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Application of atmospheric pressure chemical ionization liquid chromatography–mass spectrometry in identification of lymph triacylglycerols

Huiling Mu*, Carl-Erik Høy

Department of Biochemistry and Nutrition, Center for Advanced Food Studies, Building 224, Technical University of Denmark, DK-2800 Lyngby, Denmark

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Abstract

Atmospheric pressure chemical ionization liquid chromatography–mass spectrometry was used in the identification of triacylglycerol molecular species in lymph samples from rats given either a structured lipid or safflower oil. The structured lipid was MLM-type (M, medium-chain fatty acid; L, long-chain fatty acid) and manufactured from caprylic acid (8:0) and the oil (safflower oil or high-oleic sunflower oil). The triacylglycerol composition of lymph varied significantly between structured triacylglycerols and safflower oil. Diacylglycerol fragment ions were found for all triacylglycerols and we could also observe the ammonium adduct molecular ion $[M+NH_4]^+$ for all the triacylglycerols at the selected conditions. Protonated molecular ions were formed from triacylglycerols containing unsaturated fatty acids, and fatty acid fragment ions were also observed in the case of strong fragmentation. The lymph triacylglycerols were identified from their ammonium adduct molecular ions and diacylglycerol fragment ions. In addition to the intact MLM-type structured triacylglycerols, the MLL- and LLL-type triacylglycerols were also identified. The absorption pathway of MLM-type structured triacylglycerols is most likely the same as that of conventional long-chain triacylglycerols, i.e. they were hydrolyzed into 2-monoacylglycerol and medium-chain fatty acids, which were then used for resynthesis of triacylglycerols. The present study thereby also demonstrates the possibility to study the absorption pathway of triacylglycerol via identification of triacylglycerol species in biological samples.

Keywords: Lymph; Triacylglycerol; Lipids

1. Introduction

The most commonly used method to determine lipid absorption is to examine the fatty acid composition and the recovery of some special fatty acids in the biological samples, such as lymph, plasma, and lipoproteins [1–3]. This, however, does not provide full information on the absorption process. For instance significant amounts of medium-chain fatty acids have been detected in lymph after intragastric administration of MLM-type (M, medium-chain fatty acid; L, long-chain fatty acid) structured triacylglycerols [4,5], but it is unclear in which form these medium chain fatty acids existed. To examine

*Corresponding author. Tel.: +45-4525-2744; fax:+45-4588-6307.

E-mail address: mu@mimer.be.dtu.dk (H. Mu).

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the triacylglycerol composition in biological samples, both reversed-phase high-performance liquid chromatography (RP-HPLC) [6] and supercritical fluid chromatography [7] have been used to separate triacylglycerol molecular species, but the triacylglycerols can only be tentatively identified by their equivalent carbon numbers.

Mass spectrometry (MS) is often used in structure elucidation. For instance, LC–MS with a direct liquid inlet interface has been used for the identification of triacylglycerols in randomized butteroil [8]; HPLC-desorption chemical ionization tandem MS has been used for the identification of milk fat triacylglycerols [9]. MS with electrospray (ES) ionization has been used for structural characterization of triacylglycerols on a triple quadrupole instrument [10]. Liquid chromatography coupled with atmospheric pressure chemical ionization-mass spectrometry (LC–APCI-MS) has also been used in the analysis of triacylglycerols [11–15].

Atmospheric pressure ionization (API) offers some advantages over other approaches, for example, it avoids problems associated with the introduction of a liquid flow directly into high vacuum. Another advantage of the API-MS is the possibility to apply conventional HPLC methods directly in LC–MS for the on-line identification. The development of electrospray ionization promises to further extend MS in the analysis of nonvolatile lipid molecules. Electrospray is an ionization process that uses electrical fields to generate charged droplets and subsequent analyte ions by ion evaporation, it is based on liquid phase ionization. Therefore it is important the sample components can be easily charged in order to form the charged droplet. The APCI process begins with gas-assisted nebulization into a hot vaporizer chamber that serves to rapidly evaporate the spray droplets. The result is gas-phase HPLC solvent and analyte molecules. The gas-phase solvent and analyte molecules are ionized by the discharge from a corona needle, thus it is a gas-phase ionization process. Similar to the processes encountered in conventional chemical ionization, the protonated solvent transfers a proton to the analyte if the proton affinity of the analyte is greater than that of the solvent. APCI is only useful for those samples that can be vaporized.

API is a relatively soft ionization technique producing primarily a pseudo-molecular ion. Collision induce dissociation (CID), the process of colliding ions with neutral gas molecules to cause fragmentation is helpful for both qualitative analysis and quantification. Qualitatively, structural information about the molecule is revealed. Quantification specificity is increased by the presence of confirmatory ions. CID is, however, compound dependent, therefore the degree of fragmentation should be found experimentally. Tandem MS is a preferred method for identification of co-eluting components because of the possibility to identify the primary ions by using the daughter ions formed in the second MS. Of course the tandem MS is more expensive than single MS.

The neutral lipids were poorly ionized in our solvent system (unpublished results) when API-ES was used. Therefore in the present study we used the LC–APCI-MS to identify the triacylglycerols present in lymph samples in order to examine the new populations of triacylglycerols found during the intestinal absorption of either the structured triacylglycerols or safflower oil. The high-performance liquid chromatography (HPLC) method was modified from the method reported previously [16]. To make the method suitable for MS analysis, ammonium acetate was added as the post-column additive. Both the intact MLM- type triacylglycerol and the MLL- and LLL-type triacylglycerols were identified in the lymph samples. The results obtained in the present study demonstrate the potential applications of LC–API-MS in the analysis of biological samples and also provide new insight into the metabolic pathway of structured triacylglycerols.

2. Experimental

2.1. Chemicals

All reagents and solvents were of analytical or chromatographic grade. Acetonitrile, hexane, and isopropanol were from BDH Laboratory Supplies (Poole, England), ammonium acetate was from Sigma (St. Louis, USA, min 98%). Monoacid triacylglycerol standards (triacetin, tributyrin, tricaproin, tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin, tristearin, triolein, trilinolein, and tri-

linolenin) were from Sigma (98–99% purity). The mixture was prepared by dissolving the triacylglycerols in chloroform and the concentration was about 2.5 mg/ml for each triacylglycerol.

