Analysis of Regiospecific Distribution of FA of TAG Using the Lipase-Catalyzed Ester-Exchange

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ABSTRACT: A very simple and versatile GC method has been developed that can be utilized for quick analysis, in many samples, of the FA compositions at the *sn*-2- and *sn*-1,3-positions of TAG. By using the lipase-catalyzed, *sn*-1,3-regioselective ester-exchange reaction of TAG with ethyl acetate, followed by direct injection of the crude reaction mixture into the GC apparatus without any pretreatment, the FA located at the *sn*-2-position could accurately be analyzed as a TAG derivative in which the *sn*-1,3-positions were substituted by an acetate residue. Furthermore, the FA located at the *sn*-1,3-positions could simultaneously be analyzed as the corresponding ethyl ester derivatives using this method. The reliability of the analytical method was compared with conventional methods by analyzing the TAG consisting of caprylic acid (C) at the *sn*-2-position and oleic acid (O) at the *sn*-1,3-positions, giving comparable analytical results. The present method was applied to the analysis of the structured lipids CCD and CCE, consisting of TAG as a major component in which C and the highly unsaturated FA, DHA (D) or EPA (E), were specifically bound at the *sn*-2- and the *sn*-1,3-positions, respectively.

Paper no. J10421 in *JAOCS* **80**, 353–356 (April 2003).

KEY WORDS: Caprylic acid, DHA, EPA, ester-exchange, esterification, lipase, regiospecific distribution of fatty acids in triacylglycerols, structured lipids.

Recent studies have investigated structured lipids in which the location of the FA in the acylglycerol molecule is specified. Structured lipids containing highly unsaturated FA, such as EPA and DHA, have been extensively studied in order to capitalize on the health-promoting qualities of these FA (1–4). Development of analytical methodology to determine the purity and to identify the structure of structured lipids is very important for basic studies and the production of structured lipids consisting of TAG. A convenient and versatile method is necessary to extend the results of laboratory studies toward practical use.

GC methods (5–8), and HPLC methods using the ODS column (9,10), generally have been used for analysis of the TAG molecular species composition of a fat. However, it is difficult to distinguish the regioisomers arising from the different binding positions *sn*-1,3/*sn*-2 of the FA using these conventional methods. With regard to the method for separation of the regioisomers, HPLC methods using silver-ion columns (11–14) and chiral column (15) have been reported, but these methods are not highly versatile.

An enzymatic GC method and a ^{13}C NMR spectroscopic method have been proposed for analysis of the molecular structures of structured lipids. In the ^{13}C NMR spectroscopic method (16,17), the signals of the olefinic and carbonyl regions of the target fat are measured by the magnified spectral method, and the proportion of the FA located at the *sn*-1,3-/*sn*-2-positions are determined from chemical shifts. However, in comparison with the enzymatic GC method, as will be discussed later, the ^{13}C NMR method has drawbacks in its current state because it requires a high-resolution NMR spectrometer and because linoleic acid and linolenic acid are not resolved.

In the enzymatic GC method (18), FA are analyzed using GC after partial hydrolysis of the fat using an *sn*-1,3-regiospecific lipase, followed by separation of the resulting *sn*-2-MAG by TLC, followed by methyl esterification. This method does not require any special instruments and is highly versatile for analysis of any kind of fat that can be degraded by lipase action, but it has the drawback that somewhat complicated sequential procedures such as hydrolysis, TLC, and GC analysis are required. Furthermore, there is concern about the possible reduction in the analytical accuracy caused by the intramolecular rearrangement of FA in the DAG produced as intermediates in the enzymatic hydrolysis reaction.

We investigated a method for analysis of the *sn*-2 FA composition that is not only convenient and versatile but also free from concern about possible intramolecular rearrangement of the FA components, since it does not result in any DAG intermediates.

