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Regiospecific Analysis of Marine Oil Triacylglycerols Using Boric Acid High-Performance Thin-Layer Chromatography

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Abstract

This paper presents a smaller-sized procedure for regiospecific analysis of triacylglycerols (TAG) using boric acid high-performance thin-layer chromatography (HPTLC). Cod liver/mackerel, bonito head, and seal oils TAG (2 mg) were partially hydrolyzed by ethyl magnesium bromide, and resulting 1(3)- and 2-monoacylglycerols (MAG) were isolated by the HPTLC. Fatty acids of the 1(3)- and 2-MAG were analyzed by gas-liquid chromatography (GLC). Positional distributions of fatty acids in TAG observed for the three marine oils were similar to those previously determined by the procedure requiring 100 mg of starting TAG. The new procedure appeared to be usable for regiospecific analysis of a few milligrams of marine oils TAG.

Key words: Regiospecific analysis, High-performance thin-layer chromatography, Triacylglycerol, Fatty acid, Fish oil, Seal oil

Introduction

Method for regiospecific analysis of triacylglycerols (TAG) has been frequently investigated in order to determine positional distribution of fatty acids in TAG, i.e., contents of each fatty acid in the *sn*-1(3) and *sn*-2 positions of TAG (Luddy et al., 1964; Becker et al., 1993; Turon et al., 2002; Christie, 2003). A method for the analysis, developed as a part of stereospecific analysis, involves partial hydrolysis of TAG, isolation of 1(3)- and 2-monoacylglycerols (MAG) from the hydrolysis products, and fatty acid analysis of the MAG (Takagi and Ando, 1991; Ando, 1998). This method has been applied to the analysis of many samples of marine oils TAG (Ando et al., 1992, 2000, 2004; Ando and Narukawa, 2002). One drawback of this method is that the yields of 1(3)- and 2-MAG analytical intermediates are no more than 2–5 wt% to the starting TAG. When the 1(3)- and 2-MAG are isolated by ordinary boric acid thin-layer chromatography (TLC), 80–100 mg of starting TAG are required for clear detection.

In the present study, regiospecific analysis of 2 mg of marine oils TAG was carried out by a smaller-sized procedure. High-performance thin-layer chromatography (HPTLC) on boric acid was introduced to the isolation step of sub-milligrams of 1(3)- and 2-MAG intermediates. The aim of this study is to clarify whether the new procedure including the boric acid HPTLC is usable for regiospecific analysis of marine oil TAG. For this purpose, three marine oils were subjected to the analysis, and the results were compared with those previously reported.

Materials and methods

Materials

The three marine oils used were cod liver/mackerel oil, bonito head oil and seal oil. These oils were the same with those previously analyzed (Ando et al., 2000, 2004) and had been kept frozen at −30°C under nitrogen atmosphere. TAG were isolated by column chromatography on Silicagel 60 (Merck, Darmstadt, Germany) with the mixtures of hexane and diethyl ether for elution.

Partial hydrolysis of TAG

The procedure previously reported (Ando and Takagi, 1993) was used as follows. The marine oils TAG (2 mg), mixed with trinonadecanoylglycerol (0.5 mg; Sigma, St. Louis, USA) as an internal standard, were dissolved in 0.23 mL of dry diethyl ether, and ethyl magnesium bromide in dry diethyl ether (0.1 mL of 1M solution) was added. The mixture was shaken for 25 s, and then 2 mL of acetic acid/diethyl ether (1 : 200, v/v) was added, followed by water (1 mL) to stop the reaction. The ether layer was washed once with 2% aqueous sodium bicarbonate, and then washed with water. The ether and water remaining in the solution were

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removed at ambient temperature in a stream of nitrogen in presence of 1 mL of toluene.

Boric acid HPTLC

Boric acid (0.32 g) was dissolved in a mixture of water (10 mL) and methanol (10 mL) to produce a 1.6% solution. After predevelopment twice with methanol, Silicagel 60 HPTLC plate with concentrating zone (10× 5 cm plate including 2.5×5 cm concentrating zone, 0.2 mm thickness; Merck) was sprayed with 2.5 mL of the boric acid solution and then dried at 110°C for 30 min. The plate was immediately used after preparation.