2.2. Interesterified products

The structured triacylglycerols were produced by lipase-catalyzed interesterification in a packed-bed reactor. The interesterification procedure and parameters have been described previously [17]. Preparative HPLC was used to purify the specific structured triacylglycerols from the mixture of the interesterified product [4]. The purified 1,3-dioctanoyl-2-oleoyl-sn-glycerol (8:0/18:1/8:0) and 1,3-dioctanoyl-2-linoleoyl-sn-glycerol (8:0/18:2/8:0), as well as a reference oil i.e. safflower oil (75.5%, 18:2) were used in the animal experiments. The fatty acid profiles of purified structured triacylglycerols and safflower oil have been listed in the paper published previously [4].

2.3. Lymph collection and treatment

The animal experiments were approved by the Danish Animal Experiments Inspectorate. Male albino Wistar rats were obtained from Møllegård Breeding Center (Ll. Skensved, Denmark). The mesenteric lymph duct was cannulated with clear vinyl tubing; meanwhile a silicon tube was inserted into the stomach. More details about the animal experiments have been described previously [4]. The lymph was collected in 1-h fractions and the collection was initiated 1 h prior to the administration of lipids to obtain a baseline level of intestinal absorption of fat.

All the baseline fractions of lymph had similar triacylglycerol composition as verified by HPLC, therefore only one of them was used in the identification of triacylglycerols. The lymph fractions collected two h after intragastric administration of the lipids, representing the maximal intestinal absorption of lipids [4], were used in the identification of triacylglycerols. The total lipids from lymph samples together with the internal standard trinonanoin were extracted with chloroform and methanol [18].

2.4. High-performance liquid chromatography

A JASCO high-performance liquid chromatograph (JASCO Corporation, Tokyo, Japan) was incorporated with a SEDEX 55 evaporative light scattering detector (ELSD) (SEDERE, Alfortville, France), that was operated at 40°C at a gas pressure of 2.2 bar. The separation of triacylglycerols was performed on a Supelcosil LC-C_{18} column (l=25 cm, I.D.=4.6 mm, particle size=5 μm; Supelco, Bellefonte, USA) with a binary solvent system of acetonitrile (solvent A) and isopropanol/hexane (solvent B, 2:1, v/v) [16]. The content of solvent B in the mobile phase was increased from 25% to 40% over 15 min and then further increased to 45% over another 25 min.

2.5. Mass spectrometry

A HP 1100 Series LC/MSD system (Hewlett–Packard, Waldbronn, Germany) fitted with an atmospheric pressure chemical ionization source was used to acquire mass spectral data. The atmospheric pressure chemical ionization was used in the positive mode, the solvent vapor acted as the reagent gas. The corona voltage was 3000 V, the vaporizer temperature was 400°C, and the nebulizer gas pressure was 60 p.s.i. (1 p.s.i.=6894.76 Pa). The heated nitrogen drying gas temperature and flow-rate was 325°C and 4.0 L/min. Full mass spectra were taken in the mass range of 120–1000 m/z and the step size was 0.1 m/z. The same column and solvent as described in HPLC part was used in the separation of lymph triacylglycerols, but the solvent B was linearly increased from 10 to 60% over 60 min with a flow-rate of 1 ml/min. 50 m*M* ammonium acetate was used as the post-column additive at a flow-rate of 0.1 ml/min. System control and data evaluation were conducted by using HP ChemStation.

3. Results and discussions

3.1. Selection of APCI parameters

The triacylglycerol standard mixture was studied with LC–APCI-MS at the fragmentation potential of 60, 80, 100, 120, and 150 V. At the potential of 60 V, we observed both the ammonium adduct molecular

ions and the diacylglycerol fragment ions for all the triacylglycerols (Fig. 1A). The abundance of the $[M+NH_4]^+$ ion of m/z 236 and $[M-C2:0]^+$ ion of m/z 159 was very low because of the smaller molecular size of triacetin [19]. The total ion intensity increased with the increased fragmentation potential, meanwhile stronger fragmentation also occurred at high fragmentation potentials. At 120 V we also observed the ammonium adduct molecular ions for the triacylglycerols containing medium-chain and long-chain fatty acids (Fig. 1B), whereas at 150 V there were no molecular ions for the triacylglycerols containing fatty acids smaller than capric acid (Fig. 1C), instead more fatty acid fragment ions, such as the ion m/z 127 ($[C8:0-17]^+$) were observed at 150 V. The fragmentation potential 120 V was used in our study on lymph samples, since at this condition we observed the ammonium adduct molecular ions and the diacylglycerol fragment ions, while at the same time the ion intensity was also higher than at 60 V. Since the structured triacylglycerols given to the animals only contained medium-chain and long-chain fatty acids and we did not expect to see short chain fatty acids in the lymph triacylglycerols, the fragmentation potential 120 V was suitable for our lymph samples.

The mass spectra of the triacylglycerol standards were studied with or without the post column additive (ammonium acetate) at fragmentation potential of 120 V. We observed no molecular ions for the triacylglycerols only containing saturated fatty acids when ammonium acetate was not added in the system, but protonated molecular ions were produced from the triacylglycerols containing unsaturated fatty acids (Fig. 2A). In the presence of the post-column additive, however, we could observe the ammonium adduct molecular ions for all triacylglycerols containing medium-chain and long-chain fatty acids (Fig. 2B). This demonstrates the importance of using post column additive with the present solvent system to obtain the information about the molecular mass. Byrdwell [20] has also found the ammonium adduct molecular ions for triacylglycerols when ammonium hydroxide was incorporated into the LC system, and the triacylglycerols were identified by overall acyl chain length and degree of unsaturation since they were not resolved into molecular species.

Vaporizer temperature affects the desolvation effect and ionization process, therefore it is also an important parameter for LC–MS. Laakso and Manninen have pointed out that the vaporizer temperature has to be high enough to produce good sensitivity for the analytes [21]. Since the lipid content of biological samples is relatively low, a high sensitivity for the instrument is necessary. Therefore we also studied the effect of the vaporizer temperature on the triacylglycerol standards. The results obtained at vaporizer temperature 300, 350, and 400°C demonstrated that both the ion intensity of triacylglycerols and the level of fragmentation increased with increasing temperature. When the vaporizer temperature was above 400°C, no further significant increase of the ion intensity was observed, whereas stronger fragmentation occurred. In order to obtain both high sensitivity and the molecular ions, we chose 400°C for the vaporizer.

3.2. Identification of the triacylglycerols in the lymph from fasted animals

The animals were kept fasted one day following surgery. The triacylglycerol profile in the lymph before administration of lipids was regarded as baseline. In our previous absorption studies we investigated the fatty acid composition, but there was no information about the triacylglycerol molecular species. By studying the triacylglycerol composition we may obtain more information about the possible absorption pathway, therefore we need to identify the triacylglycerols in lymph lipids. We collected only one of the lymph baseline fractions for identification of triacylglycerols since we found similar triacylglycerol compositions for different animals during the fasted status by using HPLC with ELSD (Mu et al., unpublished results).

