Rapid Preparation of Fluorescent 9-Anthrylmethyl Esters for Fatty Acid Analysis of Small Amount of Triacylglycerols

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Abbreviations

ADAM 9-Anthryldiazomethemane

BHT Butylated hydroxytoluene

FAME Fatty acid methyl ester

GLC Gas-liquid chromatography

HPLC High-performance liquid chromatography

TAG Triacylglycerols

THF Tetrahydrofuran.

Abstract This paper proposes a one-step method for preparation of fluorescent 9-anthrylmethyl esters from triacylglycerols (TAG) ranging in amount from 0.1 to 5 μg. It involves base-catalyzed transesterification using potassium 9-anthracenemethoxide, prepared by proton exchange between 9-anthracenemethanol and potassium *tert*-butoxide. The transesterification conducted for 10 min at room temperature gave the fatty acid 9-anthrylmethyl esters in nearly maximal yields (82-85 %). The products could be analyzed by reversed-phase HPLC without purification. Excellent liner relationships were observed for standard curves of 10 to 250 pmol of TAG standards (16:0, 19:0, 18:2 and 22:6), and differences in the slopes were less than 5 % among the standards. Almost consistent compositions of the esters were observed for the products formed from 0.5-5 μg or less of fish oils TAG, and they were similar to those obtained by HPLC of ordinary multi-step synthesis products and by GLC of methyl esters. The present method is a great improvement of derivatization time, and is powerful for fatty acid analysis of small amount of natural TAG.

Keywords 9-Anthrylmethyl ester, Fatty acid, Fish oil, HPLC, Transesterification, Triacylglycerol

Introduction

Fluorescent 9-anthrylmethyl ester is one of the most widely used derivatives for fatty acid analysis by high-performance liquid chromatography (HPLC) [1-4]. Fatty acid contents and compositions of low levels of natural lipids have been determined by using this derivative, e.g., plasma and serum fatty acids, α-hydroxy fatty acids, and prostaglandins [2-4]; free fatty acids in marine phytoplankton [5]; and conjugated fatty acids in milk products [6].

For synthesis of this derivative, commercially available 9-anthryldiazomethemane (ADAM) has been widespread used. Although the ADAM reagent was pointed out to be unstable and different in activity from product to product [7], free fatty acids can be almost completely converted to their 9-anthrylmethyl esters by one-step reaction within 60 min [8]. However, when esterified fatty acids such as those of triacylglycerols (TAG) and phospholipids are analyzed, it is necessary to hydrolyze these lipids prior to derivatization. Usual methodology involves saponification, followed by acidification, extraction, washing, drying, and evaporation. It takes 1.5 h or so for the preliminary hydrolysis. In addition, such complicated treatments results in the presence of high levels of contaminants in the samples and thus compromises analytical quality and accuracy.

Recently, Destaillats and Angers [9,10] developed one-step methodology for synthesis of fatty acid picolinyl, 2-methoxyethyl, and C_2-C_4 *n*-alkyl esters from TAG, phospholipids, methyl esters, and intact lipids. It involved transesterification under base-catalyzed conditions using potassium alkoxides, which were prepared by proton exchange between potassium *tert*-butoxide and corresponding primary alcohols. Mild reaction conditions allowed complete derivatization, i.e., picolinyl ester from TAG and phospholipids, 2 min at room temperature [9]; and 2-methoxyethyl and *n*-alkyl esters from

TAG, 15 min at 40°C [10]. Dubois et al. [11] reported complete derivatization of methyl esters to picolinyl esters in 45 min at 45°C. Because 9-anthrylmethyl esters are also one of the esters of primary alcohol, this methodology is applicable to the synthesis.

This paper proposes a one-step method for rapid preparation of fatty acid 9-anthrylmethyl esters using base-catalyzed transesterification. The aim of the present study is to reveal properties of the new method and to assess its utility in fatty acid analysis of TAG in the ranges of 0.1 to 5 μg or of 0.1 to 5 nmol. For this purpose, some standard and natural TAG were subjected to this method, and the products were analyzed by reversed-phase HPLC with fluorescence detection. Fish oils TAG, containing a wide variety of fatty acids, were used as the natural TAG samples.

Materials and Methods

Reagents

Potassium *tert*-butoxide [1.0 M solution in tetrahydrofuran (THF)] and 9-anthracenemethanol (98 %) were purchased from Aldrich Chemicals (Milwaukee, WI) and Acros Organics (Geel, Belgium), respectively. THF anhydrous (99.5 %, stabilized with BHT, Kanto Chemical, Tokyo, Japan) was distilled once and stored in dark in presence of molecular sieves 4A (powder, Nacalai Tesque, Kyoto, Japan). Dichloromethane anhydrous (99.5 %) and cyclohexane (99.5 %) were products of Kanto Chemical, and the former was dried over molecular sieves 4A.

