The Triacylglycerol Structure of Olive Oil Determined by Silver Ion High-Performance Liquid Chromatography in Combination with Stereospecific Analysis

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The compositions of positions *sn*-1, *sn*-2 and *sn*-3 of triacylglycerols from "extra-virgin" olive oil (*Olea europaea*) were determined. The procedure involved preparation of diacyl-*rac*-glycerols by partial hydrolysis with ethyl magnesium bromide; 1,3-, 1,2- and 2,3-diacyl-*sn*-glycerols as (*S*)-(+)-1-(1-naphthyl)ethyl urethanes were isolated by high-performance liquid chromatography (HPLC) on silica, and their fatty acid compositions were determined. The same procedure was also carried out on the five main triacylglycerol fractions of olive oil after separation according to the degree of unsaturation by HPLC in the silver ion mode. Although stereospecific analysis of the intact triacyl-*sn*-glycerols indicated that the compositions of positions *sn*-1 and *sn*-3 were similar, the analyses of the molecular species demonstrated marked asymmetry. The data indicate that the "1-random, 2-random, 3-random" distribution theory is not always applicable to vegetable oils.

KEY WORDS: Chiral chromatography, diacylglycerols, high-performance liquid, olive oil (*Olea europaea*), silver ion chromatography, stereospecific analysis, triacylglycerols.

Olive oil is the most important vegetable oil for human consumption in Mediterranean areas, and many of its features have been extensively examined by food chemists. Among more recent papers, the oil has been subjected to separations into molecular species by reversed-phase high-performance liquid chromatography (HPLC) (1,2), silver ion thin-layer chromatography (TLC) in combination with pancreatic lipase hydrolysis (3) or with an oxidation procedure (4), high-temperature capillary gas chromatography (GC) (5,6) and silver ion TLC in combination with reversed-phase HPLC and high-resolution GC (7).

Triacylglycerols from olive oil show a small degree of asymmetry in the distribution of fatty acids among positions *sn*-1, *sn*-2 and *sn*-3 (8,9), but a single symmetric molecule (triolein) represents approximately half of the total. By combining fractionation by silver ion chromatography with stereospecific analysis, it is possible to evaluate the degree of asymmetry in other fractions. It would then be possible to see an enhancement of the differences in composition between positions *sn*-1 and *sn*-3 especially. The opportunities for such a study have been enhanced by new developments in methodology.

Silver ion chromatography has been used extensively to separate triacylglycerol molecules according to degree of unsaturation (10). Most analysts have utilized TLC methods with silica gel impregnated with silver ions to separate triacylglycerols. Recently, a cleaner and more efficient method has been developed involving HPLC with cation-exchange columns loaded with silver ions (11,12). This technique has been applied to fractionate triacylglycerols from seed oils (12–14) and more complex mixtures from North Atlantic and Baltic herring (15,16).

Natural triacyl-*sn*-glycerols have an asymmetric distribution of the fatty acids located in positions *sn*-1, *sn*-2 and *sn*-3. To obtain the fatty acid composition of each of these positions, stereospecific analysis procedures involving chemical, enzymatic and synthetic steps have been developed. They have been extensively reviewed (17,18). Recently, a new robust and simple procedure involving partial hydrolysis of triacyl-*sn*-glycerols to diacylglycerols, preparation of diastereomeric 1-(1-naphthyl)ethyl urethane derivatives and resolution of these by HPLC on silica has been developed (9,19). In this study, a combination of silver ion HPLC and chromatographic resolution of diastereomeric diacyl-*sn*-glycerol derivatives in sequence has been utilized to obtain the distribution of fatty acids in positions *sn*-1, *sn*-2 and *sn*-3 of the main triacyl-*sn*-glycerol species of olive oil.

MATERIALS AND METHODS

Samples and reagents. "Extra-virgin" olive oil, *i.e.* that produced by an extrusion process only, was from the region of Umbria, Italy. Triacylglycerol samples (100 mg) were first purified on short Florisil™ columns, eluted with hexane (3 mL) and then hexane-acetone (96:4, vol/vol; 10 mL). All solvents and reagents were Analar or HPLC grades, and were supplied by FSA Scientific (Loughborough, U.K.).