Triacylglycerols are the dominated lipids in lymph total lipids, therefore we extracted the total lipids from lymph and separated the triacylglycerols directly on a reversed-phase column. Most of the minor components from other lipid classes eluted earlier than triacylglycerols on this column, and generally did not disturb the separation of triacylglycerol species. LC–APCI-MS also discriminates the different lipid classes, for instance the sensitivity of LC–APCI-MS for free fatty acids, partial acylglycerols was much lower than that for triacylglycerols [19].

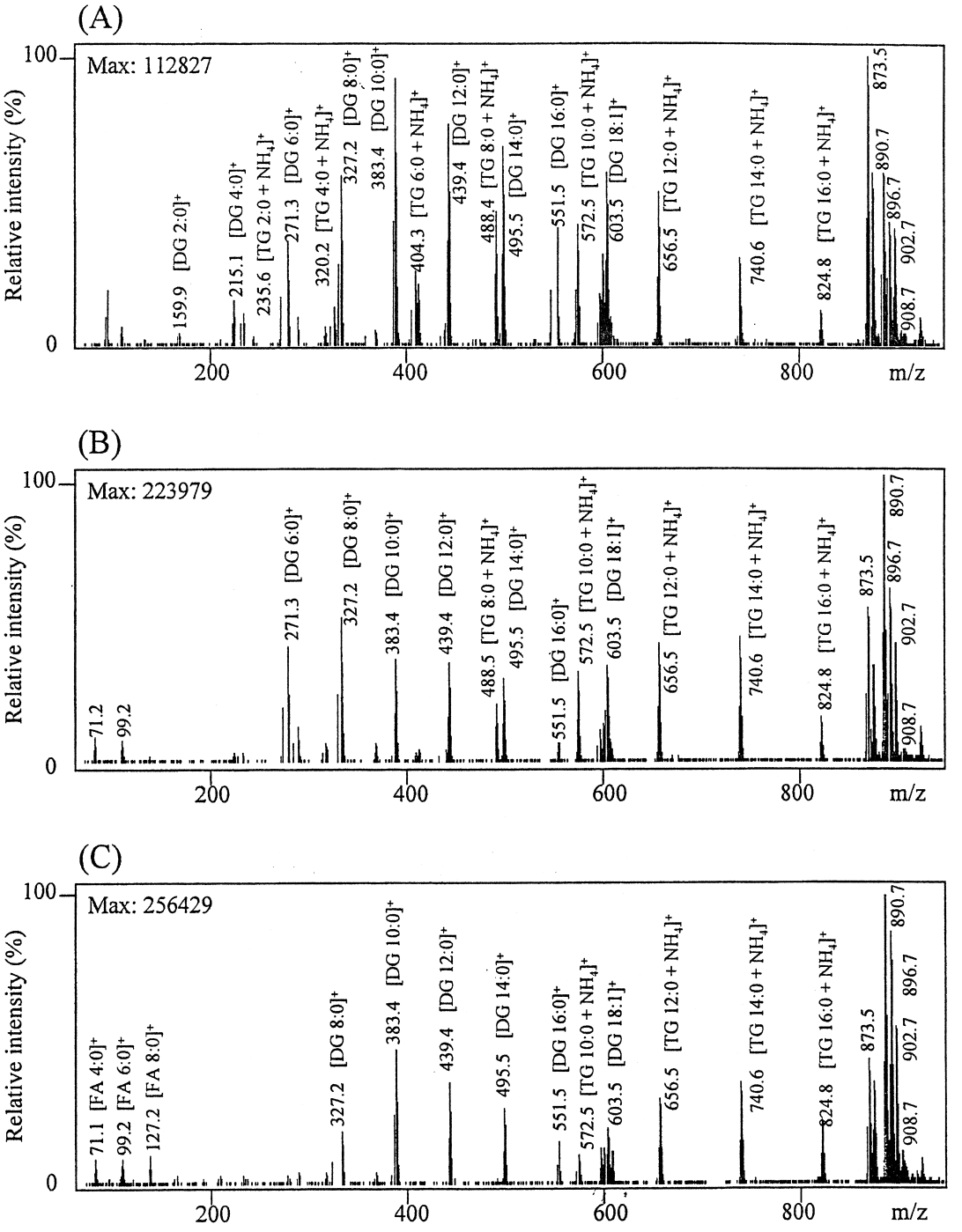

Fig. 1. APCI mass spectra of triacylglycerol standard mixture under different fragmentation potentials, (A) 60 V, (B) 120 V, and (C) 150 V. The m/z 873.5 represents the $[M+H]^+$ ion for trilinolenin, m/z 890.7, 896.7, 902.7, and 908.7 represent the $[M+NH_4]^+$ ion for trilinolenin, trilinolein, triolein and tristearin, respectively. The spectra were taken in the mass range of 60–1000 m/z with a step size of 0.1 m/z. The other experimental parameters are listed in the Experimental section.