Standard TAG used were tripalmitoylglycerol (16:0-TAG; Extrasynthèse, Genay, France), trinonadecanoylglycerol (19:0-TAG; Sigma Chemical, ST. Louis, MO), trilinoleoylglycerol (18:2-TAG; Sigma Chemical) and tridocosahexaenoylglycerol (22:6-

TAG; Nu-Chek-Prep, Elysian, MN). The 22:6-TAG was purified by thin-layer chromatography on Silica gel 60G (Merck, Darmstadt, Germany) with hexane/diethyl ether (90:10, vol/vol) for development. The other standards were used without purification. Fish oils TAG, bonito head oil TAG and cod liver/mackerel oil TAG, were isolated from industrial oils by column chromatography on Silica gel 60 (Merck) with hexane/diethyl ether (95:5 and 90:10, vol/vol) for elution [1].

General Procedure for Synthesis of 9-Anthrylmethyl Ester

9-Anthracenemethanol (60 mg) and potassium *tert*-butoxide in THF (1.0 M, 20 μL) were taken in a screw-capped glass vial (0.6 mL-volume). Anhydrous THF (200 μL) was added to the vial and vigorously mixed with a vortex mixer to produce a saturated solution of potassium 9-anthracenemethoxide. The mixture was dried over 40 mg of anhydrous calcium sulfate for 1 h at room temperature. The supernatant was used as a potassium 9-anthracenemethoxide reagent within one day.

TAG (0.1-5 μg) and 19:0-TAG (100 pmol = 93.4 ng) as an internal standard were dissolved in 10 μL of anhydrous dichloromethane in a screw-capped 0.6 mL-volume glass vial. The potassium 9-anthracenemethoxide reagent (10 μL) was added to the solution, and vigorously mixed with a vortex mixer for 10 s. After the mixture was left to stand for 10 min in dark at room temperature, 2 μL of acetic acid/dichloromethane (1:10, vol/vol) was added to stop the reaction. After removing the solvents in a stream of nitrogen, resulting fatty acid 9-anthrylmethyl esters were taken up from the residue into 160 μL of cyclohexane, and 10 μL of the solution was subjected to reversed-phase HPLC.

HPLC

Reversed-phase HPLC was done with a Hitachi L-6200 pump (Hitachi, Tokyo, Japan), a Shimadzu RF-10A_{XL} fluorescence detector (Shimadzu, Kyoto, Japan) and a Shimadzu C-R6A integrator. A column of Supersphere 100 RP-18e (25 cm×4 mm i.d., 4 μm particles, Merck) was used with HPLC-grade acetonitrile, ethanol and hexane as mobile phase at a flow rate of 1.0 mL/min. A linear gradient of acetonitrile to acetonitrile/ethanol/hexane (30:40:30, by vol) was generated over 20 min. The column temperature was held at 10°C with a Shimadzu CTO-10AS_{VP} column oven. Peaks were detected at the excitation and emission wavelengths of 365 and 412 nm, respectively. Flow cell temperature of the detector was set at 20°C.

Results and Discussion

Formation of Fatty Acid 9-Anthrylmethyl Ester

Figure 1 shows HPLC profiles of the reaction products formed from standard and fish oils TAG by the present method. Formation of fatty acid 9-anthrylmethyl esters was checked by comparison of chromatographic behaviors of the products with those of standard esters. As the standards, authentic free fatty acids were converted to 9-anthrylmethyl esters by using ADAM reagent [8]. In the HPLC of the products formed from a mixture of 16:0-, 19:0-, 18:2- and 22:6-TAG, four peaks appeared in the chromatogram (Fig. 1A). Elution times of these peaks corresponded to those of the standard 16:0, 19:0, 18:2 and 22:6 esters. It is apparent that 9-anthrylmethyl esters are synthesized from TAG by the one-step method.

Blank test of the present method including the reversed-phase HPLC was carried out by using 100 pmol of 19:0-TAG internal standard (Fig. 1B). At earlier elution times, many

unidentified large peaks appeared, whereas there was practically no peak after 8 min except for one peak of 19:0 ester. Under the HPLC conditions used in this study, all of the identified peaks eluted after 8 min (Figs. 1C and 1D). The 9-anthrylmethyl esters produced by the present method can be directly subjected to the reversed-phase HPLC without purification.

