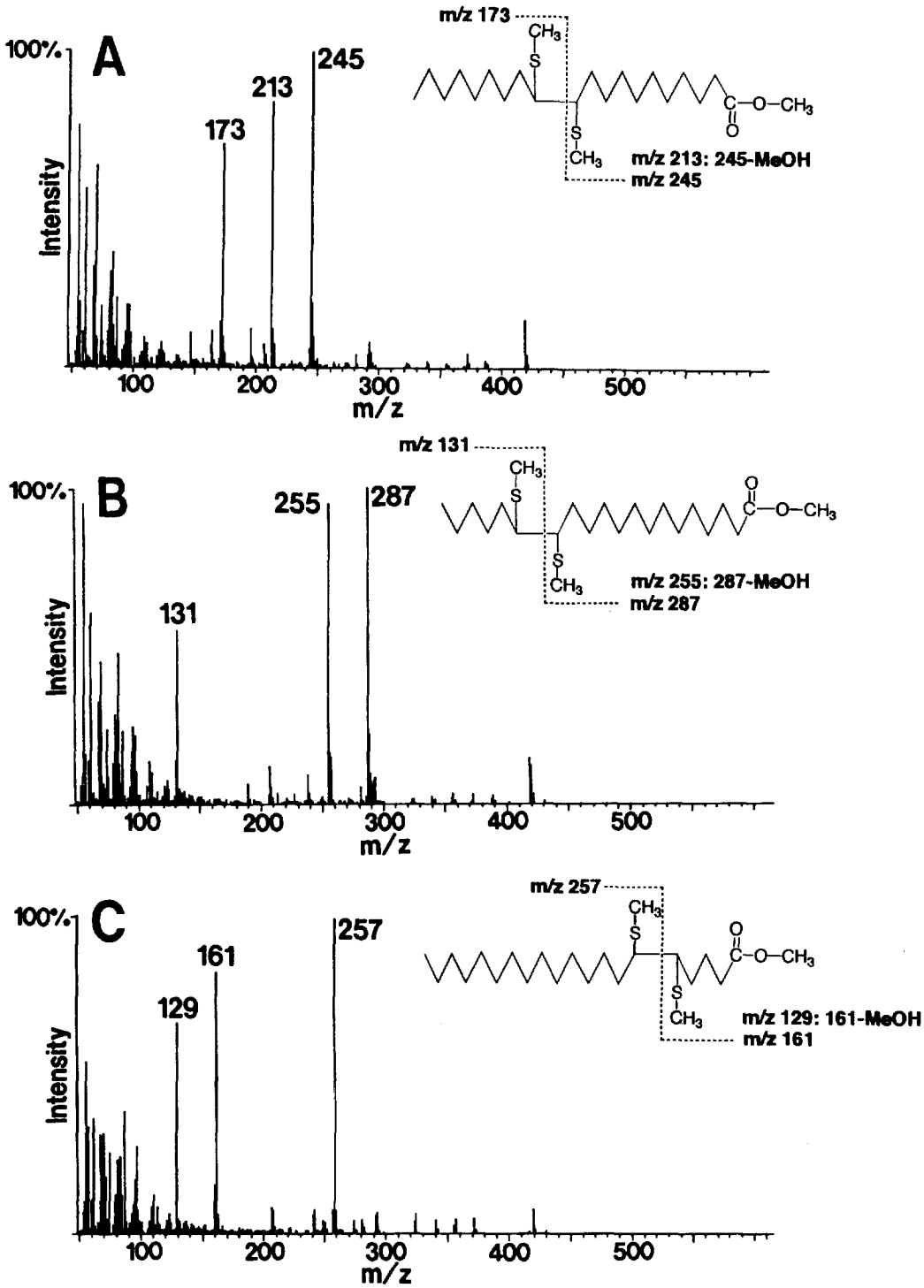

Fig. 5. Mass spectra of DMDS adducts of methyl monoenoates derived from the purified C20 methyl trienoate. Mass spectra A, B and C were obtained at the peak top of peaks 1, 2 and 3 in Fig. 4, respectively.

with podocarpic acid (all-*cis*-Δ-5,11,14/20:3) on the GC with capillary column (CBP 20) which could separate geometric isomers, such as pinolenic acid (all-*cis* Δ-5,9,12/18:3) and columbinic acid (*trans,cis,cis*-Δ-5,9,12/18:3) (data not shown). The migration of the 20:3 on AgTLC with solvent system II which could separate geometrical isomers (Table 2) also supported this conclusion. From these results, the C20 NMIFA detected in the liver of rat fed pine seed oil diet after starved–refed treatment was identified as podocarpic acid (all-*cis*-Δ-5,11,14/20:3).

As the podocarpic acid is the minor component of pine seed oil, the rat fed with a pine seed oil diet after starved–refed with a fat-free diet treatment more extensively accumulates podocarpic acid in the phospholipid fraction of the liver than the rat continuously fed with a pine seed oil diet.

It should be mentioned that the DMDS adduct of methyl Δ-11/20:1 and that of Δ-5/20:1 have similar retention times on GC with capillary column (OV-1). Therefore the separation of DMDS adducts of methyl 20:1 by normal TLC before GC–MS analysis was indispensable in our analytical procedure. This was also the case for the structural elucidation of pinolenic acid or columbinic acid by GC–MS. The DMDS adduct of methyl Δ-9/18:1 and that of methyl Δ-5/18:1 obtained by partial reduction of these C18 NMIFAs could be separated by normal TLC, but could not be separated by GC with capillary column (OV-1).

Using the combination of two AgTLC developing systems reported here, we succeeded in the isolation of C20 NMIFA incorporated in animal tissues predominantly containing usual MIFA. The methods described here are applicable for the study of the beneficial effect of NMIFAs with in vivo and in vitro experiments.

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