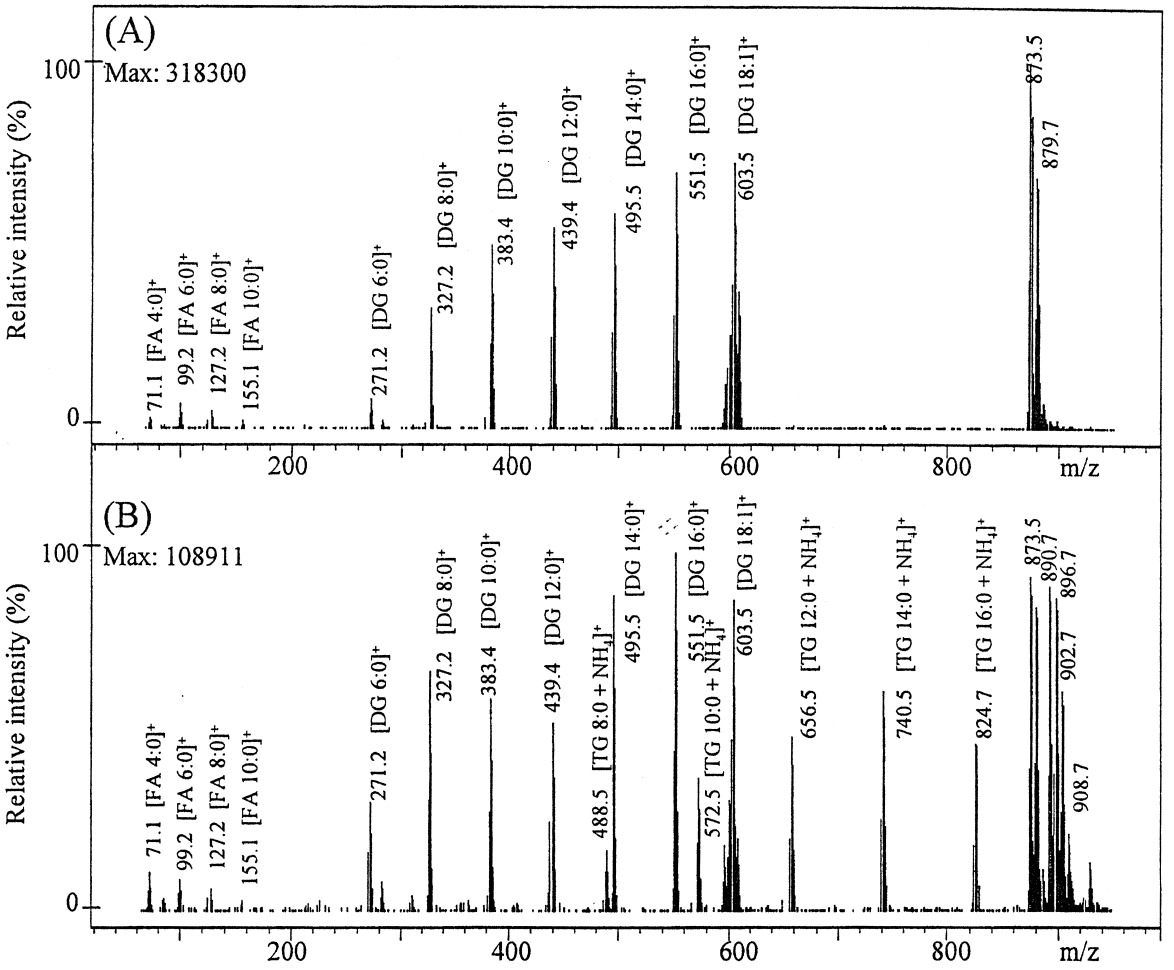

Fig. 2. APCI mass spectra of triacylglycerol standard mixture, (A) without the post-column additive ammonium acetate and (B) with the post-column additive ammonium acetate. The m/z 873.5 represents the $[M+H]^+$ ion for trilinolenin, m/z 890.7, 896.7, 902.7, and 908.7 represent the $[M+NH_4]^+$ ion for trilinolenin, trilinolein, triolein and tristearin, respectively. Fragmentation potential was 120 V, vaporizer temperature 400°C. The spectra were taken in the mass range of 60 to 1000 with a step size of 0.1 m/z.

A total ion current chromatogram (TIC) of triacylglycerols from lymph baseline fraction is shown in Fig. 3A. The triacylglycerols were identified directly from their ammonium adduct molecular ions and the diacylglycerol fragment ions. All the triacylglycerols identified in the lymph baseline fraction contained long-chain fatty acids, most of them had the equivalent carbon number from 40 to 50 (Table 1).

Equivalent carbon numbers (ECN) have often been used in tentative identification of triacylglycerols separated on a reversed-phase HPLC. There is a common rule for the elution order of triacylglycerols having the same ECN, i.e. triacylglycerols containing more double bond will elute first [22], however, co-eluting is very common when a single column is used. With the evaporative light scattering detection, it is difficult to decide if the co-eluting components are present, whereas spectra from LC–APCI-MS can be used to identify the co-eluting components. For instance the peak 17 in the Fig. 3A was identified as the triacylglycerol 16:0/16:0/18:2 by using the $[M+NH_4]^+$ ion of m/z 848.8, the $[M+H]^+$ ion of m/z 831.8, and the diacylglycerol fragment ions m/z 551.5 and m/z 575.5 by loss of