Silver ion HPLC. The HPLC system consisted of a Spectra-Physics Model 8700 solvent delivery system (Spectra-Physics, St. Albans, U.K.), a Cunow Model DDL21 light-scattering detector (Cunow SA, Cergy St. Christophe, France) and a Spectra-Physics SP 4290 integrator. An adjustable stream-splitter was installed between the column and the detector. The silver ion column "ChromSpher Lipids" was kindly donated by Dr. Stephan Rose of Chrompack Ltd. (Middelburg, Netherlands) and was prepared as described elsewhere (11). The fractionation of olive oil triacylglycerols was carried out with a ternary solvent gradient system with (A) 1,2-dichloroethane/dichloromethane (1:1, vol/vol); (B) acetone; (C) acetone/acetonitrile (9:1, vol/vol) as the three components. Linear gradients were generated from 100% A to 60% A — 40% B over 18 min and then to 90% B — 10% C over a further 25 min. The column was maintained at ambient temperature with a flow rate of 1.0 mL/min. Samples were dissolved in 1,2-dichloroethane, and aliquots of 1.0–1.5 mg triacylglycerols in 8–10 μL of 1,2-dichloroethane were injected onto the column. In order to have sufficient material available for the following stereospecific analysis procedure, the separation was repeated up to 25 times, and corresponding fractions were pooled. Fractions from one run were methylated with an internal standard for identification and quantitation by GC.

*Stereospecific analysis of triacyl-*sn*-glycerols.* A procedure described previously was used (9). Triacylglycerols

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(1–2 mg) were dissolved in dry diethyl ether (1 mL), freshly prepared 0.5 M ethyl magnesium bromide in dry diethyl ether (250 μL) was added, and the mixture was shaken for 1 min before glacial acetic acid (6 μL) in pentane (5 mL) and water (2 mL) were added to stop the reaction. The organic layer was washed twice with water (2 mL) and dried over anhydrous sodium sulfate. After evaporating the solvent in a stream of nitrogen at room temperature, the mixture of hydrolysis products was derivatized immediately.

The hydrolysis products were dissolved in dry toluene (1 μL), and (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate (12.5 μL) and 4-pyrrolidinopyridine (4 mg) were added. The mixture was heated at 50°C overnight, and the following day the solvent was removed in a stream of nitrogen. Methanol/water (95:5, vol/vol; 6 mL) was added and warmed to dissolve the products. A Bond-Elut™ ODS solid-phase extraction column (500 mg; Jones Chromatography, Hengoed, Wales) was solvated by passing 10 mL of this solvent through it. The reaction mixture was filtered through a small cotton-wool plug onto the column and washed through with a further 15 mL of solvent. The required products were then eluted with acetone (10 mL).

HPLC separation of the diastereomeric diacylglycerol derivatives was carried out with a Spectra-Physics Model 8770 isocratic pump, a Pye Unicam Model 4025 UV-detector (Pye Unicam, Cambridge, U.K.) and a Spectra-Physics SP 4290 integrator. Two columns of silica gel [Hypersil 3 μm, 25 cm × 4.6 mm i.d. (HiChrom Ltd., Reading, U.K.)] in series were utilized with 0.4% (vol/vol) *n*-propanol (containing 2% water) in hexane as mobile phase at a flow rate of 1 mL/min (10). The sample was injected in the minimum volume of hexane (5–10 μL). Detection was at 280 nm.

Gas chromatography. The methyl ester derivatives of the fatty acids from each fraction were prepared by sodium methoxide-catalyzed transesterification (9), in the presence of an internal standard (methyl nonadecanoate) only for quantitation of fractions obtained by silver ion HPLC. A Carlo Erba Model 4130 capillary gas chromatograph (Carlo Erba, Crowley, U.K.) was equipped with split/splitless injection and a capillary column (25 m × 0.22 mm i.d., film thickness 0.2 μm) of fused silica coated with Carbowax 20M (Chrompack UK Ltd., London, U.K.). The column temperature was programmed with 3 min isothermally at 175°C, then to 205°C at 4°C/min, and held at the final temperature for 20 min more. Hydrogen was the carrier gas. Components were quantitated by electronic integration.

