Regiospecific Analysis of Triacylglycerols Using Allyl Magnesium Bromide

Claus C. Becker*, Annemette Rosenquist and **Gunhild Hølmer**
Department of Biochemistry and Nutrition, Technical University of Denmark, DK-2800 Lyngby, Denmark

A method for the regiospecific analysis of triacylglycerols (TAG), using the Grignard reagent allyl magnesium bromide (AMB) to partially deacylate TAG, is described. 1,3-Distearoyl-2-oleoyl-glycerol (SOS) and 1,3-didecanoyl-2-palmitoyl-glycerol (CPC) were reacted with AMB. From the resulting mixture, the four different classes of partial acylglycerols and TAG were isolated, and the mole ratios between stearic acid and oleic acid, or decanoic acid and palmitic acid, respectively, were determined in each fraction. Different approaches of calculating the composition of the fatty acids in positions *sn*-1(3) and *sn*-2 of the original TAG were compared. For the *sn*-2 position, the best estimate was the direct determination of the fatty acid composition of 2-monoacylglycerol (MAG). Mole percentages of stearic acid and decanoic acid in the *sn*-1(*sn*-3) positions of SOS and CPC, respectively, were most accurately estimated from the fatty acid compositions of TAG and 2-MAG according to the formula: $1.5 \times \text{TAG} - 0.5 \times 2\text{-MAG}$. Using AMB and the present method of calculation, the results obtained were more accurate and showed smaller standard deviations than those obtained using other common deacylating agents, such as ethyl magnesium bromide or pancreatic lipase.
***Lipids* 28, 147-149 (1993).**

The distribution of fatty acids between the primary and secondary positions in triacylglycerols (TAG) can be determined either after pancreatic lipase degradation or after chemical degradation using a Grignard reagent (1,2). Both methods are based on the assumption that partial deacylation of TAG leads to representative mixtures of diacylglycerols (DAG) and monoacylglycerols (MAG), which can be characterized by chromatographic analysis. These methods, however, have certain limitations. The lipase assay is not reliable for TAG which contain significant amounts of short- or medium-chain fatty acids (C_{10} or less) or very long-chain polyunsaturated fatty acids (PUFA) (1). Milk fat, for instance, will not give representative acylglycerols as short- and medium-chain fatty acids are more readily hydrolyzed by the pancreatic lipase than are long-chain fatty acids (2). Some PUFA, on the other hand, have been reported to resist hydrolysis by lipase (3-5); therefore, marine oils should not be analyzed by the lipase assay either.

The reaction of TAG with ethyl magnesium bromide (EMB) can be used to obtain representative mixtures of DAG (6). However, the MAG are easily isomerized and cannot be used for regiospecific analysis of TAG. Therefore the mole percentages of the fatty acids in the *sn*-1(*sn*-3) and *sn*-2 positions are calculated from the composition of TAG and 1,2(2,3)-DAG. In the present communication it is shown that the more reactive Grignard reagent allyl magnesium bromide (AMB) yields representative 2-MAG, allowing the composition of the native TAG *sn*-2 position to be determined directly. The composition in the *sn*-1(*sn*-3) position can then be estimated from the composition of 2-MAG and TAG. The estimates obtained using AMB compare favorably to those found using EMB as they come closer to the theoretical value and have smaller standard deviations.

MATERIALS AND METHODS

1,3-Distearoyl-2-oleoyl-*sn*-glycerol (SOS) (99%+) was purchased from Sigma Chemical Company (St. Louis, MO). 1,3-Didecanoyl-2-palmitoyl-*sn*-glycerol (CPC) was synthesized according to Redgrave *et al.* (7). The structure of 1,3-didecanoyl-*sn*-glycerol was verified by ^{1}H nuclear magnetic resonance. Silicic acid 60G thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) impregnated with boric acid were used for separation of the different acylglycerol species resulting from the action of AMB on TAG. The plates were prepared by spraying with a 0.4 M aqueous boric acid solution until saturated. Then, the plates were dried at room temperature overnight and kept in a desiccator over a drying agent. Diethyl ether, analytical grade, was redistilled immediately before use. During distillation, the ether was protected from the moisture by a $CaCl_2$-tube. AMB in diethyl ether (2M) was synthesized according to Gilman and McGlumphy (8). Methanol and hexane were high-performance liquid chromatography-grade and obtained from Rathburn Chemicals (Walkerburn, Scotland). Anhydrous Na_2SO_4, 37% HCl (aq.), and boric acid were purchased from Merck. Na_2SO_4 was dried at 130°C for at least 4 h before use. Dichlorofluorescein and tripalmitoylglycerol were from Sigma.