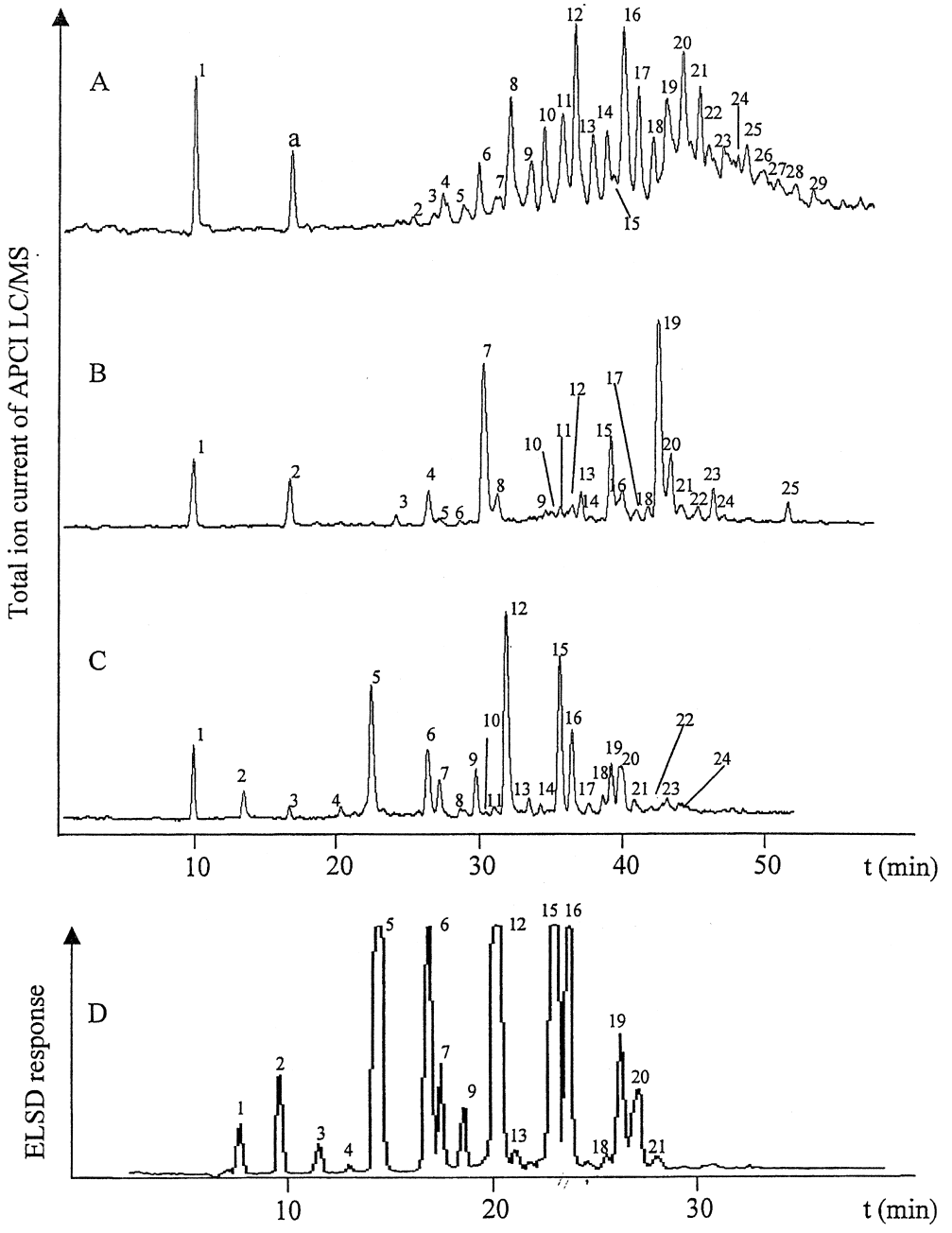

Fig. 3. Total ion current chromatogram of LC–APCI-MS for lymph lipids. (A) Lipids extracted from lymph baseline fraction, the identified triacylglycerols are listed in the Table 1. (B) Lipids from lymph collected two h after administration of structured triacylglycerol 8:0/18:1/8:0, the identified triacylglycerols are listed in the Table 2. (C) Lipids from lymph collected two h after administration of structured triacylglycerol 8:0/18:2/8:0, the identified triacylglycerols are listed in the Table 2. (D) The HPLC chromatogram of lymph lipids after administration of structured triacylglycerol 8:0/18:2/8:0, evaporative light scattering detection was used. The LC–APCI-MS and HPLC–ELSD conditions are given in the experimental section.

Table 1
The triacylglycerols identified in the lymph from fasted animal[a]

No.	t_R	ECN	TAG	MW		*m/z*				
				calculated	measured	$[M+NH_4]^+$	$[M+H]^+$	[MH−RCOOH]+		
1	9.48		i.s. TG 9:0	512.8	512.5	530.5		355.3		
2	25.26	36	18:2/20:4/22:6	951.5	950.8	968.8	951.8	623.5	647.5	671.7
3	26.80	38	18:2/18:2/20:5	901.4	900.4	918.4	901.7	599.6	621.7	
4	27.46	38	18:2/18:2/22:6	927.4	926.8	944.8	927.7	599.5	647.3	
	27.78	38	18:2/20:4/20:4	927.4	926.8	944.8	927.7	623.5	647.4	
5	28.94	40	18:2/18:2/18:3	877.4	876.8	894.8	877.7	597.6	599.6	
6	30.05	40	18:2/18:2/20:4	903.4	902.8	920.8	903.7	599.4	623.5	
7	31.60	40	18:1/18:2/22:6	929.5	928.8	946.8	929.9	601.5	649.5	647.5
		40	16:0/18:3/20:4	877.4	876.8	894.8	877.7	573.5	621.5	595.8
8	32.32	42	18:1/18:2/18:3	879.4	878.7	896.7	879.8	597.5	599.5	601.5
		42	TG18:2	879.4	878.7	896.7	879.8	599.5		
9	33.80	42	18:1/18:2/20:4	905.4	904.8	922.8	905.7	623.5	625.5	601.6
10	34.74	42	16:0/18:2/20:4	879.4	878.8	896.8	879.7	575.5	599.6	523.6
11	36.03	44	18:1/18:2/18:2	881.4	880.8	898.8	881.7	601.5	599.6	
12	36.99	44	18:2/18:2/16:0	855.4	854.8	872.8	855.7	575.6	599.5	
13	38.25	44	18:0/18:2/20:4	907.5	906.7	924.7	907.8	603.5	623.4	627.6
		44	16:0/18:1/20:4	881.4	880.6	898.6	881.8	577.6	599.5	625.6
14	39.26	44	16:0/20:4/16:0	855.4	854.7	872.7	855.8	551.5	599.5	
		46	18:2/18:2/20:1	909.5	908.8	926.8	909.7	629.5	599.5	
15	39.73	46	18:1/18:2/18:1	883.4	882.8	900.8	883.7	601.5	603.6	
16	40.48	46	18:2/18:2/18:0	883.4	882.8	900.8	883.8	599.5	603.6	
		46	18:1/18:2/16:0	857.4	856.7	874.7	857.6	575.6	577.5	601.6
17	41.55	46	16:0/18:2/16:0	831.4	830.8	848.8	831.8	551.5	575.5	
		46	18:0/18:1/20:4	909.5	908.8	926.8	909.8	605.6	625.6	627.6
18	42.66	46	18:0/20:4/16:0	883.4	882.6	900.6	883.7	579.5	599.5	627.6
		48	18:1/18:2/20:1	911.5	910.8	928.8	911.6	629.5	631.5	601.5
19	43.58	48	20:1/18:2/16:0	885.4	884.7	902.7	885.7	575.5	605.6	629.5
20	44.06	48	18:1/18:1/16:0	859.4	858.8	876.8	859.7	577.5	603.5	
21	44.76	48	18:0/18:2/16:0	859.4	858.7	876.7		575.5	579.6	603.6
22	45.98	50	20:1/18:1/18:1	913.5	912.8	930.8		631.6	603.5	
23	46.62	50	20:1/18:2/18:0	913.5	912.7	930.7	913.8	603.6	629.6	633.5
		50	20:1/18:1/16:0	887.5	886.8	904.8		577.4	605.7	631.5
24	47.63	50	18:1/18:1/18:0	887.5	886.7	904.7		603.5	605.5	
		50	16:0/16:0/20:1	861.4	860.8	878.8		551.5	605.5	
25	48.75	50	16:0/16:0/18:0	835.4	834.7	852.7		551.5	579.5	
26	49.39	52	20:1/20:1/16:0	915.5	914.9	932.9		605.6	659.5	
27	50.57	52	16:0/18:0/20:1	889.5	888.8	906.8		605.6	633.4	579.5
28	51.59	52	16:0/18:0/18:0	863.4	862.8	880.8		579.7	607.6	
		54	18:0/18:2/24:1	969.6	969.0	987.0		689.4	685.5	603.4
29	52.86	54	18:0/18:0/20:1	917.5	916.9	934.9		607.5	633.5	
		54	18:0/20:1/20:1	943.6	942.8	960.8		659.6	633.5	
30	54.10	56	16:0/20:1/24:1	971.6	971.0	989.0		716.4	661.4	605.4

[a] No, peak number in Fig. 3A; t_R, retention time; ECN, equivalent carbon number; MW, molecular mass.

linoleic acid and palmitic acid; meanwhile the triacylglycerol 18:0/18:1/20:4 was also identified by using the $[M+NH_4]^+$ ion of *m/z* 926,8, the $[M+H]^+$ ion of *m/z* 909.8, and the ion *m/z* 605.6, 625.6 and 627.6, which represent the diacylglycerol fragment ions $[M_2-20:4]^+$, $[M_2-18:0]^+$, $[M_2-18:1]^+$, respectively (Fig. 4).