Yield of 9-Anthrylmethyl Ester

Time-course changes of formation of fatty acid 9-anthrylmethyl esters were investigated by using an equimolar mixture of 16:0-, 19:0-, 18:2- and 22:6-TAG (each 100 pmol) at room temperature (20-22°C) (Fig. 2). Peak areas of the 9-anthrylmethyl esters rapidly increased, reached maxima at 2-5 min, and then tended to somewhat decrease (10 and 20 min). During the first 10 min, the 22:6 ester was found to change in a manner somewhat different from those of the other esters. The 22:6 ester reached maximum faster (2 min) and the maximum level was higher. The transesterification of 22:6-TAG seems to proceed at higher rate. In contrast, in longer reaction time (10-20 min), the four fatty acid esters were not very different in the changes. The changes were parallel to each other. Differences in the peak areas of the four esters were less than 4 % at both of 10 and 20 min.

In order to determine theoretical yield of the 9-anthrylmethyl esters, 18.75 pmol of standard 16:0 ester, corresponding to 100 % yield of this ester, was injected to the reversed-phase HPLC. The peak area was 15.8×10^4 units (mean of triplicate determinations). Theoretical yields calculated for the above four esters were 83-92 % (2 min), 85-91 % (5 min), 82-85 % (10 min), and 80-83 % (20 min). This result shows that the conversion of TAG to fatty acid 9-anthrylmethyl esters is not complete. Lower yields were observed for the reactions with no or insufficient dryness of the potassium 9-anthracenemethoxide

reagent and solvents with desiccants (data not shown). It is probable that the incomplete conversion resulted from hydrolysis (saponification) of TAG and 9-anthrylmethyl esters caused by moisture remaining in the reaction system.

Standard Curves

Detection limits of the fatty acid 9-anthrylmethyl esters were determined by using the above 10-min reaction products. The esters corresponding to each 91.5 fmol of the 22:6-, 18:2-, 16:0- and 19:0-TAG were injected to the reversed-phase HPLC. Detection limits (S/N=3) were all 26 fmol as the standard monoacid TAG. Calculated from the yields of 9-anthrylmethyl esters (82-85 %), detection limits of the esters were about 65 fmol (S/N=3).

The amount of fatty acid 9-anthrylmethyl esters as a function of the amount of starting TAG was also checked by useing the equimolar mixtures of standard TAG (Fig. 3). The reactions were conducted at room temperature for 10 min. Excellent linear relationships were observed for all TAG standards ranging from 10 to 250 pmol. The correlation coefficients of individual fatty acids were more than 0.999. The present method appears to be usable for fatty acid analysis of a mixture of 10-250 pmol of TAG molecular species. The slopes of the plots were not identical among the four standards. However, differences in the slopes were calculated to be less than 5 %.

Fatty Acid Analysis of Fish Oils TAG

The present method was applied to fatty acid analysis of bonito head oil and cod liver/mackerel oil TAG (Figs. 1C and 1D). Peak components were identified by comparing the elution times with those of authentic fatty acid esters. Table 1 shows the compositions of fatty acid 9-anthrylmethyl esters produced from 0.1-5 μg of the fish oils TAG. In both

fish oils, almost consistent compositions were observed in the analyses of 0.5-5 μg of TAG. For example, major fatty acids in the bonito head oil TAG, 22:6n-3, 18:1 and 16:0, were found at the concentrations of 31.2-31.8, 17.5-18.1, and 11.3-11.9 mole%, respectively. The 20:5n-3 ester was 8.5-8.6 mole%. In the cod liver/mackerel oil TAG, major fatty acids were found at 14.3-14.5 mole% (18:1), 12.3-12.7 mole% (16:0), 11.8-12.2 mole% (20:1) and 12.8-13.3 mole% (22:1). The 20:5n-3 and 22:6n-3 esters were 9.3-9.4 and 7.7-8.0 mole%, respectively. In the analyses of 0.1 and 0.2 μg of TAG, minor fatty acids could not be determined. However, major fatty acids showed the percentages not very different from those observed in the analyses of 0.5-5 μg of TAG.