EXPERIMENTAL PROCEDURES

Substrates and lipases. Tricaprylin of 99% purity from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) was used for this study. Rapeseed oil from The Nisshin Oil Mills, Ltd. (Tokyo, Japan) was used. Oleic acid from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) was used after purification by distillation. DHA and EPA of 98% purity from Nippon Chemical Reagents, Ltd. (Hakodate, Japan) were used. For the lipase enzymes, Lipase QL (*Alcaligenes* sp.) from Meito Sangyo Co., Ltd. (Nagoya, Japan), pancreatin (hog pancreas) from Wako Pure Chemical Industries, Ltd., and the immobilized lipase Novozym 435 (*Candida antarctica*) from Novozymes Japan Ltd. (Chiba, Japan) were used. Other reagents used in

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this study were commercially available, the purity of which were tested by the Specific Gravity method.

Preparation of TAG. The TAG POP, bearing palmitic acid (P) at the *sn-*1,3 positions and oleic acid (O) at the *sn-*2-position, respectively, was prepared by fractionation of cacao fat by HPLC using a silica gel column.

The structured fat CCO, where C represents caprylic acid, was prepared as in our previous report (19). Oleic acid (540 g) was added to tricaprylin (100 g), and after addition of Lipase QL (0.9 g), the mixture was allowed to react at 40°C for 3.5 h. After the reaction, the FA were removed by phase separation with *n*-hexane/acetonitrile, and the remaining polar substances, consisting of DAG and FA, in the *n*-hexane phase were further removed using silica gel column chromatography, giving CCO.

The structured fat CCE, where E represents EPA, and caprylic acid, specially bearing EPA at the *sn-*1- or *sn-*3-positions (Sigma-Aldrich Japan K.K., Tokyo, Japan), of the TAG, was prepared as follows. EPA (95 g) was added to tricaprylin (50 g), and, after bubbling nitrogen into the mixture for 10 min to make an adequate nitrogen atmosphere, Novozym 435 (2.9 g) was added and the mixture was allowed to react at 60°C for 23 h. After the reaction, the FA were removed by phase separation with *n*-hexane/acetonitrile, giving CCE.

The structured fat CCD, consisting of DHA (D) and caprylic acid (C), specially bearing DHA at the *sn-*1- or *sn-*3-positions of the TAG, was prepared as follows. DHA (150 g) was added to tricaprylin (50 g), and, after bubbling nitrogen into the mixture for 10 min to make an adequate nitrogen atmosphere, Novozym 435 (4 g) was added and the mixture was allowed to react at 60°C for 25 h. After the reaction, the FA were removed by phase separation with *n*-hexane/acetonitrile, giving CCD.

Analysis of the compositions of TAG and DAG. The compositions of the TAG and DAG were determined using GC with a DB-1ht (0.32 mm × 0.1 μm × 5 m) column (Agilent Technologies), under the following analytical conditions: The injection temperature was 370°C, the detector temperature was 370°C, the column temperature was raised from 200 to 370°C at a rate of 15°C/min, the split ratio was 50:1, and helium was used as the carrier gas with a constant flow rate of 6.0 mL/min. The composition of the FA was determined by calculating the corresponding percentage of the peak area.

Determination of FA at the sn-*2-position by the enzymatic ester-exchange method.* To ethyl acetate (1 mL) were added the fat sample (30 μL) and Novozym 435 (5–10%, wt/vol), and the ester-exchange reaction was carried out at room temperature (25°C) for 1–6 h using a magnetic stirrer. After the reaction, the reaction mixture was directly injected onto a DB-1ht (0.32 mm × 0.1 μm × 5 m) GC column (Agilent). The analytical conditions were as follows: The injection temperature was 370°C, the detector temperature was 370°C, the split ratio was 50:1, and helium was used as the carrier gas with a constant flow rate of 6.0 mL/min.

Determination of FA at the sn-*2-position by the enzymatic hydrolysis method.* Hydrolysis was performed according to AOCS Official Method Ch 3-91. The oil sample was purified by preparative TLC. The enzymatic reaction was carried out for 3 min at 40°C using pancreatin lipase. The obtained MAG was methylated by esterification with sulfuric acid/methanol. The GC analytical conditions were as follows: SP2340™ column (Sigma-Aldrich Japan K.K., Tokyo, Japan), and an FID detector. The column temperature was raised from 150 to 210°C (2.5°C/min), the injection temperature was 230°C, and the detector temperature was 240°C.