All of the partial hydrolysis products dissolved in diethyl ether were applied to the concentrating zone, and then the plate was developed in chloroform/acetone (96 : 4, v/v) at room temperature. After spraying with 0.2 % 2′, 7′-dichlorofluorescein-ethanol solution, separations were visualized under ultraviolet radiation. The 1(3)- and 2-MAG were recovered from the absorbents into 3 mL of diethyl ether, and converted to fatty acid methyl esters without removing boric acid.

Fatty acid analysis

Fatty acid methyl esters were prepared by reacting the 1(3)- and 2-MAG in a mixture of dichloromethane (0.1 mL), methyl acetate (4 μL) and 1 M sodium methoxide-methanol solution (4 μL) at 50°C for 20 min (Ando and Takagi, 2003). After adding acetic acid (1 μL) and removing solvents, the products were taken up in hexane. A portion of original TAG (50 μg) was also converted to methyl esters in the same manner.

Gas-liquid chromatography (GLC) of the methyl esters was performed with Shimadzu GC-17A (Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a column Omegawax 250 (30 m×0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, USA). The column temperature was programmed from 90°C (0 min) to 200°C at 10°C/min. Injector and detector temperatures were 250 and 260°C, respectively. Helium was the carrier gas. The samples were loaded onto the column for 2 min under splitless conditions, and then split ratio was held at 50 : 1. Peaks were monitored with Shimadzu C-R3A integrator.

Assignments of each fatty acid to the *sn*-1(3) and *sn*-2 positions of TAG were obtained from peak area ratio of each fatty acid to 19 : 0 formed from the trinonadecanoylglycerol internal standard, and the fatty acid composition of each position was calculated on the basis of the assignments.

Results and discussion

Separation of 1(3)- and 2-MAG by boric acid HPTLC

The 1(3)- and 2-MAG formed from the marine oils TAG were clearly resolved into two bands by boric acid HPTLC. Figure 1 shows a chromatogram of the partial hydrolysis products of cod liver/mackerel oil TAG. Rf-values of the 1(3)- and 2-MAG were 0.1 and 0.2, respectively. These components were also clearly separated from other products, diacylglycerols (Rf=0.5), *tert*-alcohols (Rf=0.65), and TAG (Rf=0.8). Similar profiles were observed for the bonito head and seal oils TAG.

In this study, the boric acid HPTLC plate was prepared by spraying with a water/methanol (1 : 1, vol/vol) solution of boric acid. Aqueous solution was also usable for the preparation. However, silicagel layer and concentrating zone often tended to be broken on the plate. When methanol solution was used, 1(3)- and 2-MAG were not sufficiently separated from each other. Mixture of water and methanol seemed the best for preparation of boric acid HPTLC plates.

The yields of 1(3)- and 2-MAG formed from 2 mg of

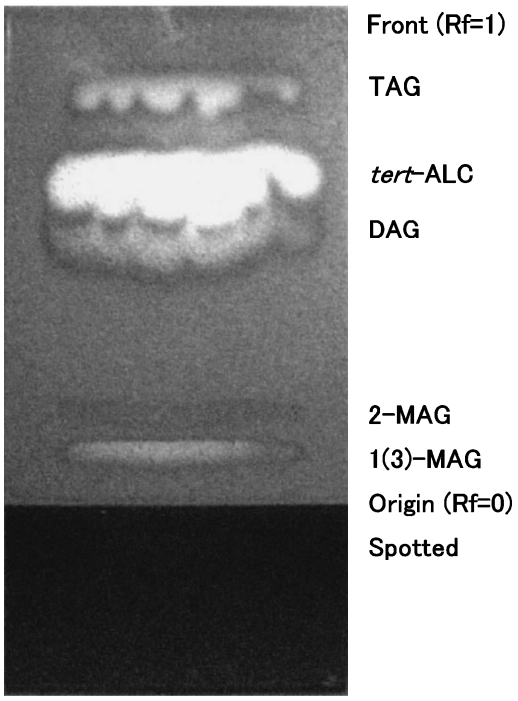

Fig. 1. Boric acid HPTLC profile of the partial hydrolysis products formed from 2 mg of cod liver/mackerel oil TAG.
TAG, triacylglycerols; *tert*-ALC, tertiary alcohols; DAG, diacylglycerols; MAG, monoacylglycerols. HPTLC conditions are as given in the text.

TAG assumed to be up to about 100 μg. It is apparent that the boric acid HPTLC is effective for separation of such levels of 1(3)- and 2-MAG.

