A Simple Method for Regiospecific Analysis of Triacylglycerols by Gas Chromatography

Paul Angers and Joseph Arul*

Department of Food Science and Nutrition and Dairy Research Center (STELA), Université Laval, Québec G1K 7P4, Canada

ABSTRACT: A simple method for regiospecific analysis of triglycerides was developed. It consists of partial deacylation of triglycerides by ethylmagnesium bromide followed by derivatization of monoglycerides with *n*-butyryl chloride, and direct analysis of dibutyrate derivatives of monoglycerides by gas chromatography. The chromatographic conditions were carried out with monoglycerides of C_{12} to C_{20} fatty acids and resulted in separation of dibutyrate derivatives between those bearing the medium- or long-chain fatty acid in the *sn*-1(3) and *sn*-2 positions of glycerol. Beef tallow and grapeseed and cotton seed oils were analyzed using this new method, and their regiospecific distributions were compared with literature data. The method does not require separation of products by thin-layer chromatography or special analytical equipment other than a standard gas chromatograph, and it can thus be used for routine regiospecific analysis of triglycerides.

Paper No. J8945 in *JAOCS 76,* 481–484 (April 1999).

KEY WORDS: Acid chloride, beef tallow, cotton seed oil, ethylmagnesium bromide, fatty acid, gas chromatography, grapeseed oil, regiospecific analysis, triglyceride.

Positional distribution of fatty acids on the glycerol moiety of triglycerides affects both the functional properties (1) and the metabolism of fats and oils (2,3). The methods used to carry out regio- or stereospecific analysis of fatty acids in triglycerides usually start with partial deacylation either by pancreatic lipase (4–7), for specific removal of fatty acid, or by a Grignard reagent (4,5,8–10). Subsequently, two approaches can be used: one consists of analyzing 2-monoglycerides, usually isolated by thin-layer chromatography, for their fatty acid composition and estimating values in the *sn*-1(3) position from the fatty acid composition of 2-monoglycerides and triglycerides (11). The second approach consists of analyzing diglycerides for their fatty acid composition and determining the fatty acid composition in the *sn*-1(3) and *sn*-2 positions from the fatty acid composition of diglycerides and triglycerides (4,5). Though these are indirect methods, they allow accurate determination of regiospecific distribution of fatty acids in triglycerides. However, use of these methods on a routine basis is laborious, since they require isolation of either mono- or diglycerides, which are time-consuming operations. Tandem mass spectrometry may also be used for regiospecific analysis of triglycerides. Though very powerful, this method is not yet widely used because of the limited availability of the instruments (12). Hence, methods used to perform positional distribution analysis of fatty acid in triglycerides are either time consuming, limiting their use on a routine basis, or require analytical techniques which are prohibitive for general use. Thus, there is a need for a simple, fast, and reliable method for regiospecific analysis of triglycerides. In this study, we developed a method for regiospecific analysis of triglycerides based on their partial deacylation by a Grignard reagent followed by derivatization of the reaction products with *n*-butyryl chloride, in presence of a base such as triethylamine, and direct analysis of the so-formed dibutyrate derivatives of monoglycerides by gas chromatography. The method was applied to beef tallow, grapeseed oil, and cotton seed oil.

MATERIALS AND METHODS

Materials. Standard monoglycerides used were: 1-monolauroyl-*rac*-glycerol, 1-monomyristoyl-*rac*-glycerol, 1-monopalmitoyl-*rac*-glycerol, 2-monopalmitoylglycerol, 1-monopalmitoleoyl-*rac*-glycerol, 1-monostearoyl-*rac*-glycerol, 1-monooleoyl-*rac*-glycerol, 1-monolinoleoyl-*rac*-glycerol, 1-monolinolenoyl-*rac*-glycerol. Standard triglycerides were: trilaurin, trimyristin, tripalmitolein, trimargarin, tristearin, triolein, trilinolein, trilinolenin, and triarachidin. All were purchased from Sigma Chemicals (St. Louis, MO). Ethylmagnesium bromide, *n*-butyryl chloride, and triethylamine were obtained from Aldrich (Milwaukee, WI). Deodorized beef tallow and refined grapeseed and cotton seed oils were obtained from a local fat and oil processor.