The TAG to be analyzed (6.0 to 6.5 mg) was dissolved in diethyl ether (5 mL) in a Teflon capped reaction tube with magnetic stirring. AMB (200 μL) was added with a pipette that had been flushed with nitrogen; the diethyl ether solution became opaque, indicating a spontaneous reaction. After one minute, additional diethyl ether (5.0 mL) was added. Then the organic phase was washed, first with an acidic buffer (4.0 mL) prepared by adding 37% HCl (1.0 vol) to a 0.4 M boric acid solution (36 vol) giving a final concentration of 0.27 M. This neutralized the $Mg(OH)_2$ formed in the reaction mixture upon addition of water. Additional washings (2×4.0 mL) with a 0.4 M boric acid solution removed excess HCl and remaining magnesium salts. The ether phase was dried briefly with about 2 g of anhydrous Na_2SO_4 before being decanted and evaporated *in vacuo*. The acylglycerol mixture was redissolved in chloroform (400 μL).

The chloroform solution was applied to a boric acid impregnated TLC plate which was developed in chloroform/acetone (96:4, vol/vol) in a saturated chamber. The fractions were visualized by spraying with 2,7-dichlorofluorescein (0.1% wt/vol) in anhydrous ethanol. The following bands were observed: 1,3-MAG ($R_f = 0.12$); 2-MAG ($R_f = 0.20$); 1,2(2,3)-DAG ($R_f = 0.50$); 1,3-DAG ($R_f = 0.62$);

*To whom correspondence should be addressed.

Abbreviations: AMB, allyl magnesium bromide; CPC, 1,3-didecanoyl-2-palmitoyl-*sn*-glycerol (10:0/16:0/10:0); DAG, diacylglycerol; EMB, ethyl magnesium bromide; MAG, monoacylglycerol; PUFA, polyunsaturated fatty acid; SOS, 1,3-distearoyl-2-oleoyl-*sn*-glycerol (18:0/18:1/18:0); TA, tertiary alcohols; TAG, triacylglycerol, TLC, thin-layer chromatography.

tertiary alcohols of the deacylated fatty acids (TA) (R_f = 0.70); and TAG (R_f = 0.82). All bands except TA were scraped off. The acylglycerols were extracted from the silicic acid with diethyl ether (2 × 2 mL). After brief centrifugation, the ether was decanted and evaporated under a stream of N_2. The residue was methylated using the alkaline transesterification procedure described by Christie (9). The methyl esters from SOS were analyzed on a Hewlett-Packard (Palo Alto, CA) Gas Chromatograph HP 5890A, equipped with a flame-ionization detector, using a HP 7673A Automatic Sampler. The fused silica column (30 m in length and 0.32 mm i.d.) coated with SP2330 (film thickness 0.25 μm) was from Supelco (Bellefonte, PA). A helium flow rate of 34 mL/min and a split ratio of 1:20 were used. The temperature of the oven was initially 140°C and was increased at a rate of 3.0°C/min until the final temperature (200°C) was reached. The methyl esters from CPC were analyzed on a Hewlett-Packard Gas Chromatograph HP 5830A equipped with a flame-ionization detector and a column (2.75 m × 2 mm) containing 10% SP 2330 on 100/120 Chromosorb WAW (on column injection) using an HP 7671A automatic sampler. A flow rate of 40 mL helium/min was employed. After the injection of a sample, the column was maintained at 100°C for 4 min before the temperature was increased to 220°C at a rate of 8°C/min. In both gas chromatographs the detector and the injector were maintained at 250°C.

The composition of the partial acylglycerols was calculated based on relative integrated peak area obtained by gas chromatography for the pure triacylglycerols.

RESULTS AND DISCUSSION

The results of the analyses of the partial acylglycerols obtained after AMB degradation of SOS and CPC, respectively, are shown in Table 1.

For SOS, the stearic acid accounted for more than 98% of the total fatty acids in the 1,3-DAG and only 2.4% of the total fatty acids in the 2-MAG, showing that a representative mixture of partial acylglycerols was obtained. In the 1(3)-MAG from SOS, oleic acid originating from isomerized 2-MAG comprised 6.8% of the fatty acids. The presence of stearic acid (54%) in 1,2(2,3)-DAG may correspondingly be explained by minor isomerization of 1,3-DAG. The remaining TAG are still representative as shown by the presence of 66.7% stearic acid.

For CPC, the 2-MAG was only contaminated with decanoic acid to an extent of 1.2%. The 1(3)-MAG, on the other hand, contained 7.7% palmitic acid which had migrated from the 2-position. As found for SOS, the diglycerides of CPC were slightly less pure.

For other TAG species, such as 1,3-dipalmitoyl-2-oleoyl-glycerol and 1,3-didecanoyl-2-stearoyl-glycerol, comparably low degrees of migration for 2-MAG and 1,2(2,3)-DAG were observed (C.C. Becker, unpublished data).