The protonated molecular ions can also be helpful for the identification of co-eluting components. Since the abundance of the protonated molecular ions

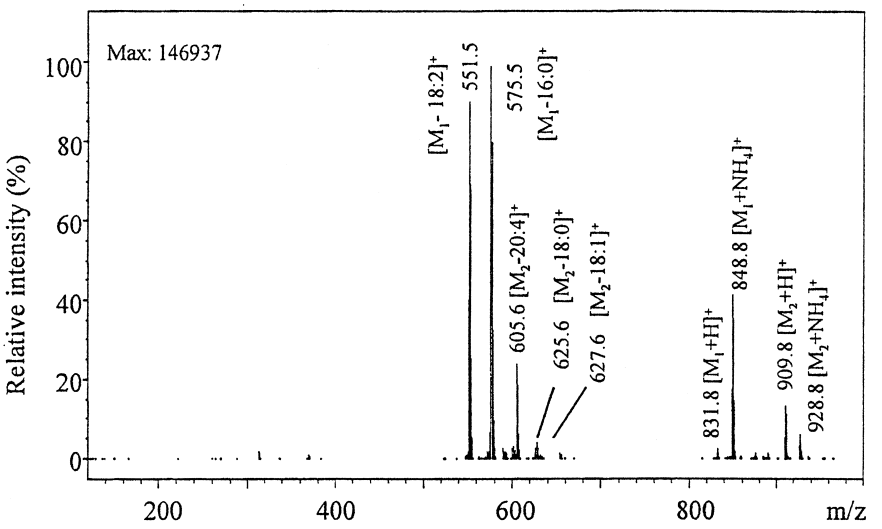

Fig. 4. Mass spectra of the peak 17 in Fig. 3A, co-eluting of triacylglycerol M_1 and M_2, which represent 16:0/16:0/18:2 and 18:0/18:1/20:4, respectively.

increases with the increase of unsaturation level of the acyl groups in the triacylglycerol [19], we may use the relation between the ammonium-adduct molecular ion and the protonated molecular ion to confirm the identification of co-eluting triacylglycerols. For instance to confirm the identities of triacylglycerol 16:0/16:0/18:2 and 18:0/18:1/20:4 (Fig. 4), we calculated the ratio of the ion intensity of $[M+NH_4]^+$ and $[M+H]^+$, it was 9.0 for the former one and 0.71 for the latter one. Since the latter one contains more double bonds, we also expected the ratio was much lower.

3.3. Identification of lymph triacylglycerols after intragastric administration of lipids

The triacylglycerol species in the lymph collected after intragastric administration of the structured triacylglycerol 8:0/18:1/8:0 or 8:0/18:2/8:0 were analyzed with LC–APCI-MS. The total ion current chromatograms of the lymph lipids (Fig. 3B and 3C) differed significantly from that of lymph baseline fraction (Fig. 3A). The new triacylglycerol species were identified based on their ammonium adduct molecular ions and diacylglycerol fragment ions. The identified triacylglycerols are listed in Table 2

Almost all the triacylglycerols contained one or more fatty acid from the administered structured triacylglycerols. The spectra of the MLM-, MLL, and LLL-type triacylglycerols related with the administered lipids and the spectra of the structured triacylglycerol used in the animal experiments are shown in Fig. 5. Comparing the ion intensity, we found that the level of the triacylglycerols in lymph increased in the order of 8:0/18:2/8:0<8:0/18:2/18:2<18:2/18:2/18:2 and 8:0/18:1/8:0<8:0/18:1/18:1<18:1/18:1/18:1.

Diacylglycerol fragment ions were the base peak for the triacylglycerol 8:0/18:1/8:0, 8:0/18:1/18:1, and 18:1/18:1/18:1. Both the $[M+NH_4]^+$ ion and the $[M+H]^+$ ion were observed for the three triacylglycerols, even though the intensity of $[M+H]^+$ ion for 8:0/18:1/8:0 was very low. The ratio between the ion intensity of $[M+NH_4]^+$ and $[M+H]^+$ were 19, 8.6, and 5.7 for triacylglycerol 8:0/18:1/8:0, 8:0/18:1/18:1, and 18:1/18:1/18:1, respectively, because of the increase of the unsaturation level. The base peak for 8:0/18:2/8:0 was also the diacylglycerol fragment ion of m/z $[M–8:0]^+$, however, the protonated molecular ion was the base peak for both 8:0/18:2/18:2 and 18:2/18:2/18:2 because linoleic acid can easily be protonated.

The same lymph lipids were also separated on a conventional HPLC with the evaporative light scat-

Table 2
Triacylglycerols identified from lymph after intragastric administration of different lipids