Deacylation of triglycerides and derivatization. Standard triglycerides, beef tallow, and grapeseed and cotton seed oils were partially deacylated with ethylmagnesium bromide, derivatized with *n*-butyryl chloride, and analyzed by gas chromatography. A typical procedure was as follows: To a stirred solution of grapeseed oil (5 mg) in anhydrous diethyl ether (0.5 mL), contained in a flame-dried flask under inert atmosphere (N_2), a solution of ethylmagnesium bromide in the same solvent (3.0 M; 20 μL, 5 eq) was added. After stirring

*To whom correspondence should be addressed.
E-mail: joseph.arul@aln.ulaval.ca

for 30 s at room temperature, glacial acetic acid (10 μL) was added, followed by 300 μL of a 10% aqueous boric acid solution. The mixture was extracted with diethyl ether saturated with boric acid (4 × 2 mL), and the organic extracts were combined, washed sequentially with sodium bicarbonate (2%), water, and brine, dried over anhydrous sodium sulfate, filtered, and concentrated under a stream of dry nitrogen. The residue was solubilized in dry chloroform (0.5 mL), triethylamine (100 μL) and *n*-butyryl chloride (50 μL) were sequentially added, and the mixture was held at 60°C for 20 min in a closed vial with constant stirring. An aliquot (100 μL) was taken and added to *n*-octane (400 μL), thus precipitating the ammonium salt which was filtered off using a PTFE membrane (0.2 μm, CSC, Montréal, Québec, Canada), and analyzed for regiospecific distribution of fatty acids. Dibutyrate derivatives of *sn*-2 isomers of monoglycerides of lauric, myristic, palmitoleic, stearic, oleic, linoleic, and linolenic acids, as well as both *sn*-1(3) and *sn*-2 isomers of margaric and arachidic acids used for determination of retention indices, were prepared using this procedure. The *sn*-1(3) isomers of the former series of fatty acids and the *sn*-2 isomer of palmitic acid were synthesized from the respective monoglycerides by derivation with *n*-butyryl chloride.

Gas chromatography of triglycerides. Analyses of triglycerides (dibutyrate derivatives of monoglycerides) were performed using a gas chromatograph (Hewlett-Packard, Palo Alto, CA, Model 5890, Series II) equipped with a flame-ionization detector and connected to a ChemStation (Hewlett-Packard). Sample volumes (1.0 μL) in *n*-octane were injected on a 65% phenylmethylsilicone capillary column (Quadrex, New Haven, CT; 30 m × 0.25 mm i.d., 0.10 μm film thickness) with a split ratio of 1:100. Injector and detector temperatures were set at 380°C, while the oven temperature was programmed from 190 to 280°C at 5°C min^{-1}, then to 360°C at 12°C min^{-1}, and held for 2 min at this temperature for a total duration of 26.67 min. The linear velocity of the carrier gas (hydrogen) was 32.5 cm s^{-1} at 190°C. Retention indices were calculated from retention times of simple even carbon number triglycerides (TG18:0, TG24:0, and TG30:0).

RESULTS AND DISCUSSION

Gas chromatographic conditions for separation of dibutyrate derivatives of 1(3)- and 2-monoglycerides of lauric, myristic, palmitic, palmitoleic, margaric, stearic, oleic, linoleic, linolenic, and arachidic acids were developed using a 65% phenylmethylsilicone capillary column for which retention indices are given in Table 1. The results showed that differences between retention index of 1(3)- and 2-monoglyceride derivatives ranged from 6 to 8 units, which corresponded to resolutions (R_S) of 1.0 to 1.2. The gas chromatogram of cotton seed oil, after partial deacylation and derivatization (Fig. 1), shows the separation of peaks of dibutyrate derivatives of monoglycerides.

Migration of acyl groups from secondary to primary positions in glycerol is a known phenomenon. In aqueous equilibrium conditions, the acyl group of monoglycerides occupies the primary position at a level of about 90% (13). This natural tendency of acyl groups in secondary position of glycerol to migrate to the primary position has to be prevented in order to obtain reliable results. Hence, it was important to determine if such migration could occur in the analytical procedure, and if it did so, to evaluate the extent of this occurrence. Consequently, the two chemical steps of the present method were tested independently for such migration, beginning with the derivatization process. Thus, standard monoglycerides 1-monopalmitoyl-*rac*-glycerol and 2-monopalmitoylglycerol were derivatized in separate experiments with *n*-butyryl chloride and analyzed by gas chromatography. The results showed complete derivatization for both monoglycerides, with no de-

TABLE 1
Retention Index (R_I) of Dibutyrate Derivatives of 1(3) and 2-Monoglycerides (MAG)

MAG	R_I[a] *sn*-1(3) Position	R_I[a] *sn*-2 Position	ΔR_I
Monolauroyl	2027	2021	6
Monomyristoyl	2253	2247	6
Monopalmitoyl	2479	2472	7
Monopalmitoleoyl	2502	2494	8
Monomargaroyl	2586	2580	6
Monostearoyl	2715	2707	8
Monooleoyl	2728	2720	8
Monolinoleoyl	2762	2755	7
Monolinolenoyl	2809	2803	6
Monoarachidoyl	2920	2914	6

[a]Values are means of three replicate analyses.