Determination of FA at the sn-*2-position by the NMR method.* By using the ^{13}C NMR magnified spectral method (20), the peaks corresponding to the carbonyl carbon atoms of the *sn-*2 oleic acid and caprylic acid were measured, and the FA composition was determined based on the peak height. The conditions of the NMR measurement were as follows: A Varian Gemini 2000 ™ (400 MHz) NMR spectrometer (Varian Technologies Japan Ltd., Tokyo, Japan) was used, and the sample concentration was 20% in CDCl$_3$ solution. The relaxation time was 40 s with a pulse width of 90 degrees, the number of scans was 1,008, the spectral width was 120–180 ppm, the acquisition time was 10 s, and the temperature was at 30°C.

Determination of the sn-*2-position FA by the HPLC method.* According to the method reported by Adlof (14), the HPLC determination of the FA at the *sn-*2-position was carried out under the following conditions; Chrom-Spher Lipids™ column (from Varian Inc., Middelburg, The Netherlands), 250 × 4.6 mm, UV detector at 206 nm, and *n*-hexane containing 0.5% of acetonitrile as a mobile phase with a flow rate at 1.0 mL/min.

RESULTS AND DISCUSSION

Lipase-catalyzed reactions in organic solvents have long been studied, but very few have been reported to exhibit lipase activity in highly polar solvents such as ethanol, acetic acid, and ethyl acetate (21). However, Novozym 435 exhibited very high activity in ethanol and acetic acid, especially in ethyl acetate (22). We considered that TAG bearing acetate residues at the *sn-*1,3-positions would be distinguishable by the FA residue located at the *sn-*2-position and, at the same time, that the FA eliminated from the *sn-*1,3-positions of the TAG could be individually separated from each other by GC analysis if we could conduct the enzyme-catalyzed ester-exchange reaction between TAG and ethyl acetate in a *sn-*1,3-regioselective manner.

Accordingly, we examined the regioselectivity of the ester-exchange reaction of TAG and ethyl acetate using Novozym 435. To 1 mL of ethyl acetate was added 100 mg of the TAG POP, in which palmitic acid is bound specifically at the *sn-*1,3-positions, and 5% of Novozym 435, and the ester-exchange reaction was carried out at room temperature (25°C) with magnetic stirring. If the ester exchange reaction occurred at the *sn-*1,3-positions, ethyl palmitate would be produced, and if it occurred at the *sn-*2-position, ethyl oleate would be produced instead. The time course of formation of ethyl palmitate and ethyl oleate is shown in Table 1. In this reaction, almost all the POP TAG disappeared after 24 h, being converted to a TAG in which 2 mol of acetic acid and 1 mol of

TABLE 1
Time Course of Formation of Ethyl Palmitate to Ethyl Oleate in the Ester-Exchange Reaction of POP and Ethyl Acetate Using Novozym 435

Reaction time (h)	Ethyl palmitate (%)	Ethyl oleate (%)
0	0.0	0.0
0.5	100	0.0
1	100	0.0
3	100	0.0
6	96.8	3.2
24	93.0	7.0

TABLE 2
Purity and *sn*-1,3/*sn*-2-Positional Selectivity of the Targeted Structured Lipids

		TAG types (%)			
TAG	Caprylic/DHA or EPA at *sn*-2 (%)	2CCE or 2CCD[a]	ECE or DCD[b]	CCC	Unknown
CCE	96.9/3.1	68.0	23.0	7.9	1.1
CCD	89.5/10.5	71.0	2.6	12.4	14.4

[a]2CE, dicapryloyl-eicosapentaenoylglycerol; 2CD, dicapryloyl-docosahexaenoyl.
[b]2EC, dieicosapentaenoyl-capryloylglycerol; 2DC, didocosahexaenoyl-capryloylglycerol.

oleic acid were incorporated, respectively. From this table, it was concluded that the ester-exchange reaction occurred at the *sn*-1,3-positions of the TAG with a very high selectivity under the reaction conditions. The results could be attributed not only to the high selectivity of the enzyme used but also to the fact that the intramolecular rearrangement of the FA did not occur under the analytical conditions because MAG and DAG were produced to an extent less than a few percent in the ester-exchange reaction conditions. Therefore, it is conceivable that the FA located at the *sn*-1,3-positions could be determined as the corresponding ethyl esters simultaneously with the FA located at the *sn*-2-position of the TAG, in which two acetate residues are bound at the *sn*-1,3-positions. In this method, complicated sequential procedures such as TLC separation and extraction are avoided, and the analytical time is shortened.

