Regioselective Analysis of the Fatty Acid Composition of Triacylglycerols with Conventional High-Performance Liquid Chromatography

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ABSTRACT: A new method for regioselective analysis of triacylglycerols *via* conventional high-performance liquid chromatography (HPLC) has been developed. The method is simple and avoids the time-consuming purification processes normally characteristic of regioselective analyses. The procedure utilizes an *sn*-1,3-specific lipase from *Rhizopus arrhizus* to deacylate the fatty acid residues located at the *sn*-1 and *sn*-3 positions of triacylglycerols. The fatty acid residues esterified at the *sn*-2 position are determined by subtraction of the results of the *sn*-1,3 analysis from an overall composition analysis based on complete saponification of the original sample. The fatty acid mixtures are converted to *p*-bromophenacyl esters and analyzed using conventional HPLC techniques. The analytical procedure has been verified using a standard structured triacylglycerol. The analytical results for three edible vegetable oils are compared with those obtained *via* the method proposed by P.J. Williams and co-workers.

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KEY WORDS: High-performance liquid chromatography, regioselective analysis, *Rhizopus arrhizus* lipase, triacylglycerols.

A substantial body of scientific evidence indicates that ingestion of specific types of fats and oils is implicated in the development of coronary heart disease. Similarly, there is increasing scientific evidence that ingestion of particular fatty acids (e.g., ω-3 fatty acids and conjugated linoleic acid) leads to reduced incidence of certain diseases (e.g., atherosclerosis and particular forms of cancer). In addition to the degree of saturation of the fatty acid residues, the positions of specific fatty acid residues in the triacylglycerol structure constitute an important factor governing the atherogenic character of a fat or oil (1). A study by Kritchevsky *et al.* (2) involving different fats containing 24% palmitic acid indicated that for rabbits, lard was significantly more atherogenic than tallow. Lard contains about 90% of its palmitic acid residues at the *sn*-2 position of the glycerol backbone. By contrast, in tallow less than 15% of the palmitic acid residues are present at the *sn*-2 position. Moreover, both physical properties (3) and oxidative stability (4) are significantly influenced by the identities and positions of the fatty acid residues on the glycerol backbone.

The fatty acid composition of triacylglycerols is usually elucidated by gas chromatography (GC), as are regio- and stereoselective analyses subsequent to a partial hydrolysis process and several purification steps. Mattson and Lutton (5) have suggested the use of a 1,3-specific lipase (pig pancreatic lipase) to carry out a partial digestion of a triacylglycerol sample, followed by purification of the mixture of *sn*-2 monoacylglycerols produced by the digestion. The fatty acid composition of this mixture of *sn*-2 monoacylglycerols is determined *via* GC. Brockerhoff (6) has reported a stereospecific analysis of triacylglycerols which utilizes a pancreatic lipase or Grignard reagent (7) to effect partial deacylation of the triacylglycerol. This reaction generates mixtures of *sn*-2 monoacylglycerols and *sn*-1,2 and *sn*-2,3 diacylglycerols when an *sn*-1,3-specific lipase is employed. By contrast, mixtures of *sn*-1,3, *sn*-1,2, and *sn*-2,3 diacylglycerols are produced when the Grignard reagent is employed. The *sn*-1,2 and *sn*-2,3 diacylglycerols are purified *via* thin-layer chromatography (TLC) and converted to phosphatides. Then the fatty acid residues located at the *sn*-2 position are liberated using a phospholipase. Newer methods avoid the use of phospholipases by employing semipreparative high-performance liquid chromatography (HPLC) to purify the different diacylglycerols (8) or to separate *sn*-1 and *sn*-3 monoacylglycerols (9). Foglia *et al.* (10) have reported a procedure for regioselective analysis of triacylglycerols that employs a 1,3-specific lipase for partial hydrolysis, followed by isolation of the free fatty acids and 1,2-(2,3-)diacylglycerols by TLC. Subsequent conversion to the corresponding fatty acid methyl esters and analysis of these esters by GC permits quantification of the positional distribution of fatty acid residues.

