Stereospecific Analysis of Triacyl-*sn*-glycerols *via* Resolution of Diastereomeric Diacylglycerol Derivatives by High-Performance Liquid Chromatography on Silica

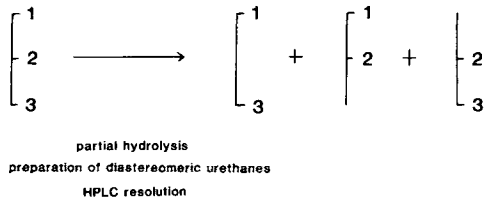
William W. Christie[a,*], **Boryana Nikolova-Damyanova**[a,1], **Päivi Laakso**[a,2] and **Bengt Herslof**[b]
[a]Hannah Research Institute, Ayr, Scotland KA6 5HL, and [b]Karlshamns LipidTeknik, S-104 65 Stockholm, Sweden

The compositions of positions *sn*-1, 2 and 3 of triacylglycerols can be determined by partial hydrolysis with ethyl magnesium bromide, derivatization of the total products with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate and isolation of the diacyl-*sn*-glycerol urethane derivatives by chromatography on solid-phase extraction columns containing an octadecylsilyl phase. The diastereomeric *sn*-1,2- and 2,3-diacylglycerol derivatives are separated by high-performance liquid chromatography on silica for determination of their fatty acids by gas chromatography. Each step in the process has been evaluated rigorously. The compositions of all three positions can be calculated with good accuracy from the analyses of these compounds and that of the total triacylglycerols. Although the 1,3-*sn*-diacylglycerol derivatives can also be isolated easily, they do not give reliable results for the composition of position *sn*-2 because acyl migration occurs during their generation. The stereospecific analysis procedure has been applied to some plant and animal triacyl-*sn*-glycerols of commercial and scientific interest, containing predominantly C_{16} and C_{18} fatty acids, *i.e.* safflower, sunflower, olive and palm oils, tallow, egg and rat adipose tissue. The method is not at present suited to the analysis of more complex triacylglycerols, such as milk fat or fish oils, and problems associated with these are discussed.

KEY WORDS: Chiral chromatography, diacylglycerols, Grignard hydrolysis, high-performance liquid chromatography, stereospecific analysis, triacylglycerols.

The procedures for stereospecific analysis of triacyl-*sn*-glycerols, *i.e.* for the determination of the compositions of the fatty acids located in each of positions *sn*-1, -2 and -3, tend to be complex and involve a number of chemical and enzymatic hydrolytic reactions in addition to synthetic steps. The intermediates for each stage in the reaction must be isolated in a high degree of purity by chromatographic means. Losses can occur at many points and the specificity or selectivity of the enzymes for particular molecular species can also introduce errors. Such procedures have been reviewed elsewhere (1,2).

Recently (3), the potential of a procedure involving partial hydrolysis of triacyl-*sn*-glycerols to diacylglycerols, preparation of diastereomeric 1-(1-naphthyl)ethyl urethane derivatives and resolution of these by high-performance liquid chromatography (HPLC) on silica was investigated, as illustrated in Scheme 1—stereospecific analysis of triacyl-*sn*-glycerols. During the final step, excellent resolution of distinct molecular species of each of the diastereomeric diacylglycerol derivatives was obtained. Others are approaching the problem in a related manner, but they use costly chiral-phase HPLC columns and reagents at present only available in Japan (4,5). In our procedure, the composition of the fatty acids in position *sn*-1 of the triacylglycerols could be calculated from the data for the triacylglycerols *per se* and that for the 2,3-*sn*-diacylglycerol derivatives isolated by HPLC. Similarly, the composition of position *sn*-2 was calculated when that of the 1,3-*sn*-diacylglycerols was determined (although this is no longer recommended as discussed below), and that for position *sn*-3 was obtained from the results for the 1,2-*sn*-diacylglycerols. The methodology utilized commonly available HPLC columns and reagents and did not require the use of enzymatic hydrolysis.



SCHEME 1

Each step has now been tested rigorously. In particular, a new procedure for purifying the urethane derivatives has been developed, eliminating the need to isolate diacylglycerols, each component of the mobile phase used to separate the diastereomers has been changed to improve the resolution, a more quantitative transesterification procedure has been developed, and it has been shown that the composition of position *sn*-2 must be determined by an indirect method. The procedure can now be considered robust for stereospecific analysis of triacyl-*sn*-glycerols.