8:0/18:1/8:0				8:0/18:2/8:0				Safflower oil			
No	tR	ECN	TAG	No	tR	ECN	TAG	No	tR	ECN	TAG
1	9.39	27	I.s. TG 9:0	1	9.43	27	I.s. TG 9:0	1	9.50	27	I.s. TG 9:0
				2	13.12	30	8:0/18:2/8:0				
2	16.50	32	8:0/18:1/8:0	3	16.48	32	8:0/18:1/8:0				
				4	20.22	34	8:0/18:2/20:4				
				5	22.35	36	8:0/18:2/18:2				
3	24.12	36	8:0/18:1/20:4								
4	26.44	38	8:0/18:1/18:2	6	26.35	38	8:0/18:2/18:1				
5	27.23	38	8:0/18:2/16:0	7	27.25	38	8:0/18:2/16:0				
						38	22:6/18:2/18:2				
6	28.71	38	8:0/20:4/18:0	8	28.79	38	8:0/20:4/18:0				
						40	18:2/18:2/18:3	2	28.90	40	18:2/18:2/18:3
				9	29.84	40	18:2/18:2/20:4	3	30.01	40	18:2/18:2/20:4
						40	8:0/18:2/20:1			40	18:1/18:2/20:5
7	30.38	40	8:0/18:1/18:1	10	30.59	40	8:0/18:1/18:1				
				11	31.17	40	8:0/18:2/18:0				
8	31.40	40	8:0/18:1/16:0								
				12	32.02	42	TG 18:2	4	32.15	42	TG 18:2
										42	18:1/18:2/18:3
				13	33.69	42	18:1/18:2/20:4	5	33.80	42	18:1/18:2/20:4
				14	34.51	42	16:0/18:2/20:4	6	34.66	42	16:0/18:2/20:4
9	34.87	42	18:1/18:1/22:6								
10	35.28	42	8:0/18:1/18:0								
11	35.90	44	18:1/18:2/18:2	15	35.88	44	18:1/18:2/18:2	7	36.00	44	18:1/18:2/18:2
12	36.78	44	16:0/18:2/18:2	16	36.76	44	16:0/18:2/18:2	8	36.88	44	16:0/18:2/18:2
13	37.42	44	18:1/18:1/20:4								
14	38.21	44	16:0/18:1/20:4								
				17	38.00	44	18:0/18:2/20:4	9	38.21	44	18:0/18:2/20:4
				18	39.02	46	18:2/18:2/20:1	10	39.20	46	18:2/18:2/20:1
15	39.63	46	18:1/18:1/18:2	19	39.63	46	18:1/18:2/18:1	11	39.79	46	18:1/18:2/18:1
16	40.35	46	16:0/18:1/18:2	20	40.35	46	16:0/18:2/18:1	12	40.59	46	16:0/18:2/18:1
										46	18:0/18:2/18:2
17	41.45	46	18:0/18:1/20:4	21	41.27	46	16:0/18:2/16:0	13	41.51	46	16:0/18:2/16:0
18	42.28	46	8:0/18:1/22:0								
				22	42.50	48	18:1/18:2/20:1	14	42.73	48	18:1/18:2/20:1
19	42.97	48	18:1/18:2/18:0	23	43.65	48	18:1/18:2/18:0				
		48	TG 18:1					15	43.84	48	18:0/18:1/18:2
20	43.95	48	16:0/18:1/18:1								
				24	44.47	48	16:0/18:2/18:0	16	44.67	48	16:0/18:2/18:0
21	44.68	48	16:0/18:1/16:0								
								17	45.86	48	16:0/16:0/16:0
22	45.83	50	18:1/18:1/20:1								
		50	18:0/18:2/20:1								
								18	46.58	50	20:1/20:1/18:2
										50	22:1/18:2/16:0
23	46.95	50	18:0/18:1/18:1								
								19	47.65	50	20:0/18:2/16:0
24	47.78	50	16:0/18:1/18:0					20	47.95	50	16:0/18:1/18:0
25	52.35	54	18:1/18:1/22:0								

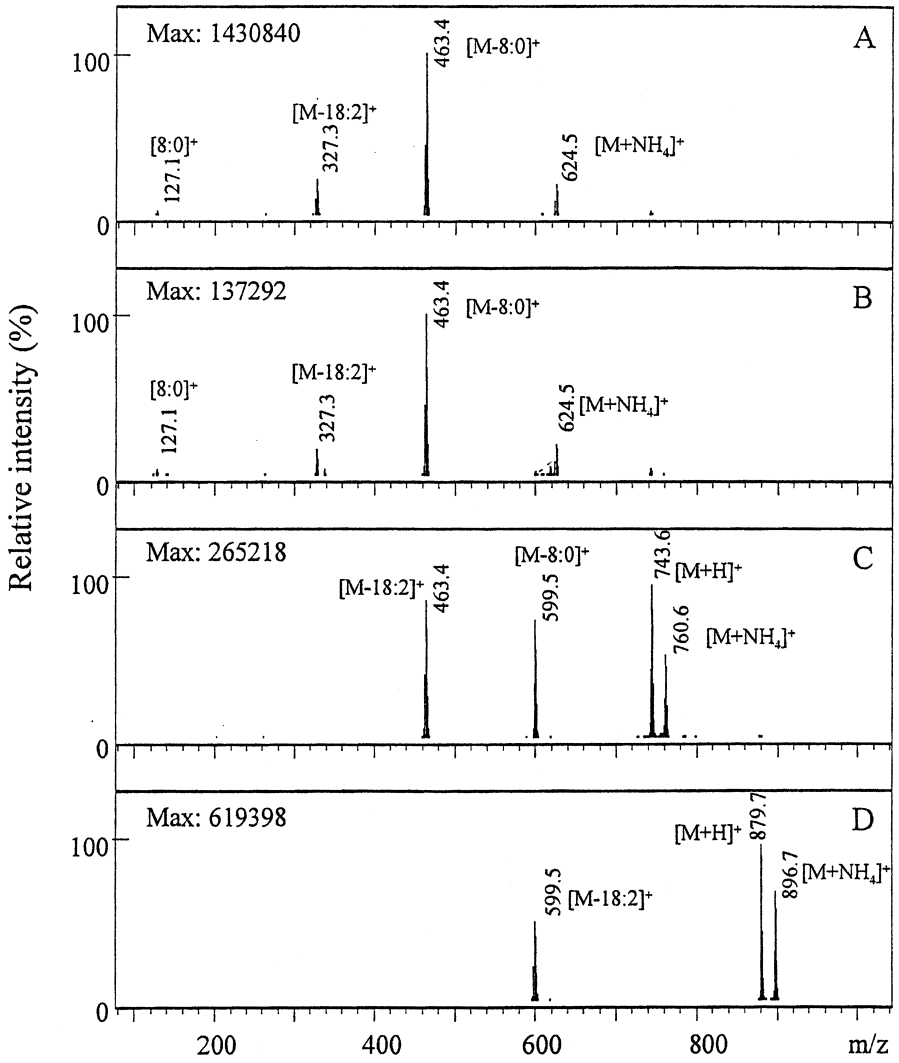

Fig. 5. (A) APCI mass spectra of the purified structured triacylglycerol 8:0/18:2/8:0 which was used in the animal experiment. APCI mass spectra of the triacylglycerol (B) 8:0/18:2/8:0, (C) 8:0/18:2/18:2, and (D) 18:2/18:2/18:2, identified in the lymph sample after feeding structured triacylglycerol 8:0/18:2/8:0.

tering detection. The HPLC chromatogram of the lipids from the lymph collected after administration of 8:0/18:2/8:0 was shown in Fig. 3D in order to compare with the LC–APCI-MS result. Even though the retention time differed because of the different solvent gradient, the two chromatograms (Figs. 3C and 3D) were quite similar. The proportions of triacylglycerols were calculated by normalization of the ELSD response. The triacylglycerol 8:0/18:1/8:0, 8:0/18:1/18:1, 18:1/18:1/18:1 constituted 9.3%, 24.0%, and 35.7% of the total triacylglycerols in lymph after administration of the structured triacylglycerol 8:0/18:1/8:0. The triacylglycerol 8:0/18:2/8:0, 8:0/18:2/18:2, 18:2/18:2/18:2 constituted 2.8%, 14.8%, and 19.4%, respectively, of the total triacylglycerols after administration of the structured triacylglycerol 8:0/18:2/8:0.