RESULTS AND DISCUSSION

In this research, the sequential combination of silver ion HPLC and stereospecific analysis (*via* resolution of diastereomeric diacylglycerols derivatives by HPLC on silica) was investigated for the structural analysis of triacyl-*sn*-glycerols of "extra-virgin" olive oil.

The intact oil was first subjected to the stereospecific analysis procedure. Diacyl-*sn*-glycerols were prepared and converted as described previously to the (*S*)-1-(1-naphthyl)-ethyl urethane derivatives. The diastereomeric forms were separated by HPLC on silica gel through isocratic elution with 0.4% *n*-propanol (containing 2% water) in hexane. It was observed that much more uniform and reproducible

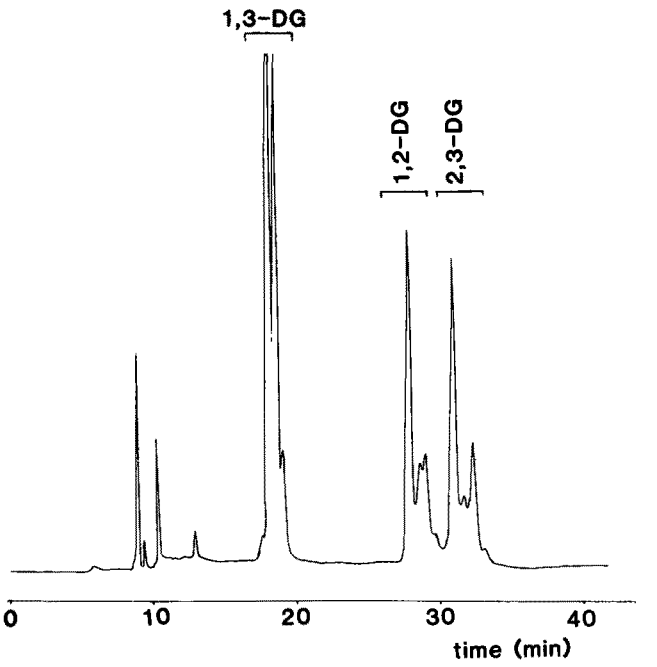

FIG. 1. Separation of the (*S*)-(+)-1-(1-naphthyl)ethyl urethane derivatives of diacylglycerols, prepared by partial hydrolysis of olive oil, by HPLC on silica gel (see Materials and Methods section for experimental details).

retention times were obtained when the helium used for degassing was passed first through a sealed flask containing the mobile phase, before being passed into the mobile phase proper; it appeared that problems described previously of increasing retention times were a consequence of selective evaporation of the polar component of the mixture (9,19). The separation of diacyl-*sn*-glycerol derivatives prepared from olive oil triacylglycerols is shown in Figure 1. The 1,3-, 1,2- and 2,3-diacyl-*sn*-glycerols were well resolved into distinct groups of peaks, and each had a dioleoyl species as the major component in each group followed by two smaller peaks. There was a suggestion of a fourth peak at the end of each group, which might be enriched in arachidic and erucic acids (or linolenic acid), but these are not considered in this paper.