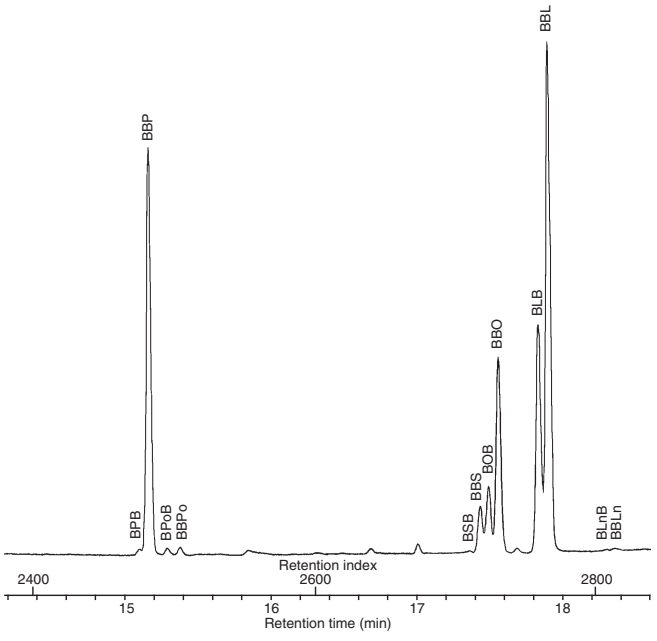

FIG. 1. Gas chromatogram of dibutyrate derivatives of monoglycerides obtained after partial deacylation of cotton seed oil by ethylmagnesium bromide, followed by derivatization with *n*-butyryl chloride. Butyric (B), 4:0; palmitic (P), 16:0; palmitoleic (Po), 16:1; stearic (S), 18:0; oleic (O), 18:1; linoleic (L), 18:2; linolenic (Ln), 18:3.

tectable migration of acyl groups. The deacylation procedure was then tested by reacting the mixed standard triglyceride 1,3-dipalmitoleyl-2-oleoyl-glycerol (POP) with ethylmagnesium bromide, followed by derivatization of the reaction products with *n*-butyryl chloride, according to the conditions reported in the experimental section. Results showed that the level at which a fatty acid present originally in the *sn*-2 position migrated to the *sn*-1(3) position was 1.6 ± 0.1% (n = 3) of the total fatty acids in the *sn*-1(3) position. However, migration from primary to secondary position in glycerol was not detected in any significant amount. Hence, the values for the secondary position were taken as obtained. Composition in the primary position of natural fats was calculated taking into account the 1.6% value for migration. It is interesting to observe a lower level of migration was obtained using the present experimental conditions in comparison to values obtained by different partial deacylation and purification conditions (nonanhydrous, large excess of Grignard reagent, and thin-layer chromatography), which were in the range of 7 to 8% (11), and this suggests that acyl migration is sensitive to the above conditions. Partial deacylation of standard triglycerides also revealed the selectivity of the deacylating reagent (ethylmagnesium bromide) toward addition to the carbonyl carbons, whether they were on fatty acids esterified to the primary or secondary positions of glycerol, to be a ratio of 3.8 to 1, exclusive of acyl migration. This ratio was observed for 1(3)-monoglycerides over 2-monoglycerides, though the theoretical value is 2 to 1. Thus, the assumption that partial deacylation of triglycerides by a Grignard reagent is nonselective, independent of the chain length of fatty acids (6,11), needs to be reconfirmed at different deacylation conditions—such as the type of Grignard reagent—since their action is susceptible to steric factors (14).