Positional distribution of fatty acids in marine oils TAG

Following fatty acid analyses of the 1(3)- and 2-

Table 1 Regiospecific Distribution of Fatty Acids in Marine Oil Triacylglycerols[a] (Mole%)

Fatty acid	Cod liver/mackerel oil			Bonito head oil			Seal oil		
	TAG	*sn*-1(3)	*sn*-2	TAG	*sn*-1(3)	*sn*-2	TAG	*sn*-1(3)	*sn*-2
14 : 0	6.9	7.9	4.8	2.7	2.4	3.4	4.7	2.3	8.9
14 : 1	0.1	0.1	0.1	0.1	0.1	0.1	0.7	0.5	1.2
iso-15 : 0	0.3	0.3	0.2	0.1	0.1	0.1	0.2	0.2	0.2
15 : 0	0.5	0.5	0.4	0.7	0.7	0.6	0.3	0.2	0.5
anteiso-16 : 0	0.2	0.2	0.3	0.0	0.0	0.0	0.0	0.1	0.0
16 : 0	13.5	15.7	9.3	12.7	14.1	9.8	11.7	10.2	14.4
16 : 1n-7	7.2	7.0	7.6	7.3	7.9	5.9	14.9	9.7	24.3
16 : 1n-5	0.4	0.4	0.3	0.2	0.2	0.2	0.3	0.2	0.4
iso-17 : 0	0.3	0.3	0.3	0.2	0.3	0.1	0.3	0.4	0.1
anteiso-17 : 0+16 : 2n-6	0.2	0.1	0.2	0.2	0.1	0.2	0.1	0.1	0.2
16 : 2n-4+phytanic	1.5	1.7	1.0	1.8	1.4	2.7	0.6	0.7	0.4
17 : 0	0.2	0.3	0.2	0.5	0.5	0.3	0.1	0.1	0.2
16 : 3n-4+17 : 1	0.3	0.3	0.5	1.0	0.9	1.2	0.6	0.4	0.9
16 : 3n-3+17 : 1	0.4	0.4	0.5	0.2	0.2	0.2	0.1	0.1	0.1
iso-18 : 0	0.2	0.3	0.1	0.2	0.2	0.1	0.2	0.3	0.1
16 : 4n-1	0.5	0.4	0.7	0.1	0.1	0.2	0.3	0.3	0.3
18 : 0	2.0	2.6	0.9	1.7	2.3	0.4	1.2	1.5	0.7
18 : 1n-9[b]	11.5	11.7	11.1	16.9	21.6	7.0	24.3	21.7	29.3
18 : 1n-7	2.9	3.8	1.3	2.7	3.5	1.2	4.9	5.3	4.0
18 : 1n-5	0.7	0.8	0.3	0.2	0.2	0.2	0.6	0.6	0.6
18 : 2n-6	1.3	1.3	1.3	2.4	2.6	2.1	1.1	0.6	2.0
18 : 2n-4	0.2	0.2	0.1	0.2	0.3	0.2	0.1	0.1	0.2
18 : 3n-3	0.9	0.7	1.1	0.7	1.0	0.1	0.6	0.4	1.0
18 : 4n-3	2.7	2.4	3.4	1.2	1.0	1.6	1.3	1.6	0.8
18 : 4n-1	0.2	0.2	0.3	0.0	0.0	0.0	0.2	0.2	0.2
20 : 0	0.2	0.2	0.1	0.1	0.1	0.0	0.1	0.1	0.0
20 : 1n-11+20 : 1n-13	8.1	8.6	7.3	0.4	0.5	0.3	4.2	5.8	1.3
20 : 1n-9	3.5	3.9	2.6	0.9	1.2	0.3	2.7	3.9	0.6
20 : 1n-7	0.4	0.5	0.1	0.1	0.1	0.1	0.3	0.5	0.1
20 : 1n-5	0.2	0.2	0.1	0.1	0.0	0.0	0.1	0.1	0.1
20 : 2n-6	0.2	0.2	0.4	0.2	0.3	0.1	0.1	0.1	0.0
20 : 4n-6	0.8	0.8	0.9	2.0	1.9	2.3	0.5	0.6	0.3
20 : 3n-3	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.1
20 : 4n-3	0.7	0.7	0.9	0.6	0.7	0.3	0.6	0.8	0.4
20 : 5n-3	8.1	6.3	11.5	8.2	8.5	7.7	6.4	9.1	1.5
22 : 1n-11+22 : 1n-13	11.7	13.0	9.3	0.4	0.6	0.2	1.7	2.3	0.5
22 : 1n-9	1.4	1.5	1.3	0.1	0.1	0.0	0.4	0.6	0.1
22 : 1n-7	0.2	0.3	0.2	0.0	0.0	0.0	0.1	0.1	0.0
21 : 5n-3	0.5	0.3	0.8	0.2	0.2	0.2	0.3	0.5	0.1
22 : 5n-6	0.0	0.0	0.0	1.4	1.2	1.9	0.1	0.1	0.0
22 : 5n-3	1.1	0.4	2.3	1.4	1.3	1.7	4.1	5.8	1.0
22 : 6n-3	6.4	2.8	13.3	28.8	20.8	45.5	8.1	11.1	2.8
24 : 1n-9	0.8	0.2	1.8	0.5	0.4	0.7	0.2	0.3	0.1
Others (<0.1%)	0.7	0.5	0.8	0.5	0.4	0.9	0.6	0.4	0.6