The objective of a regiospecific analysis is to determine the fatty acid composition of positions *sn*-1(3) and *sn*-2 in the original TAG. This can be done by direct determination or by calculation based on the composition of the different acylglycerols, as demonstrated in Tables 2 and 3.

For both SOS and CPC the most accurate estimate for the composition of 1(3)-MAG was obtained by the formula: 1.5 × TAG − 0.5 × 2-MAG. The estimates were 98.8 ± 0.7 percent stearic acid in SOS and 99.4 ± 1.4 percent decanoic acid in CPC. For the 2-MAG obtained from the deacylation of TAG, only a low level of migration had occurred, and the composition of the 2-position could therefore be determined directly. During the synthesis of the TAG used in this investigation, a limited isomerization of the intermediate 1,3-DAG may have taken place. The values calculated for the *sn*-1(3) positions are therefore probably even less than one percent from the true value. For both 1(3)-MAG and 2-MAG it should be noted that the procedures giving the best estimates of the fatty acid composition in 1(3)-MAG and 2-MAG are also the procedures with the lowest standard deviation.

It is surprising that while the 1(3)-MAG resulting from the Grignard deacylation is slightly contaminated with former 2-MAG, the 2-MAG obtained appears to be almost completely representative. This could be explained by the greater stability of 1(3)-monoacylglycerols; thus an equilibrium mixture of monoolein in an aqueous system consists of about 90% of the 1-isomer and 10% of the 2-isomer (10).

When the EMB method is used, the composition of

TABLE 1

Fatty Acid Composition (mole% decanoic acid or stearic acid) of the Acylglycerols from 1,3-Distearoyl-2-oleoyl-glycerol (99% +) (SOS), and 1,3-Didecanoyl-2-palmitoyl-glycerol (97% +) (CPC) After Deacylation by the Grignard Reagent Allyl Magnesium Bromide[a]

TAG analyzed	Acylglycerol formed	%18:0 (for SOS)[b] %10:0 (for CPC)[b]	%18:1 (for SOS)[c] %16:0 (for CPC)[c]
SOS	1(3)-MAG	93.2 ± 3.8	6.8
	2-MAG	2.4 ± 0.8	97.6
	1,2(2,3)-DAG	53.9 ± 3.6	46.1
	1,3-DAG	98.3 ± 1.7	1.7
	TAG	66.7 ± 0.4	33.3
CPC	1(3)-MAG	92.3 ± 1.4	7.7
	2-MAG	1.2 ± 1.3	98.8
	1,2(2,3)-DAG	51.7 ± 1.0	48.3
	1,3-DAG	93.8 ± 2.1	6.2
	TAG	66.7 ± 0.8	33.3

[a]Abbreviations: MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.
[b]Values are means ± SD for four experiments.
[c]Standard deviations as given for 18:0 and 10:0.

TABLE 2

Calculation of the Fatty Acid Composition (mol%) of the *sn*-1(3) Positions of 1,3-Distearoyl-2-oleoyl-glycerol (99% +) (SOS), and 1,3-Didecanoyl-2-palmitoyl-glycerol (97% +) (CPC) from the Composition of Other Acylglycerols Obtained by Deacylation with Allyl Magnesium Bromide[a]

Formulas for calculation	SOS 1(3)-MAG % 18:0[a,b]	CPC 1(3)-MAG %10:0[a,b]
1(3)-MAG (from Table 1)	93.2 ± 3.8	92.3 ± 1.4
1.5 × TAG − 0.5 × 2-MAG[c]	98.8 ± 0.7	99.4 ± 1.4
2 × 1,2(2,3)-DAG − 2-MAG	105.5 ± 7.2	102.1 ± 2.4
1,3-DAG (from Table 1)	98.3 ± 1.7	93.8 ± 2.1
3 × TAG − 2 × 1,2(2,3)-DAG	92.1 ± 7.3	96.7 ± 3.1

[a]Abbreviations: See Table 1.
[b]Values are means ± SD for four experiments.
[c]The best estimate for the fatty acid composition of the *sn*-1(*sn*-3) positions of the triacylglycerols.

TABLE 3

Calculation of the Fatty Acid Composition (mol%) of the *sn*-2 Positions of 1,3-Distearoyl-2-oleoyl-glycerol (99% +) (SOS), and 1,3-Didecanoyl-2-palmitoyl-glycerol (97% +) (CPC) from the Composition of Other Acylglycerols Obtained by Deacylation with Allyl Magnesium Bromide[a]