Each fraction was collected and transesterified for fatty acid analysis by gas chromatography (GC), and the data were used to calculate the results for each of the three positions; these are listed in Table 1, *i.e.* the composition of position *sn*-1 was calculated from the data for the intact triacylglycerols and the 2,3-diacyl-*sn*-glycerol derivatives, that for position *sn*-3 from the 1,2-diacylglycerols and that for position *sn*-2 by difference. The composition of position *sn*-2 obtained from the analysis of the 1,3-diacyl-*sn*-glycerols is listed separately as a check, but is relatively unimportant because of acyl migration, which affects only this fraction during the hydrolysis step (9,19). Because of the indirect method of calculation, small negative values are occasionally obtained for minor components. Olive oil, like other vegetable oils, does not exhibit a markedly asymmetric distribution of fatty acids between positions *sn*-1 and *sn*-3. It has higher proportions of oleate and low concentrations of saturated fatty acids in position *sn*-2,

TABLE 1

Fatty Acid Compositions (mole % of the total fatty acids) of the Triacyl-*sn*-glycerols from Olive Oil and of Positions *sn*-1, *sn*-2 and *sn*-3 of the Triacylglycerols

Fatty acid	Total	Positions			
		sn-1[a]	*sn*-2[b]	*sn*-3[c]	*sn*-2[d]
16:0	13.6	16.7	2.0	22.1	5.6
16:1	0.6	0.7	0.8	0.4	0.4
18:0	2.0	1.7	2.2	2.0	0.5
18:1(n-9)	75.4	70.0	86.3	69.8	82.8
18:1(n-7)	0.9	1.2	−0.3	1.9	0.4
18:2	7.6	9.7	9.1	3.9	10.3

[a] 3 × TG − 2 × 2,3-DG; [b] 3 × TG − (a + c); [c] 3 × TG − 2 × 1,2-DG; [d] 3 × TG − 2 × 1,3-DG where TG = triacylglycerols; DG = diacyl-*sn*-glycerols.

with small differences only in the relative compositions of positions *sn*-1 and *sn*-3, *i.e.* a higher proportion of 16:0 in position *sn*-3 and of 18:2(n-6) in position *sn*-1. The results obtained here were rather similar to those obtained previously (8,9).

Silver ion HPLC was used to fractionate the triacylglycerols according to the degree of unsaturation with the fractions being collected *via* the stream-splitter. For analytical purposes, the nine fractions shown in Figure 2 were obtained initially. The more saturated molecules eluted first and the degree of unsaturation increased, with the elution order of the components being SSM, SMM, SSD, MMM, SMD, MMD, SDD, MDD and MMT, where S = saturated, M = monoenoic, D = dienoic and T = trienoic fatty acyl moieties. Fractions were identified by their retention times relative to material of known composition. In most recent preparative studies of olive oil by silver ion TLC (3,4,7), the trienoic fractions, *i.e.* MMM, SMD and SST, were not resolved.

For preparative purposes, five main fractions (SSM, SMM, MMM, SMD and MMD) were separated. These amounted to more than 95% of the total. The fractionation was repeated several times to obtain enough material from the least abundant fractions for stereospecific analysis. Each fraction was transesterified with sodium methoxide in the presence of an internal standard for identification and quantitation of the fatty acids by GC. The fatty acid composition of these fractions is listed in Table 2. Some fatty acid specificity was evident in that a relatively high proportion of 16:1 was collected in the MMD component, but it was not detectable in the SMD fraction; more of the 18:0 was in the SMM and SMD fractions in comparison to that in the SSM fraction. As a check, the data from the fractions were used to compute the composition of the original intact triacylglycerols; good agreement was obtained.

The fractions obtained by silver ion HPLC were subjected to the procedure for stereospecific analysis of triacyl-*sn*-glycerols. The partial hydrolysis step was performed as before, but the amount of ethyl magnesium bromide was reduced to one-quarter of the original because of the small size of the samples (1 to 2 mg). The results are shown in Table 3. When possible (depending on the amount of material available), the analysis was repeated twice and the results obtained agreed with those shown here.