Different solvent gradients were used for HPLC–

ELSD and LC–APCI-MS because of the background problem for LC–APCI-MS when the solvent gradient from the conventional HPLC was applied, and we could not obtain the same resolution for triacylglycerols. The reason for this may be that LC–APCI-MS can produce ions from components in low concentration that would not allow for detection with ELSD, and the ions produced from those components resulted in background increase. LC–APCI-MS is thus more sensitive than HPLC–ELSD.

Similar to other lymph lipids, the triacylglycerol species in the lymph collected after intragastric administration of safflower oil were also identified with LC–APCI-MS (Fig. 6A) and the results are listed in Table 2. Most of the triacylglycerol species were same as the triacylglycerols from the safflower oil (Fig. 6B), but some new triacylglycerols were also detected, such as 18:2/18:2/20:4.

3.4. Intestinal absorption of the MLM-type structured triacylglycerols

It is well known that the triacylglycerols containing long-chain fatty acids are hydrolyzed into sn-2-monoacylglycerols and free fatty acids before absorption, the long-chain fatty acids are activated to their CoA derivatives through the action of fatty acyl-CoA ligase and resynthesized to triacylglycerols [23]. Medium-chain fatty acids are predominantly transported through portal vein since the ligase has low activity towards the medium-chain fatty acids [24,25]. However, when medium-chain fatty acids were administered in the form of MLM-type structured triacylglycerols, the recovery of medium-chain fatty acids in the lymphatics was much higher [4]. It was unclear if the medium-chain fatty acids in the structured lipids were absorbed in the form of intact

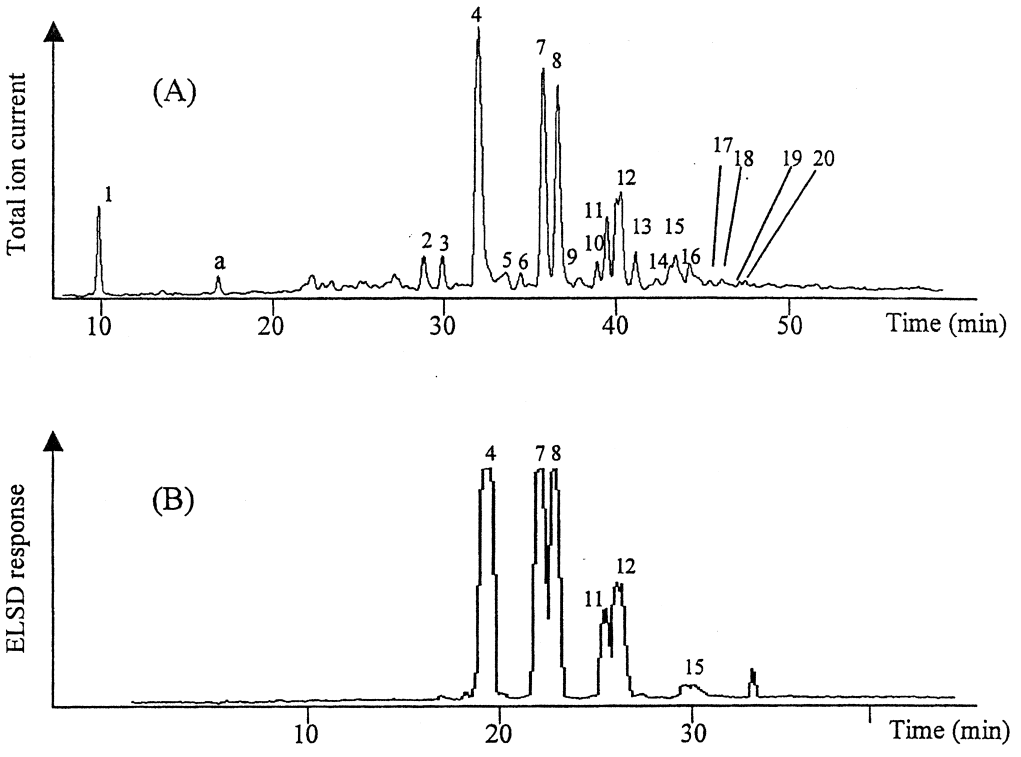

Fig. 6. (A) Total ion current chromatogram of LC–APCI-MS for lipids from the lymph obtained two h after administration of safflower oil, and (B) HPLC chromatogram of safflower oil with evaporative light scattering detection (ELSD). The LC–APCI-MS and HPLC–ELSD conditions are given in the experimental section.

triacylglycerols or they had the similar absorption pathway as long-chain triacylglycerols.

In the present study, we identified the triacylglycerol molecular species with the help of LC–APCI-MS and found that several triacylglycerols were related to the caprylic acid from the structured triacylglycerols. Not only the intact MLM-type structured triacylglycerols were found in the lymph, MLL-type and LLL-type triacylglycerols were also identified, actually there was a group of new triacylglycerols containing caprylic acid. This result suggests that the MLM-type structured triacylglycerols most likely are absorbed by the same pathway as conventional long-chain triacylglycerols, i.e. they are hydrolyzed into sn-2-monoacylglycerol (containing 18:1 and 18:2) and caprylic acid, and part of the caprylic acid released from the structured triacylglycerols was used for resynthesis of triacylglycerols. Therefore several new triacylglycerol molecular species containing caprylic acid were present in lymph. We cannot exclude the possibility that part of the MLM-type triacylglycerols found in the lymph was absorbed in the intact form.

The lymph triacylglycerols formed after intragastric administration of MLM-type structured triacylglycerols and safflower oil were significantly different. Most of the lymph triacylglycerols identified after administration of safflower oil were similar to the triacylglycerols present in the oil, whereas we found several fatty acids after administration of one single triacylglycerol 8:0/18:1/8:0 or 8:0/18:2/8:0. This result indicates that when a single triacylglycerol was administered, we may expect a significant contribution of endogenous fatty acids in the lymph.

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