EXPERIMENTAL PROCEDURES

Standards, reagents and lipid samples. 1,3-Dioleoyl-2-palmitoylglycerol, 1,2-dioleoyl-3-palmitoylglycerol and 1,3-dipalmitoyl-2-oleoylglycerol were purchased from the Sigma Chemical Co. (Poole, U.K.). (*S*)-(+)-1-(1-naphthyl) ethyl isocyanate was obtained from the Aldrich Chemical Co. (Gillingham, U.K.). The solvents used were HPLC grade and were obtained from Fisons Ltd. (Poole, U.K.).

Chromatographically pure safflower, sunflower, olive, palm, coconut, palm kernel and fish oils and tallow were supplied by Karlshamns LipidTeknik AB (Stockholm, Sweden). Milk fat was obtained from the EEC Bureau of Standards (Brussels, Belgium). Lipids were extracted from the yolk of an egg from a chicken (*Gallus gallus*) and from

*To whom correspondence should be addressed.
[1]Present address: Institute of Organic Chemistry, Centre of Phytochemistry, Sofia 1113, Bulgaria.
[2]Present address: University of Turku, Dept. of Chemistry and Biochemistry, Laboratory of Food Chemistry, 20500 Turku, Finland.

parametrial adipose tissue of a female Wistar rat with chloroform-methanol (2:1, v/v). Lipid samples were first purified on short Florisil™ columns, eluted with hexane-diethyl ether (4:1, v/v), after which triacylglycerols were obtained by preparative HPLC on a silica column (Spherisorb™ S5W, 25 cm × 8 mm i.d.) with 2% tetrahydrofuran in hexane as the eluent at a flow-rate of 2 mL/min, and with refractive index detection (Knauer, Oberursel/Taunus, Germany).

Partial hydrolysis of triacylglycerols. Triacylglycerols (10 mg) were dissolved in dry diethyl ether (2 mL), freshly prepared 0.5 M ethyl magnesium bromide in dry diethyl ether (0.5 mL) was added, and the mixture was shaken for 1 min before glacial acetic acid (50 μL) in hexane (5 mL) and water (2 mL) were added to stop the reaction (6). The solvent layer was washed twice with water and dried over anhydrous sodium sulphate for 20 min. After evaporating the solvent in a stream of nitrogen at room temperature, the mixture of hydrolysis products was derivatized immediately.

Preparation of 1-(1-naphthyl)ethyl urethane derivatives. The hydrolysis products were dissolved in dry toluene (0.5 mL) and (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate (12.5 μL) and 4-pyrrolidinopyrridine (4 mg) were added. The mixture was heated at 50°C overnight, then the solvent was removed in a stream of nitrogen. Methanol-water (95:5, v/v; 6 mL) was added and warmed to dissolve the products as far as possible. 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). (If further purification is needed, it can be achieved by elution through a short Florisil column with hexane-acetone (10 mL; 96:4, v/v). The ODS columns have been re-used a further five times following resolvation with the starting solvent (10 mL).

HPLC resolution of diastereomers. HPLC separation of the diastereomeric diacylglycerol urethane derivatives was carried out with a Spectra-Physics (St. Albans, U.K.) Model 8770 isocratic pump together with a Pye Unicam (Cambridge, U.K.) PU 4025 UV-detector and a Spectra-Physics Model 4270 integrator. For optimum resolution, two columns of silica gel (Hypersil™ 3μ, 25 cm × 4.6 mm i.d. (HiChrom Ltd., Reading, U.K.) in series were utilized with 0.4 to 0.33% (v/v) 1-propanol (containing 2%water) in isooctane as mobile phase at a flow-rate of 1 mL/min. The sample was injected in the minimum volume of isooctane (5–10 μL). Detection was at 280 nm.

Pancreatic lipase hydrolysis. Triacylglycerols were subjected to hydrolysis with pancreatic lipase essentially as described by Luddy *et al.* (7). The 2-monoacyl-*sn*-glycerols were separated by HPLC on a column of silica gel (Hypersil 5μ; 25 cm × 4.6 mm i.d.) with hexane-tetrahydrofuran (7:3, v/v) at 1 mL/min as the mobile phase (1). They were detected with a Cunow Model DDL 21 light-scattering detector (Severn Analytical Ltd, Shefford, U.K.) and were collected *via* a stream splitter between the column and the detector.