By combining silver ion HPLC and stereospecific analysis of triacyl-*sn*-glycerols from olive oil in this way, it was

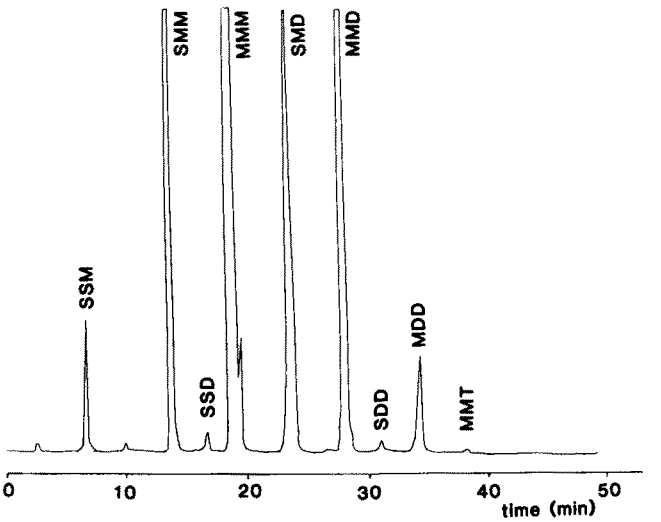

FIG. 2. Fraction of the triacylglycerols of olive oil by HPLC in the silver ion mode (see Materials and Methods section for experimental details).

possible to obtain a better estimate of the true degree of molecular asymmetry. The treatment was again restricted to the six main fatty acids. The MMM fraction was almost exclusively constituted by triolein. As olive oil contains more than 70% oleic acid [18:1(n-9)], it was to be expected that the MMM fraction comprised nearly half the total. The minor monoenoic fatty acids tended to be concentrated in the primary positions. In most fractions, oleic acid was predominantly in position *sn*-2, but in the MMD fraction it was more concentrated in position *sn*-3 followed in sequence by *sn*-2 and *sn*-1. In the SSM fraction, there was more oleate in position *sn*-3 than in position *sn*-1, but the opposite was true of the SMM and SMD fractions. This specificity was not apparent from the analysis of the total triacyl-*sn*-glycerols, where there appeared to be little difference in the compositions of positions *sn*-1 and *sn*-3.

Palmitic acid (16:0) was distributed mainly in positions *sn*-1 and *sn*-3 compared to position *sn*-2 in all fractions containing saturated fatty acids. In the SMM and SMD fractions, palmitic acid showed a preference for position *sn*-3 compared to position *sn*-1 (as in the intact triacyl-*sn*-glycerols) with ratios of 46.9 to 34.6 and 51.5 to 27.0,

TABLE 2

Fatty Acid Compositions (mole % of the total fatty acids) and Proportions of the Main Triacylglycerol Fractions Obtained by Preparative Silver Ion HPLC from Olive Oil

Fatty acid	Total	Fraction					Recombined composition
		SSM	SMM	MMM	SMD	MMD	
16:0	13.6	63.5	30.1		30.9		13.2
16:1	0.6	0.5	0.3	1.1	trace	2.7	1.0
18:0	2.0	3.9	3.6		3.2		1.4
18:1(n-9)	75.4	32.1	65.5	98.4	33.2	64.6	77.4
18:1(n-7)	0.9	trace	0.5	0.5	trace	1.1	0.5
18:2	7.6				32.7	31.7	6.4
Fraction amount	(mole %)[a]	3.2	31.0	45.8	6.0	14.0	

[a]Normalized to 100% to ignore minor components present in insufficient amounts for further analysis.

TABLE 3

Fatty Acid Compositions (mole % of the total) of Triacyl-*sn*-glycerol Fractions (from olive oil) Obtained by Preparative Silver Ion HPLC, and of Positions *sn*-1, *sn*-2 and *sn*-3