We analyzed the FA compositions at the *sn*-2-position using the structured fat CCO and the enzymatic ester-exchange method. The structured fat CCO consisted of 89.4% dicapryloyl-oleoylglycerol (2CO), 4.6% tricaprylin (CCC), 5.0%
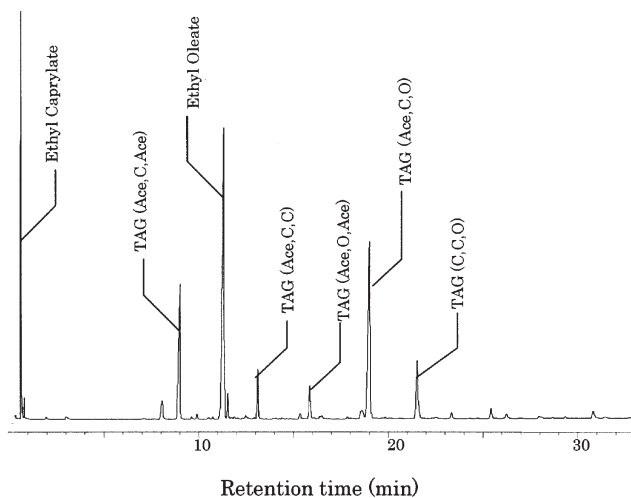
FIG. 1. Gas chromatogram of the reaction mixture of structured fat CCO subjected to the enzymatic ester-exchange method for 2 h. Conditions: The column was a DB-1ht (0.32 mm × 0.1 μm × 5 m; Agilent), the injection temperature was 370°C, the detector temperature was 370°C. The column temperature was raised from 50 to 370°C (10°C/min), the split ratio was 50:1, and helium was used as the carrier gas with a constant flow rate 6.0 mL/min. Each peak was identified by comparison with corresponding reference material and also by determination using other analytical methods. C, caprylic acid; O, oleic acid; Ace, acetic

dioleoyl-capryloylglycerol (2OC), and 1.0% unknown. The relative ratio of caprylic acid to oleic acid in the FA esterified at the *sn*-2-position was 83.8:16.2 (%/%) determined from the ratio of the peak areas of the TAG (Acetate, C, Acetate) and TAG (Acetate, O, Acetate). The chromatogram is shown Figure 1.

Next, for the structured fat CCO, the relative ratio of oleic acid to caprylic acid in the FA esterified at the *sn*-2-position was calculated using the enzymatic hydrolysis, NMR, and HPLC methods. The results were: enzymatic ester-exchange, 83.8:16.2 (caprylic/oleic at *sn*-2, expressed as %/%); enzymatic hydrolysis, 88.8/11.2; NMR, 90.7:9.3. It is evident that results obtained by means of the enzymatic ester-exchange method using the regioselective lipase are consistent with those obtained by the other analytical methods. Although variations due to FA specificity cannot be completely eliminated under the present analytical conditions, MAG and/or DAG levels were less than a few percent. Therefore, the perturbation of the analytical values caused by intramolecular rearrangement of the FA could be eliminated, providing a very convenient and time-saving method that might be useful for analyzing many test samples.

The acylglycerols CCD and CCE were prepared and analyzed by the enzymatic ester-exchange method. The results are shown in Table 2. The results indicate that the structured fat that was prepared using the enzymatic ester-exchange reaction contained the highly unsaturated FA residues DHA and EPA specifically located at the *sn*-1- or 3-positions.