Fatty acid analysis. The naphthylethylurethane derivatives were dissolved in 1,2-dichloroethane (0.5 mL), methyl acetate (25 μL) and 1M sodium methoxide (25 μL) were added, and the mixture was heated for 1 hr at 50°C (8). Acetic acid (6 μL) was added and the solvents were evaporated under vacuum. Other lipids were methylated similarly except that a shorter reaction time was utilized. The products were taken up in hexane and filtered before gas chromatography (GC) analysis. When necessary (*e.g.* to check the yield of diacylglycerols), methyl nonadecanoate was added as an internal standard (9). A Carlo Erba Model 4130 gas chromatograph [MSE Scientific Instruments (Fisons), Crawley, U.K.], equipped with a split/splitless injection system, was used for fatty acid analyses with a fused silica column coated with Carbowax 20M™ (25 m × 0.22 mm i.d., d_f = 0.2 μm (Chrompack UK Ltd, London, England)). The temperature of the oven was maintained at 160°C for 3 min, then it was raised at 4°C/min to 190°C. Hydrogen was the carrier gas.

RESULTS AND DISCUSSION

Method development. In any robust method for stereospecific analysis of triacyl-*sn*-glycerols, the number of steps should be kept to the minimum, and each one should be studied rigorously as part of the overall scheme. Partial hydrolysis with ethyl magnesium bromide is the first step. This must be carried out under controlled conditions and the products taken on to the next stage immediately to minimize the opportunities for acyl migration to occur. The usual practice is to isolate the required 1,3-, 1,2- and 2,3-*sn*-diacylglycerols by thin-layer chromatography on boric acid-impregnated plates (1,2), prior to converting them to stable derivatives. However, in this study, it was determined that this step was unnecessary as the hydrolysis mixture could be converted directly to the (*S*)-(+)-1-(1-naphthyl)ethyl urethane derivatives, which could be purified with relative ease by chromatography on solid-phase extraction columns packed with an octadecylsilyl bonded phase. The other products, such as tertiary alcohols, do not form derivatives and are eluted separately from the column together with the excess of the derivatizing reagents before the required derivatives are eluted. It was demonstrated earlier that no acyl migration of the diacylglycerols occurs during the derivatization procedure *per se* (3).

The related diastereomeric (*S*)-1-(1-phenyl)ethyl urethane derivatives were also prepared but were less well resolved by HPLC.

In the initial work (3), it was observed that an HPLC mobile phase of hexane containing a small amount of the polar modifier, isopropanol, gave both good resolution of diastereomers and of molecular species with silica as the stationary phase. It has now been observed that other alcohols can be effective as modifiers and that primary alcohols are preferable to secondary. Short-chain alcohols, such as methanol or ethanol, tended to give better resolution of diastereomers but poorer resolution of molecular species. Also, in the earlier work (3), difficulties were experienced with obtaining reproducible retention times, which tended to lengthen appreciably during the working day. This problem could be partially alleviated by adding 2% water to the polar modifier, 1-propanol, and by making up only sufficient mobile phase for each analysis, replacing this completely for the second and subsequent analyses each day. Isooctane gave slightly better results than hexane.

Some of these effects appeared to be dependent on the nature of the silica surface and varied a little with different columns, even when they were from the same batch from the manufacturer. All columns gave excellent results when eluted for two-hour periods with hexane and 0.5% isopropanol (containing 2% water), first with the addition of 0.1% ethylamine, then on its own, then with 0.1% acetic acid, and finally with the neutral mobile phase again. No resolution was obtained with alumina or spherical carbon as the stationary phase, nor with bonded phases with octadecyl, diol, amine, sulphonic acid or nitrile moieties.

Finally, the accuracy of the procedure when applied to natural triacylglycerols is highly dependent on the precision of the GC analysis of methyl esters prepared from the component fatty acids. The naphthylurethane derivatives were transferred more slowly than most lipids, but reproducible and quantitative esterification was obtained by prolonging the reaction time.

Application to standards. To test the methodology, it was first applied to the synthetic standards, 1,3-dioleoyl-2-palmitoylglycerol, 1,2-dioleoyl-3-palmitoyl-glycerol and 1,3-dipalmitoyl-2-oleoylglycerol. Pancreatic lipase hydrolysis was used to determine the composition of position *sn*-2, and we confirmed that the compounds contained no more than 1–2% of other molecular species. The standards were then hydrolyzed partially with ethyl magnesium bromide, derivatized and resolved by HPLC as described above. Generally, 10-μg samples were first subjected to analytical HPLC, then 300- to 400-μg samples were separated and fractions were collected manually for fatty acid analysis. There was no significant loss of resolution with the larger samples. The chromatographic traces are illustrated in Figure 1, and the results of the fatty acid analysis are listed in Table 1. In each instance, some early peaks represented small amounts of nonlipid by-products; unchanged triacylglycerols might also be present but would not be detected at 280 nm. That these and any other non-ultraviolet (UV) absorbing by-products did not interfere or were absent was confirmed by HPLC under identical elution conditions but with light-scattering detection. Peaks for the 1,3-, 1,2- and 2,3-*sn*-diacylglycerol derivatives were always clearly delineated.