Table 1 also shows the compositions obtained by other methodologies. One of them was determined by HPLC of 9-anthrylmethyl esters prepared by ordinary multi-step methodology, where 10 mg of the TAG were saponified by the method of Christie [1] and a potion of resulting free fatty acids (50 μg) were converted to 9-anthrylmethyl esters in a methanolic solution of ADAM reagent [8]. The other one was determined by gas-liquid chromatography (GLC) of fatty acid methyl esters (FAME) prepared by transmethylation of 2 mg of TAG using sodium methoxide in methanol [1]. The compositions of fatty acid 9-anthrylmethyl esters prepared by the present one-step method were similar to those determined by the ADAM-HPLC and FAME-GLC methods. This result indicates that fatty acid composition of fish oil TAG determined by way of the present one-step synthesis is comparable to those determined by ordinary methods. It is apparent that fatty acid analysis of 0.5-5 μg or less of TAG can be carried out by using the one-step transesterification.

Advantages of the Present One-Step Method

The present method is based on the methodology developed for rapid preparation of fatty

acid picolinyl esters [9,11], C_2-C_4 *n*-alkyl esters and 2-methoxyethyl esters [10]. However, some improvements were also required. Differing from the preceding cases, 9-anthracenemethanol is a solid alcohol. Because of its limited solubility, concentration of the potassium 9-anthracenemethoxide reagent is much lower than those of the previous alkoxides. Moisture in the solid 9-anthracenemethanol was removed by adding anhydrous calcium sulfate after preparation of the reagent. The transesterification was stopped by adding acetic acid in accordance with the methods for transmethylation [1]. The reagent was precipitated by exchanging the solvent (mixture of dichloromethane and THF) to a nonpolar solvent (cyclohexane), and 9-anthrylmethyl esters were taken up in the cyclohexane. Repeated injections of this solution did not cause negative influence to the reversed-phase HPLC.

In the present study, the new method was revealed to be usable for synthesis of fatty acid 9-anthrylmethyl esters from TAG. It was also revealed that the resulting 9-antherylmethyl esters are usable for fatty acid analysis of 0.1-5 μg of natural TAG including those from fish oils. The esters can be synthesized in 10 min, and subjected to HPLC after a few minutes. Comparing with multi-step methodology taking about 1.5 h for preliminary hydrolysis, the present method seems to be great improvement of the derivatization time. Precision of fatty acid analysis also seems to be improved, because of much less use of reagents, solvents, apparatuses, and their handling. The present method is the most rapid and convenient synthesis of 9-anthrylmethyl esters from TAG, and very effective to facilitate fatty acid analysis of small amount of natural TAG.

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Figure Legends

Fig. 1 HPLC profiles of fatty acid 9-anthrylmethyl esters produced from standard and fish oils TAG. A, mixture of 16:0-, 19:0-, 18:2- and 22:6-TAG (each 100 pmol); B, 19:0-TAG (100 pmol) used as an internal standard; C, bonito head oil TAG (0.5 μg) mixed with 19:0-TAG (100 pmol); and D, cod liver/mackerel oil TAG (1 μg) mixed with 19:0-TAG (100 pmol). The numbered peaks were identified by using standard esters as follows: 1, 18:4n-3+18:4n-1; 2, 20:5n-3; 3, 22:6n-3; 4, 18:3n-3+18:3n-6+16:2+12:0; 5, 20:4n-3; 6, 22:5n-3; 7, 20:4n-6; 8, 22:5n-6; 9, 16:1+18:2n-6+20:3+22:4n-6; 10, 14:0; 11, 17:1n-8; 12, 15:0; 13, 20:2n-6; 14, 18:1; 15, 16:0; 16, 17:0; 17, 20:1; 18, 18:0; 19, 19:0; 20, 22:1; 21, 20:0; and 22, 24:1.

Fig. 2 Time-course change of formation of fatty acid 9-anthrylmethyl esters from TAG. A mixture of 16:0-, 19:0-, 18:2- and 22:6-TAG (each 100 pmol) was converted to the 9-anthrylmethyl esters at room temperature (20-22°C). Peak areas were obtained by HPLC under the conditions described in the text. Data represent mean ± standard deviation of triplicate reactions.

Fig. 3 Plots of the amounts of 9-anthryl methyl esters *vs.* TAG subjected to the synthesis. Equimolar mixture of 16:0-, 19:0-, 18:2- and 22:6-TAG were subjected to the reactions conducted for 10 min at room temperature (20-22°C). Peak areas were obtained by HPLC under the conditions described in the text. Data represent mean ± standard deviation of triplicate reactions.