Although most of the aforementioned methods permit stereoespecific analysis of triacylglycerols, they generally involve several time-consuming purification processes involving TLC or HPLC that may cause migration of fatty acid residues between positions in the glycerol backbone. Recently, Williams *et al.* (11) developed a simple technique for regioselective analysis of triacylglycerols avoiding the purifi-

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cation steps. The technique involves parallel, selective methylation of duplicate samples for subsequent GC analyses.

We have developed a simple method that permits use of standard HPLC techniques to determine the fatty acid composition of acylglycerols in mixtures of free fatty acids and acylglycerols. The procedure takes advantage of the fact that in a mixture of acylglycerols and free fatty acids, only free fatty acids are derivatized by 2,4-dibromoacetophenone in the presence of an 18-crown-6-ether as a catalyst. A 1,3-specific lipase from *Rhizopus arrhizus* is employed to specifically remove the fatty acid residues located at the *sn*-1,3 positions of the triacylglycerol. Although this method does not permit one to conduct a complete stereospecific analysis of triacylglycerols, it does permit one to determine which fatty acid residues are located in the *sn*-2 position of the triacylglycerol and their relative proportions. This result is very important from a nutritional point of view. The method is rapid and simple and, to the best of our knowledge, is the first technique that permits one to employ conventional HPLC for this purpose.

EXPERIMENTAL PROCEDURES

Materials. A lipase from *R. arrhizus* was obtained from Roche (Indianapolis, IN). 1,2-Dioleoyl-3-palmitoyl-*rac*-glycerol, Triton X-100, and all the fatty acids used as standards for the HPLC analysis (nonanoic, decanoic, lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidonic acid) were from Sigma (St. Louis, MO). 18-Crown-6-ether, 2,4-dibromoacetophenone (PBPB), and olive oil were purchased from Aldrich (St. Louis, MO). All solvents employed (methanol, chloroform, and acetonitrile) were HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA). Corn oil and safflower oil were kindly donated by Archer Daniels Midland (Springfield, IL).

Reaction in the presence of the 1,3-specific lipase. A slightly modified version of the method proposed by Fisher *et al*. (12) was employed to effect the lipolysis reaction. Approximately 10 mg of the triacylglycerol was mixed with approximately 10 mg of Triton X-100 in a 4-mL vial. Next, 50 μL of a Tris-HCl buffer (40 mM, and pH = 7.2) containing 50 mM sodium borate was added. The mixture was vortexed for 1 min. Then, 20 μL of the lipase solution (1000 units) was added, and the mixture was vortexed for another 10 s. The mixture was then held for 25 min in an orbital shaker at 22°C. The reaction was quenched by adding 0.5 mL of 0.1 M acetic acid. Next, 0.5 mL of the aforementioned Tris buffer solution was added. The lipid fraction was then extracted once with 3 mL of a 2:1 (vol/vol) mixture of chloroform/methanol and twice with 2 mL of the same mixture. If the solution thereby obtained was not clear, the mixture was centrifuged for 5 min at 2820 × *g*, and the upper, aqueous, phase was discarded. Then, two 2.5-mL aliquots (A and B) of the extract were subjected to evaporation under N_2. Aliquot A was directly derivatized to produce the corresponding *p*-bromophenacyl esters of the free fatty acids liberated by lipolysis in the presence of the *sn*-1,3 specific lipase, while aliquot B was saponified prior to derivatization to form the *p*-bromophenacyl esters.

Saponification reaction. Approximately 5 mg of the triacylglycerol and 100 μL of a 1 N solution of KOH in methanol were placed in a 4-mL vial. The resulting mixture was incubated at 80°C for 30 min and later cooled to room temperature. Then, 120 μL of a 1 M HCl solution was added to convert the fatty acid salts to their acidic form. Next, 1 mL of distilled water was added. The mixture of free fatty acids was then extracted once with 2 mL and twice with 1 mL of a 2:1 (vol/vol) mixture of chloroform/methanol. The solvent was evaporated under N_2, and the fatty acid was derivatized to form the corresponding *p*-bromophenacyl ester.