With 1,3-dioleoyl-2-palmitoylglycerol as the substrate, a single peak would be expected for the 1,3-form consisting of the dioleoyl species. However, a substantial subsidiary peak was observed, which evidently was the palmitoyloleoyl species from the fatty acid data; it could only arise by acyl migration from the 1,2- and 2,3-*sn*-diacylglycerols. Related effects were seen with 1,2-dioleoyl-3-palmitoylglycerol and 1,3-dipalmitoyl-2-oleoylglycerol as substrates. On the other hand, the 1,2- and 2,3-forms were essentially single peaks, *i.e.* the 1-oleoyl-2-palmitoyl and 1-palmitoyl-2-oleoyl species, respectively, when 1,3-dioleoyl-2-palmitoylglycerol and 1,3-dipalmitoyl-2-oleoylglycerol were the substrates. The small amounts of other species detected were present in amounts consistent with those anticipated from pancreatic lipase hydrolysis data. With 1,2-dioleoyl-3-palmitoylglycerol as the substrate, two peaks of approximately equal intensity were seen for each of the 1,2- and 2,3-diastereomers, and they presumably are the dioleoyl and oleoyl-palmitoyl species. The 1-oleoyl-3-palmitoyl- and 1-palmitoyl-3-oleoyl-*sn*-glycerol derivatives, which were also presumably formed, were not separated from each other.

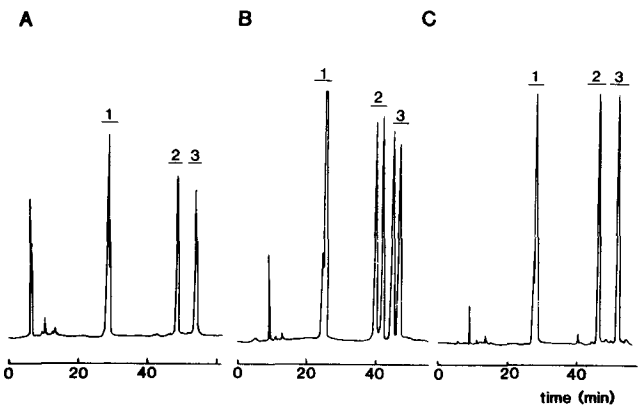


FIG. 1. HPLC resolution of (*S*)-(+)-1-(1-naphthyl)ethyl urethane derivatives of diacyl-*sn*-glycerols prepared from 1,3-dioleoyl-2-palmitoylglycerol (A), 1,2-dioleoyl-3-palmitoylglycerol (B) and 1,3-dipalmitoyl-2-oleoylglycerol (C). The mobile phase was isooctane with 0.4% 1-propanol (containing 2% water). Other conditions were as in the Experimental section. The numbers 1, 2 and 3 above peaks refer to the 1,3-, 1,2- and 2,3-*sn*-diacylglycerol derivatives, respectively.

Isomerization of the diacylglycerols produced by hydrolysis with Grignard reagents has been observed previously (6,10–12). As in this instance, only the composition of 1,3-*sn*-diacylglycerols appeared to be affected while the 1,2- and 2,3-diastereomers were not. The mechanism of this isomerization has been discussed by Brockerhoff (12). We confirmed his results and were unable to find conditions in which the degree of isomerization was lower than that reported here, *e.g.* by the use of alcohols or other acids to stop the reaction. Even under some experimental conditions when the degree of contamination of the 1,3-*sn*-diacylglycerols by isomerized 1,2- /2,3-forms approached 50%, no significant contamination of the 1,2- and 2,3-forms was observed.

These findings have important implications when the compositions of the various diacylglycerol isomers and diastereomers are used in stereospecific analyses. As can be seen from Table 1 for each of the standard triacylglycerols, the compositions of positions *sn*-1 and *sn*-3 can be obtained with high precision by arithmetic means from the data for the total triacylglycerols and that for the 2,3- and 1,2-*sn*-diacylglycerols, respectively. The composition of position *sn*-2 calculated in a similar way from the contaminated 1,3-*sn*-diacylglycerols is greatly in error when compared to the result obtained by pancreatic lipase hydrolysis. On the other hand, a close approximation to the true result for position *sn*-2 can be obtained by calculation from the data for the total triacylglycerols and those obtained for positions *sn*-1 and *sn*-3.