Fatty acid	Total	Positions		
		sn-1[a]	*sn*-2[b]	*sn*-3[c]
Fraction SSM				
16:0	59.3	88.4	10.4	78.9
18:0	7.6	7.7	3.9	11.1
18:1(n-9)	33.2	3.8	85.6	10.0
Fraction SMM				
16:0	28.7	34.6	4.5	46.9
16:1	0.6	0.5	0.2	0.9
18:0	4.2	3.1	2.3	7.1
18:1(n-9)	66.6	61.7	93.0	45.1
Fraction MMM				
16:1	1.0	1.7	0.1	1.0
18:1(n-9)	97.8	96.7	100.0	96.6
18:1(n-7)	1.3	1.5	−0.2	2.4
Fraction SMD				
16:0	27.3	27.0	3.3	51.5
18:0	3.7	2.0	2.2	6.9
18:1(n-9)	41.2	38.7	53.4	31.5
18:2	27.8	32.3	41.1	10.1
Fraction MMD				
16:1	0.3	0.4	0.1	0.3
18:1(n-9)	65.7	49.3	68.8	79.1
18:1(n-7)	1.1	1.1	−0.2	2.3
18:2	32.9	49.2	31.2	18.3
Check on the composition of the total triacyl-*sn*-glycerols[d]				
16:0	13.2	15.2	1.9	20.2
16:1	1.0	1.0	0.1	0.8
18:0	1.4	1.3	0.9	3.0
18:1(n-9)	77.4	72.8	90.3	71.5
18:1(n-7)	0.5	0.8	−0.1	1.4
18:2	6.4	8.8	6.8	3.2

[a]$3 \times TG - 2 \times 2,3\text{-DG}$; [b]$3 \times TG - (a + c)$; [c]$3 \times TG - 2 \times 1,2\text{-DG}$ where TG = triacylglycerols; DG = diacyl-*sn*-glycerols.

[d]Calculated by recombining the above indicated data and the previously described (Table 2) % amount of the considered fractions. Example: % amount of 16:0 in position *sn*-1; $(88.4 \times 3.2 + 34.6 \times 31.0 + 27.0 \times 6.0)/100 = 15.2\%$.

respectively, but in the SSM fraction there was a slight preference for position *sn*-1 (88.4 to 78.9). Stearic acid (18:0) was mainly present in position *sn*-3 in all the relevant fractions, followed by position *sn*-1 and then position *sn*-2. However, in the SMD fraction, there were comparable amounts of stearic acid in positions *sn*-1 and *sn*-2. Linoleic acid (18:2) had an unusual distribution in that in fraction SMD, the highest proportion was in position *sn*-2 (41.1%) with less in position *sn*-1 (32.3%) and least in position *sn*-3 (10.1%). In the MMD fraction, in contrast, position *sn*-1 was favored (49.2%), followed by positions *sn*-2 (31.2%) and *sn*-3 (18.9%). Such results would not have been predicted from the results of the stereospecific analysis of the whole triacyl-*sn*-glycerols. In the fractions in which they were present, the distributions of the minor components, 16:1 and 18:1(n-7) appeared to be parallel to those in the intact triacyl-*sn*-glycerols.

As a check on the accuracy of these data, they were used to compute the fatty acid compositions of the intact triacylglycerols and those of positions *sn*-1, *sn*-2 and *sn*-3. The agreement between these results and those in Table 1 represents a further demonstration of the reliability of the experimental procedures.

With many natural triacyl-*sn*-glycerol samples, it has proved possible to obtain good agreement between the compositions of molecular species calculated from stereospecific analysis data on the basis of a "1-random, 2-random, 3-random" distribution theory (20) and the actual proportions determined by experiment. The topic has been reviewed elsewhere (17,18). With olive oil, the above data demonstrate that this theory is not valid.

These results demonstrate that, by combining silver ion HPLC resolution of molecular species of triacyl-*sn*-glycerols with stereospecific analysis, it is possible to obtain a different picture of structural features than can be obtained from analyses of the intact oil alone. With olive oil, stereospecific analysis indicated relatively little difference between the compositions of the primary positions. The differences in the positional distributions in the fractions compared to those anticipated must in some way be a reflection of the biosynthetic process. The "1-random, 2-random, 3-random" theory implies that fatty acids are esterified to the triacylglycerols with noncorrelative specificity (20), *i.e.* that intermediates in the biosynthetic pathway are esterified from distinct pools of fatty acids for each position without regard for the fatty acid composition of the intermediates *per se.* In olive oil, the acyltransferases involved in biosynthesis must proceed with high specificity for the monoacyl and diacyl intermediates.

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