Preparation of p*-bromophenacyl esters.* The method employed was a modified version of that proposed by Durst *et al*. (13); 0.5 mL of a 1 mg/mL solution of nonanoic acid in chloroform (used as an internal standard) was added to the mixture of fatty acids. Then, 100 μL of a 0.5 mg/mL solution of 18-crown-6-ether in acetonitrile was added to 3 mL of a solution containing 0.15 mg/mL of PBPB in acetonitrile. Next, 300 μL of the solution containing the fatty acids and the internal standard was combined with the mixture of PBPB and the 18-crown-6-ether together with approximately 200 mg of K_2CO_3. The mixture was held at 80°C in a sealed vial for 30 min and then cooled in an ice bath for 15 min. Then the mixture was filtered through a 0.45 μm membrane filter and analyzed by HPLC.

Analysis of mixtures of fatty acids by HPLC. The HPLC analysis of the mixture of fatty acids was conducted following the method described by Garcia *et al*. (14) as modified (Lessard, P.L., and C.G. Hill, Jr., unpublished data). The HPLC system was equipped with a photodiode array detector and a Nova-Pak C18 column (150 × 3.9 mm) (Waters, Milford, MA).

Methylation reactions for the GC analyses. The procedure employed in the preparation of these samples was identical with that described previously for the reaction in the presence of the 1,3-specific lipase through the point at which the samples are extracted with the 2:1 (vol/vol) mixture of chloroform/methanol. The organic phase was then evaporated to give a solution containing approximately 12 mg/mL of lipids. Then two 50-μL aliquots were taken. The solvent was evaporated from these aliquots under nitrogen. Then 500 μL of internal standard (C17) in a 2:1 (vol/vol) mixture of chloroform/methanol was added to each aliquot. Methylation of the esterified fatty acids in the first aliquot was accomplished by addition of 1 mL of 2 N sodium methoxide. After 20 min of reaction at room temperature, 100 μL of distilled water was added, and the fatty acid methyl esters were extracted with 2 mL of hexane. The second aliquot was employed to determine the distribution of total fatty acids, both free and in residue form. The methylation procedure for this aliquot required addition of 1 mL of 1 N methanolic HCl. The resulting mixture was then held at 80°C for 30 min. The fatty acid methyl esters formed were extracted with 2 mL of hexane.

Analysis of the products of the methylation reactions by GC. Sample (1 μL) was injected into a Hewlett-Packard

model 6890 gas chromatograph fitted with a 30-m HP-Innowax column (0.32 mm i.d.) (Palo Alto, CA). Injector and detector temperatures were set at 220 and 230°C, respectively. The temperature program was as follows: 100°C for 1 min, then heating from 100 to 145°C at 20°C/min; a second heating ramp was programmed from 145 to 200°C at 30°C/min; then a third ramp of 50°C/min was used from 200 to 250°C. The column was held at the final temperature (250°C) for 4 min.

Calculations. The relative abundances (expressed as mole fractions) of the different fatty acid residues located at the *sn*-1,3 positions were calculated from the data obtained from aliquot A, which had been derivatized to form the bromophenacyl esters subsequent to the enzymatic reaction.

The relative abundances of the fatty acid residues located at the *sn*-2 position of the triacylglycerol were determined by subtraction of the molar fractions of fatty acid residues located at the *sn*-1,3 positions from the fractions corresponding to the initial overall triacylglycerol composition. The initial composition was obtained by analysis of aliquot B subsequent to the saponification process. Thus

$$2\text{-FA}_X = [\text{FA}_X - (\text{FA}\cdot 0.6667\cdot 1,3\text{-FA}_X)]/2\text{-FA} \qquad [1]$$

where 2-FA_X = mole fraction of the total fatty acid residues located at the *sn*-2 position, which corresponds to fatty acid X; FA_X = total number of moles of fatty acid X present in the triacylglycerol sample (obtained from aliquot B); FA = total number of moles of all fatty acids present in the triacylglycerol sample (obtained from aliquot B); $1,3\text{-FA}_X$ = mole fraction of fatty acid X located at the *sn*-1,3 positions (obtained from aliquot A); and 2-FA = total number of moles of fatty acid residues located at the *sn*-2 position.