TABLE 1
Fatty Acid Compositions of Fish Oils TAG, Obtained by the Present and Other Methods (Mole%, Mean ± Standard Deviation)

	Present one-step method (n=3)							
Fatty acid	0.1 µg	0.2 µg	0.5 µg	1.0 µg	2.5 µg	5.0 µg	ADAM[a] (n=3)	FAME[b] (n=3)
Bonito head oil TAG								
18:4n-3[c]	0.8 ± 0.6	0.8 ± 0.6	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.0	1.2 ± 0.0
20:5n-3	8.8 ± 0.4	8.3 ± 0.0	8.5 ± 0.1	8.6 ± 0.0	8.6 ± 0.0	8.6 ± 0.0	8.5 ± 0.1	8.3 ± 0.0
22:6n-3	31.4 ± 1.3	30.5 ± 0.4	31.6 ± 0.9	31.8 ± 0.2	31.4 ± 0.1	31.2 ± 0.1	31.0 ± 0.2	30.1 ± 0.1
18:3n-3+16:2[d]	ND[g]	0.2 ± 0.1	0.9 ± 0.0	1.2 ± 0.2	1.3 ± 0.0	1.3 ± 0.0	1.0 ± 0.2	2.8 ± 0.0[j]
20:4n-3	ND	Tr	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.0
22:5n-3	0.9 ± 0.2	0.9 ± 0.0	1.4 ± 0.0	1.6 ± 0.1	1.7 ± 0.0	1.7 ± 0.0	1.6 ± 0.0	1.5 ± 0.0
20:4n-6	2.0 ± 0.1	2.0 ± 0.0	2.2 ± 0.0	2.3 ± 0.1	2.5 ± 0.0	2.5 ± 0.0	2.4 ± 0.0	2.1 ± 0.0
22:5n-6	1.5 ± 0.0	1.4 ± 0.0	1.5 ± 0.1	1.6 ± 0.1	1.8 ± 0.0	1.8 ± 0.0	1.7 ± 0.0	1.5 ± 0.0
16:1+18:2n-6[e]	9.9 ± 0.7	10.5 ± 0.4	9.8 ± 0.1	9.6 ± 0.0	9.4 ± 0.0	9.4 ± 0.0	9.6 ± 0.1	9.6 ± 0.1
14:0	3.7 ± 0.8	4.5 ± 0.3	3.8 ± 0.1	3.7 ± 0.0	3.6 ± 0.1	3.7 ± 0.0	3.7 ± 0.0	3.2 ± 0.1
17:1n-8	0.9 ± 0.3	1.4 ± 0.1	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.3 ± 0.0	1.1 ± 0.0
15:0	0.2 ± 0.3	0.9 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.0
20:2n-6	ND	ND	0.2 ± 0.0	NR	NR	NR	0.1 ± 0.1	0.2 ± 0.0
18:1	19.9 ± 1.0	19.0 ± 0.6	18.1 ± 0.4	17.8 ± 0.1[h]	17.6 ± 0.1[h]	17.5 ± 0.1[h]	17.8 ± 0.2	18.9 ± 0.0
16:0	14.0 ± 0.5	13.2 ± 0.3	11.9 ± 0.2	11.6 ± 0.1	11.3 ± 0.0	11.3 ± 0.0	11.8 ± 0.2	12.2 ± 0.1
17:0	Tr	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
20:1	1.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.0	1.5 ± 0.1
18:0	2.4 ± 0.2	2.1 ± 0.2	1.7 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.7 ± 0.1	1.7 ± 0.0
22:1	Tr	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.4 ± 0.0
20:0	ND	ND	Tr	Tr	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1	ND	ND	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
Others	2.0 ± 0.2	1.5 ± 0.5	1.4 ± 0.2	1.6 ± 0.2	1.9 ± 0.0	1.9 ± 0.0	2.2 ± 0.1	1.7 ± 0.1