Results of regiospecific analysis of grapeseed oil, cotton seed oil, and beef tallow are shown in Table 2. The results obtained with grapeseed oil identified oleic and linoleic acids as the major fatty acids at the *sn*-2 position, with about three times more linoleic than oleic acid. Linoleic acid was still the major fatty acid at the *sn*-1(3) position, along with oleic acid. However, palmitic and stearic acids were nearly absent at the *sn*-2 position. The results are comparable to the literature data (15), except that our sample had a higher level of linoleic acid at the *sn*-1(3) position (67.4%), compared to 53 to 56%. Cotton seed oil was also high in linoleic acid. The composition of its fatty acids at the *sn*-2 position was comparable to that of grapeseed oil, with 75.3% linoleic acid and oleic as the other main acid. However, at the *sn*-1(3) position, the lower level of linoleic acid in comparison to grapeseed oil is compensated by a higher level of palmitic acid, which constitutes slightly over a third of the fatty acids at this position. These values are in agreement with those found in the literature (16). Palmitic, stearic, and oleic acids were the major fatty acids at the *sn*-1(3) position for beef tallow, with somewhat equimolar distribution, while at the *sn*-2 position, oleic acid made up 49.9% of the fatty acids, with palmitic acid as the other major acid. The fatty acid distribution of beef tallow obtained by this method was comparable to published values (17).

The present method is simpler because it avoids the use of time-consuming thin-layer chromatography, consequently facilitating regiospecific analysis of triglycerides on a routine basis using common gas chromatographic technique. The method is sensitive, allowing quantitation of low levels of

TABLE 2
Experimental[a] and Literature[b] Values for Fatty Acid Distribution Between *sn*-1,3 and *sn*-2 Positions of Grapeseed Oil, Cotton Seed Oil, and Beef Tallow

	Composition (mol%)[c]									
Fat source	12:0	14:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:0
Grapeseed										
sn-1/3	—[d]	—	10.7 ± 0.2	0.2 ± 0.1	—	5.4 ± 0.1	15.5 ± 0.3	67.4 ± 0.5	0.8 ± 0.1	—
	(—)	(—)	(14–19)	(—)	(—)	(7–9)	(15–25)	(53–56)	(—)	(—)
sn-2	—	—	0.7 ± 0.2	0.8 ± 0.3	—	0.2 ± 0.1	24.1 ± 0.4	73.7 ± 1.7	0.7 ± 0.2	—
	(—)	(—)	(—)	(—)	(—)	(—)	(22–28)	(71–77)	(—)	(—)
Cotton seed										
sn-1/3	—	0.1 ± 0.1	35.4 ± 0.3	0.8 ± 0.1	—	3.5 ± 0.1	16.5 ± 0.2	43.6 ± 0.3	0.2 ± 0.1	—
	(—)	(—)	(33.4)	(0.6)	(—)	(2.4)	(15.4)	(46.7)	(—)	(—)
sn-2	—	0.1 ± 0.1	2.0 ± 0.3	0.7 ± 0.3	(—)	0.4 ± 0.1	21.3 ± 0.3	75.3 ± 0.6	0.3 ± 0.1	—
	(—)	(0.1)	(6.7)	(0.6)	(—)	(1.5)	(28.0)	(63.2)	(—)	(—)
Beef tallow										
sn-1/3	0.2 ± 0.1	2.4 ± 0.1	34.3 ± 0.6	3.3 ± 1.2	1.8 ± 0.1	26.8 ± 0.6	28.3 ± 0.4	2.6 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
	(—)	(0–3.8)	(32.6–41.5)	(0.4–7.5)	(1.0–3.9)	(7.5–28.4)	(24.9–42.7)	(0–2.9)	(0–1.5)	(—)
sn-2	—	7.1 ± 0.9	21.1 ± 0.2	4.0 ± 0.5	1.0 ± 0.2	9.4 ± 0.1	49.9 ± 0.8	5.8 ± 0.2	0.7 ± 0.1	1.0 ± 0.1
	(—)	(0.6–3.9)	(15.5–21.5)	(2.1–5.6)	(0.3–0.6)	(7.6–21.2)	(51.2–63.6)	(1.5–6.2)	(0–0.6)	(—)

[a]Values are means of five replicate analyses ± standard deviation.
[b]Literature data are in parentheses. References are grapeseed oil (14), cotton seed oil (15), and beef tallow (16).
[c]The percentages represent the sum of all the isomers with the same number of carbon atoms and unsaturations.
[d]Concentrations lower than 0.1% are not reported.

dibutyrate derivatives of monoglycerides, and it permits direct evaluation of acyl migration during both partial deacylation and derivatization, which, under the conditions used, was low. In addition, both the limited number of manipulations and reaction conditions that exclude the presence of air may prevent the facile oxidation of polyunsaturated fatty acids, thus improving the accuracy of the results.