Formulas for calculation	SOS 2-MAG % 18:0[b]	CPC 2-MAG %10:0[b]
2-MAG (from Table 1)[c]	2.4 ± 0.8	1.2 ± 1.3
4 × 1,2(2,3)-DAG − 3 × TAG	15.7 ± 14.4	6.6 ± 4.7
3 × TAG−2 × 1,3-DAG	3.4 ± 3.7	12.5 ± 4.9
3 × TAG−2 × 1(3)-MAG	13.7 ± 7.7	15.4 ± 3.7
2 × 1,2(2,3)-DAG − 1(3)-MAG	14.7 ± 8.1	11.0 ± 2.5
2 × 1,2(2,3)-DAG − 1,3-DAG	9.6 ± 7.4	9.5 ± 2.5

[a]Abbreviations: See Table 1.
[b]Values are means ± SD for four experiments.
[c]The best estimate for the fatty acid composition of the *sn*-2 positions of the triacylglycerols.

sn-1(*sn*-3) and *sn*-2 is estimated from the composition of TAG and 1,2(2,3)-DAG. The equations employed are:

$$1(3)\text{-MAG} = 3 \times \text{TAG} - 2 \times 1,2(2,3)\text{-DAG} \quad [1]$$

$$2\text{-MAG} = 4 \times 1,2(2,3)\text{-DAG} - 3 \times \text{TAG (6)} \quad [2]$$

The results obtained with these equations are shown in Tables 2 and 3. The equations suggested for the EMB method showed larger standard deviations than the equations used with the AMB method due to larger factors and, in the case of 2-MAG, more terms. Furthermore, the results from the AMB method were closer to the theoretical value.

The possible discrimination between the primary and secondary positions in TAG by the present method was also examined, using 1,2,3-tripalmitoylglycerol. With 7% of the tripalmitin degraded, the ratio between 1(3)-MAG and 2-MAG, and between 1,2(2,3)-DAG and 1,3-DAG, respectively, approached the theoretical value of 2. This means that the positional discrimination was of minor importance, at least for palmitic acid. However, the calculations are based on mole% of fatty acid in each class of acylglycerol, and not the amount of these; therefore a certain positional discrimination will not influence the results obtained with the present method.

The amount of AMB added is theoretically enough to deacylate all ester bonds of about 250 mg of TAG. That only a minor proportion of the TAG was deacylated is partly due to the content of azeotropic water in ether. The amount of water should be minimized as it increases the polarity of the solvent, and hence, in principle, the rate of acyl migration. Therefore, an excess of AMB must be added to compensate for the reaction with the water present. This reaction produces magnesium salts which further increases the polarity of the solvent. In order to minimize isomerization, boric acid is added to the washing solutions (11).

In conclusion, the method presented offers a fast and simple way to carry out regiospecific analyses of triacylglycerols. Sample sizes of about 6 mg were used, but smaller amounts (2 mg) can also be analyzed. This makes the method well suited for biological samples. The method can be used for most fats, including those for which the pancreatic lipase assay is unreliable. The results obtained are also more accurate and have smaller standard deviations than when EMB is used. In our laboratory the AMB method has proven to be particularly useful for the analysis of triacylglycerols, such as marine oils, that contain long-chain polyunsaturated fatty acids and for seed oils that contain medium-chain fatty acids (C_8–C_{12}).

ACKNOWLEDGMENTS

We would like to thank Dr. T. Holm and Dr. I. Lundt, Institute of Organic Chemistry, Technical University of Denmark, for valuable discussions. Doug Sarney kindly corrected the English.

REFERENCES

1. Gunstone, F.D. (1967) *An Introduction to the Chemistry and Biochemistry of Fatty Acids and Their Glycerides*, 2nd edn., Science Paperbacks, London.
2. Entressangles, B., Pasero, L., Savary, P., Sarda, L., and Desnuelle, P. (1961) *Bull. Soc. Chim. Biol. 43*, 581-585.
3. Lawson, L.D., and Hughes, B.G. (1988) *Lipids 23*, 313-317.
4. Brockerhoff, H. (1965) *Arch. Biochem. Biophys. 110*, 586-590.
5. Bottino, N.R., Vandenburg, G.A., and Reiser, R. (1967) *Lipids 2*, 489-493.
6. Brockerhoff, H. (1971) *Lipids 6*, 942-956.
7. Redgrave, T.G., Kodali, D.R., and Small, D. (1988) *Am. Soc. Biochem. Mol. Biol. 28*, 5118-5123.
8. Gilman, H., and McGlumphy, J.H. (1928) *Bull. Soc. Chim. 43*, 1322-1325.
9. Christie, W.W. (1989) *Gas Chromatography and Lipids*, 1st edn., p. 72, The Oily Press, Ayr.
10. Larsson, K. (1986) in *The Lipid Handbook* (Gunstone, F.D., Harwood, J.L., and Padley, F.B., eds.) pp. 364-367, Chapmann and Hall, London and New York.
11. Thomas, A.E., Scharoun, J.E., and Ralston, H. (1965) *J. Am. Oil Chem. Soc. 42*, 789-792.

[Received August 10, 1992; Revision accepted November 13, 1992]