[a] The analysis started with 2 mg of TAG ($n=1$).

[b] Including 18 : 1n-11 isomer in the seal oil TAG.

MAG, positional distributions of fatty acids in TAG were calculated. Table 1 shows the results in the form of fatty acid compositions of the *sn*-1(3) and *sn*-2 positions in TAG.

In the cod liver/mackerel oil, major fatty acids at more than 5 mole% of total fatty acids were 14 : 0, 16 : 0, 16 : 1n-7, 18 : 1n-9, 20 : 1n-11+20 : 1n-13, 20 : 5n-3, 22 : 1n-11+22 : 1n-13 and 22 : 6n-3. Of these fatty acids, 14 : 0, 16 : 0, 22 : 1n-11+22 : 1n-13 showed higher concentrations in the *sn*-1(3) position, whereas 20 : 5n-3 and 22 : 6n-3 were higher in the *sn*-2 position. The other acids, 16 : 1n-7, 18 : 1n-9, 20 : 1n-11+20 : 1n-13, were almost even in the two positions.

Bonito head oil is characterized by high content of 22 : 6n-3. This acid was higher in the *sn*-2 position. Other major fatty acids (>5 mole%) were 16 : 0, 16 : 1n-7, 18 : 1n-9, and 20 : 5n-3. Concentrations of 16 : 0, 16 : 1n-7 and 18 : 1n-9 were higher in the *sn*-1(3) position, and 20 : 5n-3 was even in the two positions.

In the seal oil TAG, 16 : 0, 16 : 1n-7, 18 : 1n-9+18 : 1n-11, 20 : 5n-3 and 22 : 6n-3 were the major fatty acids (>5 mole%). The former three fatty acids were higher in the *sn*-2 position, whereas 20 : 5n-3 and 22 : 6n-3 were found in the *sn*-1(3) position at much higher concentrations.

The three marine oils contained 22 : 6n-3 (DHA) at the concentrations of 6.4-28.8 mole%. Concentrations of this acid observed in the *sn*-1(3) and *sn*-2 positions were 2.8 and 13.3 mole% (cod liver/mackerel), 20.8 and 45.5 mole% (bonito head), and 11.1 and 2.8 mole% (seal), respectively. In general, 22 : 6n-3 is rich in the *sn*-2 position of marine fish TAG and in the *sn*-1(3) position

Table 2 Comparison of the Regiospecific Analyses Starting with 2 and 100 mg of Marine Oil Triacylglycerols (TAG) (Mole%)