Values of FA_X and FA could also be determined after the saponification of another fresh triacylglycerol sample, before the action of the lipase. In that case, it would not be necessary to separate the two fractions after the enzymatic hydrolysis. However, the method first described permits one to determine the extent of hydrolysis obtained with the enzymatic reaction by ascertaining the difference in the number of moles of a particular fatty acid corresponding to each of the two fractions.

The calculations for the GC method were accomplished using the procedure reported by Williams *et al.* (11).

RESULTS AND DISCUSSION

Analysis of structured triacylgycerols. To validate the aforementioned analytical procedure, analyses of a structured triacylglycerol (1,2-dioleoyl-3-palmitoyl-*rac*-glycerol) standard were conducted. The mole percentages of the various acid residues present in the structured triacylglycerol were determined by saponification, followed by generation of the *p*-bromophenacyl esters. Inspection of the data in Figure 1 indicates excellent agreement between the experimental values (33.1% palmitic acid and 66.9% oleic acid) and the expected values (33.3% palmitic acid and 66.7% oleic acid). This result implies that the technique described in this manuscript could be employed to elucidate regiospecific fatty acid compositions of many fats and oils *via* HPLC. The only requirement is that an adequate HPLC method exist for determination of the various fatty acids of interest. For example, the technique developed by Garcia *et al.* (14) could be employed for this purpose. If the sample to be analyzed consists of a mixture of fatty acids and triacylglycerols, two parallel derivatizations should be conducted. Direct derivatization of the sample will lead to a result corresponding to the free fatty acids present in the mixture. The composition of the fatty acid residues of the triacylglycerol can be determined by subtracting the values corresponding to the results for the free fatty acids from the values obtained *via* saponification of the original mixture followed by derivatization.

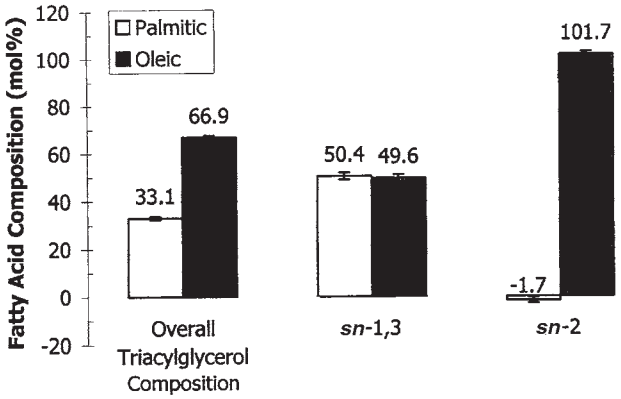

FIG. 1. Fatty acid composition and positional distribution of 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol.

The regiospecific analysis contained in Figure 1 indicates that the experimental values (50.4% palmitic acid and 49.6% oleic acid for the *sn*-1,3 positions, and –1.7% palmitic acid and 101.7% oleic acid for the *sn*-2 position) are in very good agreement with the values expected for the structured lipid employed to validate the analytical technique. The experimental error expressed as a standard deviation (see the error bars in Fig. 1) is very low. Hence, the method has good reproducibility.

The negative value obtained for the palmitic acid residue located at the *sn*-2 position does not have physical significance. This result is merely a consequence of the numerical procedure employed in the analysis. When one takes the difference between two large numbers, the result may be negative and have an uncertainty range that encompasses zero.