Amount (nmol)[f]	0.28 ± 0.01	0.57 ± 0.03	1.51 ± 0.02	3.11 ± 0.01	7.79 ± 0.05	15.14 ± 0.08	—	—
Cod liver/mackerel oil TAG								
18:4n-3[c]	3.7 ± 0.4	4.0 ± 0.1	3.6 ± 0.0	3.6 ± 0.1	3.7 ± 0.2	3.7 ± 0.1	3.5 ± 0.1	3.3 ± 0.0
20:5n-3	9.9 ± 0.2	9.7 ± 0.2	9.3 ± 0.2	9.4 ± 0.0	9.4 ± 0.0	9.3 ± 0.0	9.2 ± 0.1	8.9 ± 0.1
22:6n-3	6.9 ± 0.3	7.2 ± 0.4	7.7 ± 0.2	7.9 ± 0.1	8.0 ± 0.0	7.9 ± 0.0	7.4 ± 0.0	7.2 ± 0.1
18:3n-3+16:2[d]	0.2 ± 0.2	0.7 ± 0.4	1.6 ± 0.1	1.7 ± 0.0	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.0	2.7 ± 0.0[j]
20:4n-3	Tr	0.2 ± 0.3	0.8 ± 0.0	0.8 ± 0.1	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.0
22:5n-3	0.6 ± 0.0	0.7 ± 0.2	1.4 ± 0.0	1.4 ± 0.1	1.5 ± 0.0	1.5 ± 0.0	1.4 ± 0.0	1.2 ± 0.0
20:4n-6	Tr	Tr	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.3 ± 0.0
22:5n-6	ND	ND	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	Tr
16:1+18:2n-6[e]	9.7 ± 0.2	9.5 ± 0.1	8.9 ± 0.0	8.8 ± 0.0	8.7 ± 0.0	8.6 ± 0.0	8.8 ± 0.1	8.8 ± 0.1
14:0	8.8 ± 0.2	8.3 ± 0.1	7.7 ± 0.0	7.6 ± 0.0	7.8 ± 0.2	7.7 ± 0.1	7.6 ± 0.0	7.6 ± 0.1
17:1n-8	ND	Tr	1.2 ± 0.1[i]	1.2 ± 0.0[i]	1.2 ± 0.0[i]	1.2 ± 0.1[i]	0.5 ± 0.0	0.7 ± 0.0
15:0	0.1 ± 0.2	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
20:2n-6	ND	ND	0.1 ± 0.0	NR	NR	NR	0.2 ± 0.0	0.2 ± 0.0
18:1	15.5 ± 0.1	15.0 ± 0.2	14.4 ± 0.1	14.5 ± 0.1[h]	14.3 ± 0.1[h]	14.3 ± 0.0[h]	14.2 ± 0.1	14.5 ± 0.1
16:0	14.2 ± 0.1	13.8 ± 0.2	12.7 ± 0.0	12.6 ± 0.0	12.4 ± 0.1	12.3 ± 0.0	12.5 ± 0.1	13.1 ± 0.1
17:0	ND	ND	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:1	12.9 ± 0.1	12.8 ± 0.1	12.2 ± 0.0	12.1 ± 0.1	11.9 ± 0.1	11.8 ± 0.0	12.0 ± 0.1	11.7 ± 0.1
18:0	2.3 ± 0.2	2.1 ± 0.1	2.1 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	1.9 ± 0.0
22:1	14.2 ± 0.2	14.2 ± 0.2	13.3 ± 0.0	13.1 ± 0.1	12.9 ± 0.1	12.8 ± 0.0	12.9 ± 0.1	12.9 ± 0.1
20:0	ND	ND	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
24:1	0.2 ± 0.3	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	0.7 ± 0.0
Others	0.8 ± 0.8	0.4 ± 0.5	0.8 ± 0.3	0.9 ± 0.1	1.1 ± 0.1	1.4 ± 0.0	2.7 ± 0.1	2.6 ± 0.1
Amount (nmol)[f]	0.28 ± 0.00	0.57 ± 0.01	1.48 ± 0.01	3.00 ± 0.02	7.69 ± 0.04	15.27 ± 0.04	—	—