ACKNOWLEDGMENTS

We acknowledge the financial support of the Natural Sciences and Engineering Research Council (NSERC) of Canada.

REFERENCES

1. J.A. Love, Animal Fats, in *Bailey's Industrial Oil and Fat Products*, 5th edn., edited by Y.H. Hui, Wiley, New York, 1996, Vol. 1, pp. 10–13.
2. Redgrave, T.G., D.R. Kodali, and D.M. Small, The Effect of Triacyl-*sn*-glycerol Structure on the Metabolism of Chylomicron and Triacylglycerol-Rich Emulsions in the Rat, *J. Biol. Chem. 263*:5118–5123 (1988).
3. Mortimer, B.C., W.J. Simmonds, C.A. Joll, R.V. Stick, and T.G. Redgrave, Regulation of the Metabolism of Lipid Emulsion Model Lipoproteins by Saturated Acyl Chain at the 2-Position of Triacylglycerol, *J. Lipid Res. 29*:713–720 (1988).
4. Brockerhoff, H., Stereospecific Analysis of Triglycerides, *Lipids 6*:942–956 (1971).
5. Christie, W.W., The Positional Distribution of Fatty Acids in Triglycerides, in *Analysis of Fats and Oils*, edited by R.J. Hamilton and J.B. Rossell, Elsevier, New York, 1986, pp. 313–339.
6. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman, and R.W. Reimenschneider, Pancreatic Lipase Hydrolysis of Triglycerides by a Semimicro Technique, *J. Am. Oil Chem. Soc. 41*:693–696 (1964).
7. Williams, J.P., M.U. Khan, and D. Wong, A Simple Technique for the Analysis of Positional Distribution of Fatty Acids on Di- and Triacylglycerols Using Lipase and Phospholipase A2, *J. Lipid Res. 36*:1407–1412 (1995).
8. Damiani, P., F. Santinelli, M.S. Simonetti, M. Castellini, and M. Rosi, Comparison Between Two Procedures for Stereospecific Analysis of Triacylglycerols from Vegetable Oils—I: Olive Oil, *J. Am. Oil Chem. Soc. 71*:1157–1162 (1994).
9. Takagi, T., and Y. Ando, Stereospecific Analysis of Triacyl-*sn*-glycerols by Chiral High-Performance Liquid Chromatography, *Lipids 26*:542–547 (1991).
10. Kuksis, A., Analysis of Positional Isomers of Glycerolipids by Non-enzymatic Methods, in *Advances in Lipid Methodology*, edited by W.W. Christie, The Oily Press, Dundee, 1996, Vol. 7, pp. 1–36.
11. Becker, C.C., A. Rosenquist, and G. Høimer, Regiospecific Analysis of Triacylglycerols Using Allyl Magnesium Bromide, *Lipids 28*:147–149 (1993).
12. Kallio, H., and P. Rua, Distribution of the Major Fatty Acids of Human Milk Between *sn*-2 and *sn*-1,3 Positions of Triacylglycerols, *J. Am. Oil Chem. Soc. 71*:985–992 (1994).
13. Larsson, K., and P.J. Quinn, Physical Properties: Structural and Physical Characteristics, in *The Lipid Handbook*, 2nd edn., edited by F.D. Gunstone, J.L. Harwood, and F.B. Padley, Chapman and Hall, London and New York, 1994, pp. 443–446.
14. Canonne, P., D. Bélanger, and G. Lemay, Steric Effects in the Reaction of Di(bromomagnesio)alkanes with Carboxylic Esters, *Tetrahedron Lett. 22*:4995–4998 (1981).
15. Morin, O., Maïs et Pépins de Raisin, in *Manuel des Corps Gras*, edited by A. Karleskind, Lavoisier, Paris, 1992, pp. 140–144.
16. Bézard, J., M.A. Ouedraogo, G. Sempore, and L. Schreiber, Étude de la Répartition Stéréospécifique des Acides Gras dans les Triglycérides de l'Huile de Coton, *Rev. Fr. Corps Gras 37*:83–90 (1990).
17. Smith, S.B., A. Yang, T.W. Larsen, and R.K. Tume, Positional Analysis of Triacylglycerols from Bovine Adipose Tissue Lipids Varying in Degree of Unsaturation, *Lipids 33*:197–207 (1998).

[Received July 13, 1998; accepted December 20, 1998]