Migration of fatty acid residues does not appear to be a problem with this technique, probably because of the low temperature employed for the enzymatic reaction. Moreover, migration that might take place after the enzymatic reaction (e.g., during the extraction process) does not affect the analysis since the relative abundances of the fatty acid residues located at the *sn*-2 position are determined by differences between the total fatty acid composition of the triacylglycerol and the values corresponding to the fatty acid residues located

TABLE 1
Comparative Fatty Acid Compositions (mol%) and Positional Distributions of Fatty Acid Residues on the Triacylglycerols of Corn Oil[a]

Fatty acid	Overall triacylglycerol composition		Residue composition at the *sn*-1,3 positions			Residue composition at the *sn*-2 position		
	HPLC	GC	HPLC	GC	(6)	HPLC	GC	(6)
C12	0	0.3	0	0	0	0	0.2	0
C14	0	0.2	0	0.2	0	0	0.3	0
C16	11.6 ± 0.5	11.5	17.4 ± 0.4	14.5	19.3	0.4 ± 0.4	3.0	1.8
C16:1	0	0	0	0	0.6	0	0.3	0.3
C18	2.6 ± 0.4	2.1	3.3 ± 0.07	2.7	4.0	1.3 ± 0.5	1.0	0.4
C18:1	24.9 ± 0.7	25.6	25.6 ± 0.7	27.7	29.1	24.1 ± 0.5	27.8	26.7
C18:2	58.9 ± 0.9	59.4	52.4 ± 0.4	54.5	45.7	73.5 ± 1.8	67.3	69.8
C18:3	0.6 ± 0.01	0.9	0.7 ± 0.05	0.4	1.3	0.5 ± 0.20	0.1	1.0
C20	0.5 ± 0.10	0	0.6 ± 0.02	0	0	0.2 ± 0.20	0	0
Other	0.9	0	0	0	0	0	0	0

[a]Data developed using our method of high-performance liquid chromatography (HPLC), the gas chromatography (GC) method developed by Williams *et al.* (11), and data from Brockerhoff (6).

at the *sn*-1,3 positions which are released by the enzymatic reaction. The lipase from *R. arrhizus* exhibits a high selectivity for the *sn*-1,3 positions of the triacylglycerol. Ota *et al*. (15) have reported a positional specificity index of 98.9% for this lipase.

Analysis of the products of the enzymatic reaction indicated that the percentage of the fatty acid residues in the original acylglycerols that were released by hydrolysis was approximately 50%.

Analyses of vegetable oils. The technique described previously was used to carry out regiospecific analyses of the triacylglycerols from three edible oils, namely, olive, corn, and soybean oils (see Tables 1–3). The experimental results obtained with our HPLC procedures are compared with results obtained in our laboratory following the GC procedure described by Williams *et al*. (11) for the regioselective analysis of triacylglycerols. In addition, the experimental values are compared with data obtained from the literature. For natural products, the fatty acid composition of triacylglycerols can vary depending on their geographic origin, the local climatic conditions under which the precursor is grown, the stage of development and particular strain of the vegetable, and the particular processing conditions to which it has been subjected, such as extent of pressing, fractionating, and refining.

The first step in our regioselective analytical protocol is a saponification procedure that permits one to determine the overall fatty acid composition of the triacylglycerol. Inspection of the entries in Tables 1–3 indicates that the HPLC analyses of the three vegetable oils are in very good agreement with those obtained *via* methylation followed by GC analysis according to the procedure of Williams *et al*. (11). Hence, saponification followed by formation of the *p*-bromophenacyl esters can be employed to obtain an overall distribution of the fatty acid residues present in a variety of triacylglycerols.

TABLE 2
Comparative Fatty Acid Compositions (mol%) and Positional Distributions of Fatty Acid Residues on the Triacylglycerols of Soybean Oil[a]