[a] HPLC of the 9-anthrylmethyl esters prepared by hydrolysis of the TAG and esterification with ADAM reagent.

[b] GLC of the fatty acid methyl esters prepared by transmethylation of the TAG.

[c-e] Including 18:4n-1[c]; 18:3n-6 and 12:0[d]; and 20:3 and 22:4n-6[e].

[f] Amount of total fatty acids calculated from peak area ratios to internal standard (100 pmol of 19:0-TAG = 300 pmol of 19:0 acid).

[g] ND, not detected; Tr, trace amount; NR, not resolved.

[h,i] Including 20:2n-6[h] and unidentified fatty acid[i] not resolved from the peaks.

[j] Including branched-chain fatty acids inseparable from 16:2 by the GLC.

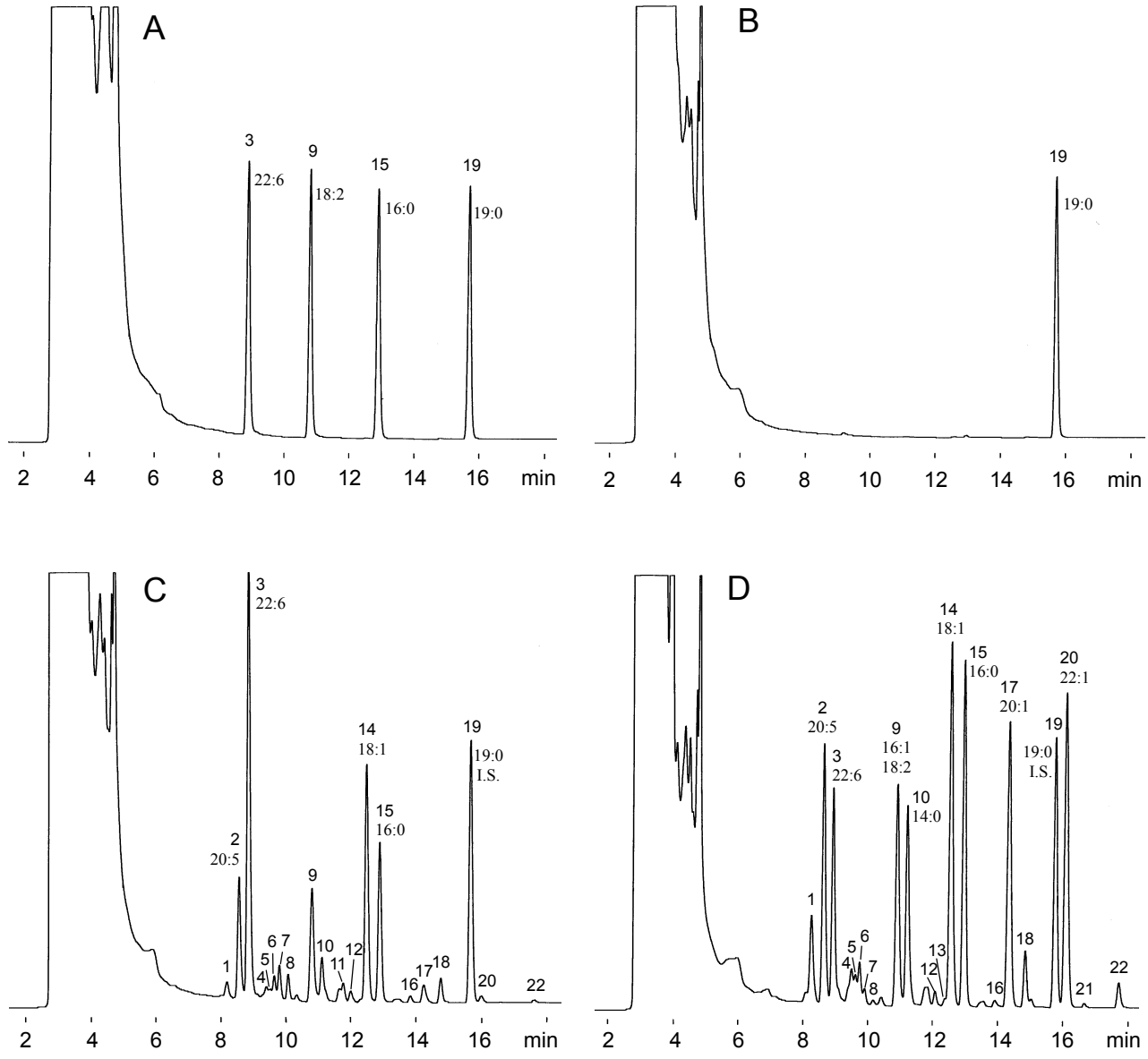

FIG. 1 Ando et al.