Next, as an application of the enzymatic ester-exchange method to analysis of common oil, rapeseed oil was analyzed and the analytical data were compared with those obtained by the enzymatic hydrolysis method. In this experiment, only ethyl esters that were produced by ester-exchange at the *sn*-1,3-positions could be resolved by GC because of small M.W. differences of the FA contained in rapeseed oil. Furthermore, as shown in Figure 2, ethyl linoleate and ethyl linolenate were not resolved from each other. However, in regard to the analyzable FA compositions, almost the same result as that of the enzymatic hydrolysis method was obtained (Table 3), although the analytical accuracy was found to be worse. As improvements in accuracy can be expected in the future with upgrading of separation performance of GC columns, this convenient analytical method using the enzymatic ester-exchange reaction might be useful for analysis of FA compositions of various kinds of TAG.
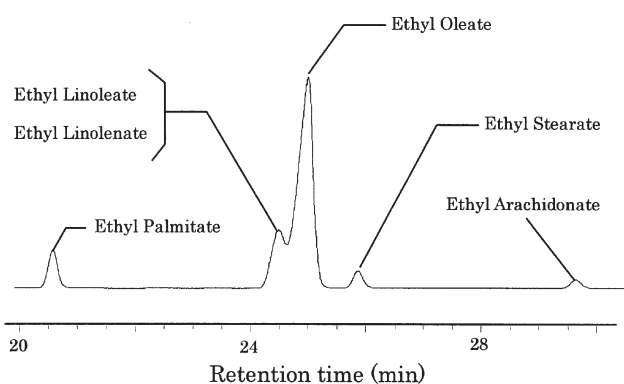
FIG. 2. Gas chromatogram of the reaction mixture of rapeseed oil subjected to the enzymatic ester-exchange method for 3 h. Conditions: The column was a DB-1ht (0.32 mm × 0.1 μm × 5 m; Agilent), the injection temperature was 370°C, the detector temperature was 370°C. The column temperature was raised from 50 to 370°C (3°C/min), the split ratio was 50:1, and helium was used as the carrier gas with a constant flow rate of 6.0 mL/min. Each peak was identified by comparison with corresponding reference material and also by determination using other analytical methods.

TABLE 3
Comparison of the *sn*-1,3-Acyl Groups in Rapeseed Oil as Obtained by the Enzymatic Ester-Exchange and Enzymatic Hydrolysis Methods[a]

	sn-1,3-Position (%)	
Acyl group	Enzymatic ester-exchange	Enzymatic hydrolysis
14:0	Not detected	0.2
16:0	7.8	6.4
16:1	Not detected	0.3
17:0	Not detected	0.3
18:0	3.6	3.1
18:1	69.8	67.7
18:2	16.9	12.8
18:3		4.9
20:0	1.9	1.1
20:1	Not detected	2.0
24:0	Not detected	0.3
24:1	Not detected	0.3

[a]Calculated by *sn*-2-acyl group and total acyl group.