In contradiction with a previous suggestion (3), it was not possible to separate by HPLC species in which the position only of the fatty acids differed, *e.g.* 1-palmitoyl-2-oleoyl- and 1-oleoyl-2-palmitoyl diacylglycerol derivatives.

Application to natural fats and oils. The method was then applied to a number of natural oils and fats of plant

TABLE 1

Fatty Acid Compositions (mol% of the total) of the Synthetic Triacylglycerols (TG), the Chiral Diacyl-*sn*-glycerols (DG) Obtained by Partial Hydrolysis and Positions *sn*-1, -2 and -3

Fatty acid	TG	1,3-DG	1,2-DG	2,3-DG	Position*					
					sn-1[a]	*sn*-2[b]	*sn*-2[c]	*sn*-2[d]	*sn*-3[e]	*sn*-1/3[f]
				1,3-dioleoyl-2-palmitoylglycerol						
16:0	34.6	15.7	50.7	50.7	2.2	72.3	99.1	99.4	2.4	2.2
18:1	65.4	84.3	49.3	49.3	97.8	27.7	0.9	0.6	97.6	97.8
				1,2-dioleoyl-3-palmitoylglycerol						
16:0	34.1	43.6	25.8	26.3	49.7	15.1	1.8	3.0	50.8	49.7
18:1	65.9	56.4	74.2	73.7	50.3	84.9	98.2	97.0	49.2	50.3
				1,3-dipalmitoyl-2-oleoylglycerol						
16:0	66.0	87.6	50.2	49.3	99.6	22.9	0.9	1.7	97.6	98.2
18:1	34.0	12.4	49.8	50.7	0.4	77.1	99.1	98.3	2.4	1.8

*a = 3 × TG − 2 × 2,3-DG; b = 3 × TG − 2 × 1,3-DG; c = 3 × TG − (a + e); d = pancreatic lipase; e = 3 × TG − 2 × 1,2-DG; f = (3 × TG − d)/2.

and animal origin that contained C_{16} and C_{18} fatty acids predominantly. When separated by HPLC, several peaks (usually three in number) representing different molecular species were obtained for each of the diacylglycerols, but no attempt was made to collect individual species for analysis. Rather, all the peaks in the 1,3-diacylglycerol region were collected together and so forth. The results are listed in Table 2; the separation of the diacylglycerol derivatives from palm oil are shown in Figure 2 as an example of the application to a vegetable oil. Results for each of the three positions were calculated from the data for the compositions of the intact triacyl-*sn*-glycerols and of the 1,2- and 2,3-*sn*-diacylglycerols, and from these data and those for position *sn*-2 obtained by pancreatic lipase hydrolyses. The compositions of position *sn*-2 obtained from the analyses of the 1,3-*sn*-diacylglycerols are listed separately.

The chromatographic traces obtained for safflower and sunflower oils tended to resemble that published previously for maize oil (3), while that for olive oil had dioleoyl species as the major peak in each diacylglycerol group followed by two smaller ones. With palm oil (Fig. 2), the central peak in each group, which is presumably the palmitoyloleoyl species, was the largest. In this instance, there was a suggestion of a fourth peak at the end of the 2,3-*sn*-diacylglycerol group, which might be enriched in myristic acid; a similar peak in the 1,2-group would be submerged. As with the standard triacylglycerols, the results for position *sn*-2 obtained by utilizing the composition of the contaminated 1,3-diacylglycerols in the calculation gave less reliable results than those obtained by calculation from the 1,2- and 2,3-*sn*-diacylglycerols. On the other hand, with the safflower, sunflower and olive oils, the results obtained for position *sn*-2 by all methods might be considered acceptable, probably because these do not exhibit a markedly asymmetric distribution of fatty acids among the positions or because a single fatty acid comprises more than 60% of the total in each; it illustrates the importance of testing new methodology with appropriate model compounds. With all of the seed oils, good agreement was obtained with the two methods for calculating positions *sn*-1 and *sn*-3.