Major fatty acid	2 mg of TAG[a)]			100 mg of TAG[b)]		
	TAG	*sn*-1(3)	*sn*-2	TAG	*sn*-1(3)	*sn*-2
Cod liver/mackerel oil						
14 : 0	6.9	7.9	4.8	7.6	8.8	5.2
16 : 0	13.5	15.7	9.3	13.0	15.5	8.1
16 : 1n-7	7.2	7.0	7.6	7.3	7.6	6.9
18 : 1n-9	11.5	11.7	11.1	10.7	11.0	10.1
20 : 1n-11+20 : 1n-13	8.1	8.6	7.3	7.4	8.1	6.2
20 : 5n-3	8.1	6.3	11.5	9.6	6.7	15.2
22 : 1n-11+22 : 1n-13	11.7	13.0	9.3	10.8	12.5	7.7
22 : 6n-3	6.4	2.8	13.3	7.3	2.2	16.9
Bonito head oil						
14 : 0	2.7	2.4	3.4	3.2	2.8	3.8
16 : 0	12.7	14.1	9.8	12.3	13.5	9.4
16 : 1n-7	7.3	7.9	5.9	7.2	7.8	5.9
18 : 1n-9	16.9	21.6	7.0	15.8	19.9	7.0
18 : 1n-7	2.7	3.5	1.2	2.8	3.5	1.3
18 : 2n-6	2.4	2.6	2.1	2.4	2.5	2.3
20 : 5n-3	8.2	8.5	7.7	8.1	8.3	8.0
22 : 6n-3	28.8	20.8	45.5	29.3	22.1	44.5
Seal oil						
14 : 0	4.7	2.3	8.9	5.0	2.2	10.1
16 : 0	11.7	10.2	14.4	11.3	9.4	14.9
16 : 1n-7	14.9	9.7	24.3	14.3	9.0	23.7
18 : 1n-9+18 : 1n-11	24.3	21.7	29.3	22.3	19.3	27.8
18 : 1n-7	4.9	5.3	4.0	4.9	5.2	4.4
20 : 1n-11+20 : 1n-13	4.2	5.8	1.3	4.3	6.2	1.2
20 : 5n-3	6.4	9.1	1.5	6.6	9.8	0.9
22 : 6n-3	8.1	11.1	2.8	8.7	12.9	1.2

[a)] Analyzed in the present study.
[b)] Calculated from the data reported by Ando et al. (2000) (bonito head oil) and Ando et al. (2004) (cod liver/mackerel and seal oils).

of sea mammal TAG (Brockerhoff et al., 1968; Litchfield, 1968). Distribution patterns of 22 : 6n–3 observed in this study were the same with those of the general tendency.

Comparison with the analysis starting with 100 mg of TAG

Previously positional distribution of fatty acids in the same marine oils was investigated by regiospecific analysis as a part of stereospecific analysis (Ando et al., 2000, 2004). The analysis started with 100 mg of TAG, and 1(3)– and 2–MAG formed from the TAG were isolated by ordinary boric acid TLC (10 wt% of boric acid to Silicagel 60G, 20×20 cm plate, 0.5 mm thickness).

Table 2 compares the distributions found in the present analysis (2 mg) and previous analysis (100 mg). In all three oils, fatty acid compositions of the *sn*–1(3) and *sn*–2 positions were similar between the 2– and 100–mg analyses. For example, the concentrations of 16 : 0, 18 : 1n–9 and 22 : 6n–3 observed in the 2–mg analysis of bonito head oil TAG were 14.1, 21.6 and 20.8 mole% in the *sn*–1(3) position and 9.8, 7.0 and 45.5 mole% in the *sn*–2 position, respectively. These values were close to those obtained by the 100–mg analysis, i.e., 13.5, 19.9 and 22.1 mole% [*sn*–1(3) position] and 9.4, 7.0 and 44.5 mole% [*sn*–2 position]. Fatty acids of the seal oil also resembled in compositions between the two analyses. In the cod liver/mackerel oil, somewhat lower values were observed for 20 : 5n–3 and 22 : 6n–3 in the *sn*–2 position of the 2–mg analysis. However, these fatty acids were also lower in the TAG used for the 2–mg analysis than those for the 100–mg analysis. It is probable that these resulted from different preparations of TAG subjected to the two analyses. Similarity in the analytical results observed for all marine oils indicates a view that positional distribution obtained by the present procedure is not different from that obtained by the analysis of 100 mg of TAG.

Advantages of regiospecific analysis using boric acid HPTLC

In the present study, 2 mg of TAG were subjected to regiospecific analysis using boric acid HPTLC. The results were similar to those previously obtained by the analysis of 100 mg of TAG. The three oils applied to the present analysis were typical and popular marine oils originating from fish and sea mammals. Distribution patterns of 22 : 6n–3 observed were also similar to those of general tendency. Therefore, it is concluded that the new procedure is usable for regiospecific analysis of a few milligrams of marine oils TAG. Such level is comparable to that of some other methodologies, e.g. enzymatic method of Luddy et al. (1964) (5 mg of starting TAG) and chemical method of Becker et al. (1993) (6.0–6.5 mg).

The use of boric acid HPTLC has also another advantage. The analysis can be carried out with small apparatuses and much less use of organic solvents. The present procedure is also a rapid, convenient, and harmless procedure for regiospecific analysis of marine oil TAG.

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