ACKNOWLEDGMENT

REFERENCES

1. Shimada, Y., A. Sugihara, K. Maruyama, T. Nagano, S. Nakayama, H. Nakano, and Y. Tominaga, Production of Structured Lipid Containing Docosahexaenoic and Caprylic Acids Using Immobilized *Rhizopus delemar* Lipase, *J. Ferment. Bioeng. 81*:299–303 (1996).
2. Irimescu, R., K. Hata, Y. Iwasaki, and T. Yamane, Comparison of Acyl Donors for Lipase-Catalyzed Production of 1,3-Dicapryloyl-2-eicosapentaenoylglycerol, *J. Am. Oil Chem. Soc. 78*:65–70 (2001).
3. Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki, and T. Yamane, Utilization of Reaction Medium-Dependent Regiospecificity of *Candida antarcica* Lipase (Novozym 435) for the Synthesis of 1,3-Dicapryloyl-2-docosahexaenoyl (or eicosapentaenoyl) Glycerol, *Ibid. 78*:285–289 (2001).
4. Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki, and T. Yamane, Two-Step Enzymatic Synthesis of Docosahexaenoic Acid-Rich Symmetrically Structured Triacylglycerols *via* 2-Monoacylglycerols, *Ibid. 78*:743–748 (2001).
5. Takagi, T., and Y. Itabashi, Gas Chromatographic Separation of Triacylglycerols Based on Their Degree of Unsaturation, *Lipids 12*:1062–1068 (1977).
6. Frega, N., F. Bocci, and G. Lercker, High-Resolution Gas-Chromatographic Determination of Diacylglycerols in Common Vegetable Oils, *J. Am. Oil Chem. Soc. 70*:175–177 (1993).
7. Sassano, G.J., and B.S.J. Jeffrey, Gas Chromatography of Triacylglycerols in Palm Oil Fractions with Medium-Polarity Wide-Bore Columns, *Ibid. 70*:1111–1114 (1993).
8. Lin, S.W., and N.W. Lam, Analysis of Lipids in Palm Oil by On-Column Capillary Gas–Liquid Chromatography, *J. Chromatogr. Sci. 32*:185–189 (1994).
9. Bergqvist, M.H.J., and P. Kaufmann, A Multivariate Optimization of Triacylglycerol Analysis by High-Performance Liquid Chromatography, *Lipids 28*:667–675 (1993).
10. Murui, T., and J. Shouge, Detemination of Triacylglycerol Composition of Vegetable Oil and Application to Identifying the Components of Oil Admixture, *Yukagaku 45*:29–36 (1996).
11. Smith, E.C., A.D. Jones, and E.W. Hammond, Investigation of the Use of Argentation High-Performance Liquid Chromatography for the Analysis of Triacylglycerols, *J. Chromatogr. 188*:205–212 (1980).
12. Takano, S., and Y. Kondo, Triacylglycerol Analysis by Combined Argentation/Nonaqueous Reversed Phase High-Performance Liquid Chromatography, *J. Am. Oil Chem. Soc. 64*:380–383 (1987).
13. Jeffrey, B.S.J., Silver-Complexation Liquid Chromatography for Fast, High-Resolution Separations of Triacylglycerols, *Ibid. 68*:289–293 (1991).
14. Adlof, R.O., Analysis of Triacylglycerol Positional Isomers by Silver Ion High-Performance Liquid Chromatography, *J. High Resolut. Chromtogr. 18*:105–107 (1995).
15. Takagi, T., and Y. Ando, Stereospecific Analysis of Triacyl-*sn*-glycerols by Chiral High-Performance Liquid Chromatography, *Lipids 26*:542–547 (1991).
16. Wollenberg, K.F., Quantitative High-Resolution ^{13}C Nuclear Magnetic Resonance of the Olefinic and Carbonyl Carbons of Edible Vegetable Oils, *J. Am. Oil Chem. Soc. 67*:487–494 (1990).
17. Miyake, U., and K. Yokomizo, Determination of 2-Acyl Positional Fatty Acid Distribution in Vegetable Oils by High-Resolution ^{13}C Nuclear Magnetic Resonance Spectroscopy, *Yukagaku 47*:179–185 (1998).
18. Brockerhoff, H., Stereospecific Analysis of Triacylglycerols: An Alternative Method, *J. Lipid Res. 8*:167–169 (1967).
19. Negishi, S., S. Shirasawa, Y. Arai, J. Suzuki, and S. Mukataka, Activation of Powdered Lipase by Cluster Water and the Use of Lipase Powders for Commercial Esterification of Food Oils, *Enzyme Microb. Technol. 32*:66–70 (2003).
20. Miyake, Y., K. Yokomizo, and N. Matsuzaki, Determination of Unsaturated Fatty Acid Composition by High-Resolution Nuclear Magnetic Resonance Spectroscopy, *J. Am. Oil Chem. Soc. 75*:1091–1094 (1998).
21. Dordick, J.S., Enzymatic Catalysis in Monophasic Organic Solvents, *Enzyme Microb. Technol. 11*:194–211 (1989) .
22. Irimescu, R., M. Yasui, Y. Iwasaki, N. Shimizu, and T. Yamane, Enzymatic Synthesis of 1,3-Dicapryloyl-2-eicosapentaenoylglycerol, *J. Am. Oil Chem. Soc. 77*:501–506 (2000).