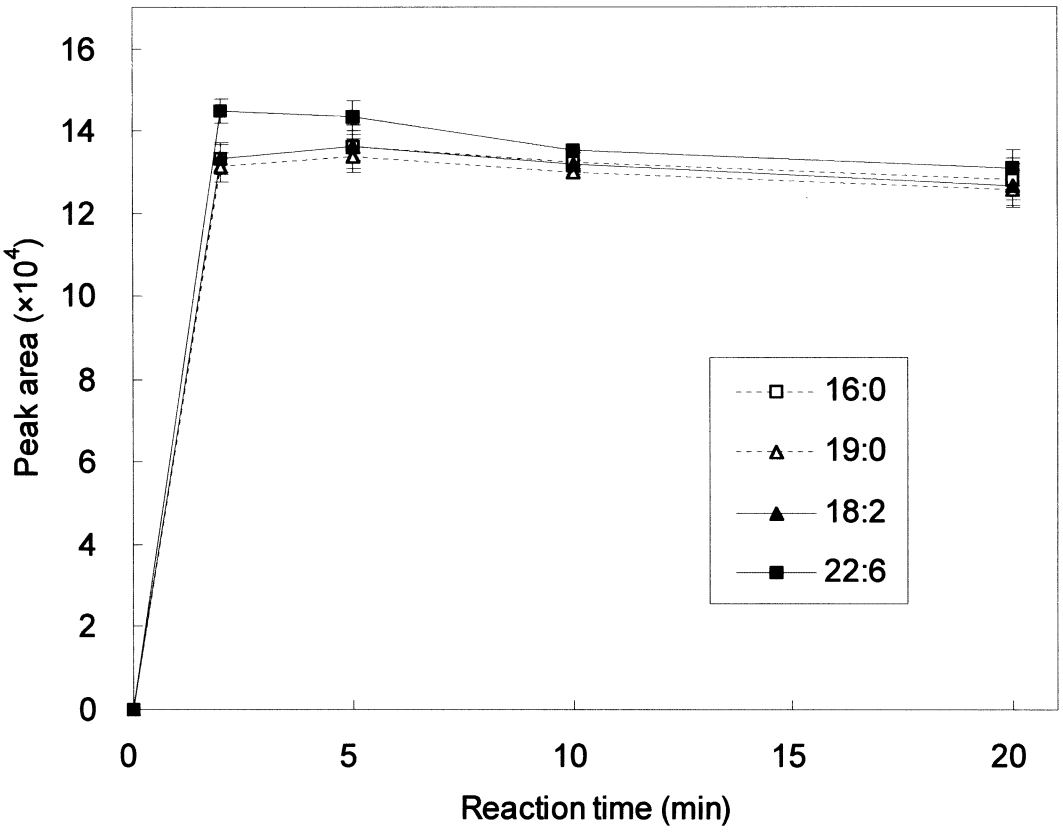

FIG. 2 Ando et al.

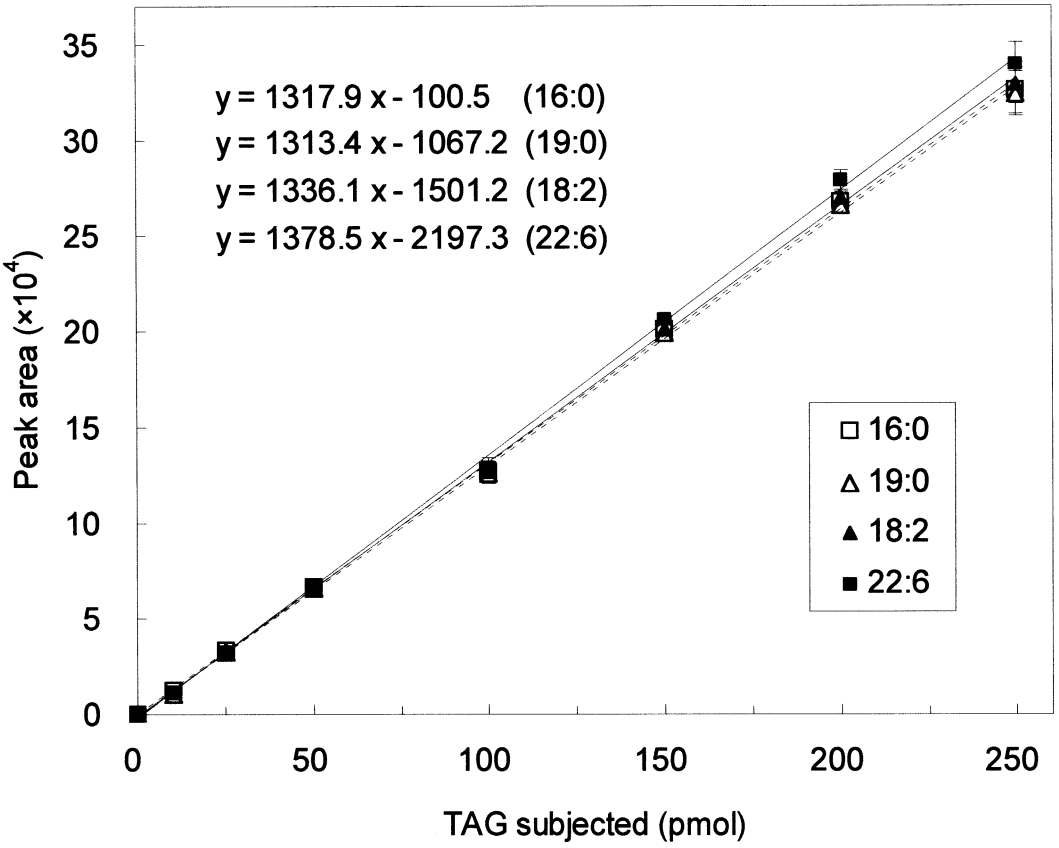

FIG. 3 Ando et al.