Fatty acid	Overall triacylglycerol composition		Residue composition at the *sn-1,3* positions			Residue composition at the *sn*-2 position		
	HPLC	GC	HPLC	GC	(6)	HPLC	GC	(6)
C12	0.5 ± 0.04	0.2	0.0	0.1	0	0.8	0.2	0
C14	0	1.0	0	0.1	0	0	0.3	0
C16	11.7 ± 0.30	11.6	17.7 ± 0.24	16.5	16.4	0.9 ± 0.26	5.2	2.2
C16:1	0	0	0	0.2	0.1	0	0.1	0.1
C18	4.9 ± 0.35	4.7	6.8 ± 0.05	6.9	5.0	0.9 ± 0.8	2.4	0.3
C18:1	23.0 ± 0.45	23.4	23.8 ± 0.4	29.5	24.6	22.6 ± 0.9	25.2	23.6
C18:2	52.5 ± 0.60	52.2	44.8 ± 0.3	42.5	46.7	69.6 ± 1.3	61.0	68.1
C18:3	6.1 ± 0.19	6.5	6.5 ± 0.13	4.3	6.7	5.8 ± 0.7	5.7	5.7
C20	0.4 ± 0.09	0.4	0.4	0	0.5	0.2	0	0
Other	1.8	0	0	0	0	0	0	0

[a]Data developed using our method of HPLC, GC method developed by Williams *et al.* (11), and data from Takagi and Ando (9). For abbreviations see Table 1.

TABLE 3
Comparative Fatty Acid Compositions and Positional Distributions of Fatty Acid Residues on the Triacylglycerols of Olive Oil[a]

Fatty acid	Overall triacylglycerol composition		Residue composition at the *sn*-1,3 positions			Residue composition at the *sn*-2 position		
	HPLC	GC	HPLC	GC	(6)	HPLC	GC	(6)
C12	0	0.1	0	0	0	0	0.2	0
C14	0	0	0	0	0	0	0	0
C16	11.1 ± 0.1	11.4	16.1 ± 0.28	13.0	17.0	1.6 ± 0.4	4.9	1.9
C16:1	0.6 ± 0.05	0.6	0.8 ± 0.08	0.6	0.9	0.5 ± 0.3	0.6	0.1
C18	3.5 ± 0.08	3.5	5.1 ± 0.04	4.1	2.1	0.4 ± 0.3	1.8	0.9
C18:1	77.5 ± 1.1	78.0	73.2 ± 0.45	77.2	74.0	89.6 ± 0.2	84.9	90.3
C18:2	5.3 ± 0.05	6.1	4.2 ± 0.11	4.8	6	7.6 ± 0.2	7.5	6.8
C18:3	0	0.3	0	0.4	0	0	0.1	0
C20	0.5 ± 0.15	0	0.6 ± 0.05	0	0	0.3 ± 0.02	0	0
Other	1.5	0	0	0	0	0	0.2	0

[a]Data developed using our method of HPLC, the GC method developed by Williams *et al.* (11), and data from Santinelli *et al.* (17). Values expressed as mole percentages. For abbreviations see Table 1.

Tables 1–3 also contain data concerning the regioselective analyses of the triacylglycerols contained in corn, soybean, and olive oil, respectively. As a general rule, oil seeds containing common fatty acid residues are characterized by selective placement of unsaturated fatty acids at the *sn*-2 position. By contrast, long-chain or anomalous fatty acids are often selectively enriched at the *sn*-3 position (16). The data for the acyl groups located at the *sn*-1,3 and *sn*-2 positions of the triacylglycerols obtained using HPLC and GC are consistent with one another. Either method could be employed to conduct regioselective analyses of triacylglycerols while avoiding time-consuming purification processes. The main disparities between the results are those associated with long-chain saturated fatty acids, especially for palmitic acid. The values obtained with the method proposed by Williams *et al*. (11) indicate a lower relative abundance of this fatty acid at the *sn*-1,3 positions (and a concomitant higher relative abundance of this fatty acid at the *sn*-2 position) than those obtained with our method. Williams *et al*. also reported lower amounts of palmitic and stearic acids at the *sn*-1,3 positions than the values expected for a regioselective analysis of a standard triacylglycerol (1-palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol). The literature also indicates a relatively low abundance of long-chain saturated fatty acid residues at the *sn*-2 position of these edible oils. The low abundance of these residues at the *sn*-2 position in the oils considered here may, at least in part, be responsible for the relatively large uncertainties in the values for these residues.

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