Only olive oil appears to have been subjected to stereospecific analysis before by the original Brockerhoff method involving the use of lipases (13). The results obtained here are rather similar. Maize (corn) (3,13), soybean (13) and evening primrose oils (14) appear to be the only high-linoleate oils to have been subjected to stereospecific analysis. In general, these have higher proportions of linoleate and low concentrations of saturated fatty acids in position *sn*-2, with small differences only in the relative compositions of positions *sn*-1 and *sn*-3. This is also true of the results reported here for safflower and sunflower oils, although there were some differences, notably that there was more linoleic acid and less oleic acid in position *sn*-3 relative to position *sn*-1 with the former. The opposite was found with sunflower oil. The composition of position *sn*-2 only of palm oil seems to have been reported previously (15), and the result recorded here was comparable. Positions *sn*-1 and *sn*-3 differed more perhaps than has been seen with some other seed oils, in that there was somewhat more palmitic acid in position *sn*-3 and more oleic acid in position *sn*-1. Only the result for the distribution of myristic acid may be untrustworthy, because of poor resolution of diastereomeric species enriched in this acid, but it was a relatively minor component.

Triacyl-*sn*-glycerols of animal origin would be expected to be a greater challenge for the new methodology for stereospecific analysis because they tend to have highly asymmetric fatty acid distributions. The asymmetry of the diacylglycerol derivatives generated from egg triacylglycerols is evident from the HPLC trace illustrated in Figure 3, when the heights of the three peaks in the 1,2- and 2,3-*sn*-diacylglycerol groups are compared. The main component of the former was the palmitoyloleoyl species while the dioleoyl species predominated in the latter. The results for the distribution of the fatty acids on the three positions of the glycerol moiety, obtained by the two methods, were in good agreement with each other and with a previous analysis (16), in that palmitic acid was concentrated in position *sn*-1 while most of the oleic acid was in positions *sn*-2 and -3. Again, the result for position *sn*-2 obtained from the analysis of the 1,3-diacylglycerols was appreciably in error. A new finding in this study was

TABLE 2

Fatty Acid Compositions (mol% of the total) of Natural Triacylglycerols and of Positions *sn*-1, *sn*-2 and *sn*-3

Fatty acid	TG	Position			Position			
		sn-1[a]	*sn*-2[b]	*sn*-3[c]	*sn*-1[d]	*sn*-2[e]	*sn*-3[f]	*sn*-2[g]
				Safflower seed oil				
16:0	7.9	14.2	2.9	6.8	16.5	0.6	9.1	0.9
18:0	2.1	2.9	0.2	3.1	2.7	0.4	3.0	−0.7
18:1	13.7	14.4	15.3	11.4	14.0	15.7	11.0	11.2
18:2	76.3	68.6	81.5	78.7	66.8	83.3	76.9	88.6
				Sunflower seed oil				
16:0	7.2	10.6	1.3	9.7	11.2	0.7	10.4	2.0
18:0	4.6	3.3	1.1	9.2	4.0	0.4	9.9	1.6
18:1	21.9	16.6	21.5	27.6	17.7	20.4	28.7	20.6
18:2	66.3	69.5	76.0	53.5	67.0	78.5	51.0	75.7
				Olive oil				
16:0	12.5	15.2	2.5	19.6	16.5	1.3	20.9	4.5
16:1	1.0	1.3	0.2	1.4	0.7	0.7	0.8	1.4
18:0	2.9	2.9	0.6	5.2	3.1	0.3	5.4	0.6
18:1(n-9)	70.7	68.6	81.0	62.6	68.2	81.4	62.2	78.1
18:1(n-7)	1.3	0.9	1.1	1.8	0.8	1.2	1.6	0.5
18:2	11.7	11.0	14.6	9.4	10.6	15.1	9.0	14.9
				Palm oil				
14:0	1.3	0.4	0.6	2.9	0.7	0.4	3.1	2.5
16:0	48.4	60.1	13.3	71.9	60.1	13.3	71.9	31.4
16:1	0.2	0.0	0.5	0.1	0.4	0.1	0.5	0.3
18:0	3.7	3.4	0.2	7.6	3.1	0.5	7.3	0.6
18:1	36.3	26.8	67.9	14.4	27.1	67.6	14.7	48.7
18:2	10.0	9.3	17.5	3.2	8.6	18.2	2.5	16.5
				Egg				
14:0	0.5	0.8	0.0	0.7	0.6	0.2	0.4	0.4
16:0	27.3	68.2	4.8	8.9	69.6	3.5	10.2	10.6
16:1(n-7)	1.4	3.9	0.4	0.0	4.1	0.1	0.3	1.5
16:1(n-5)	4.0	5.4	0.7	6.0	2.9	3.1	3.6	2.7
18:0	4.6	6.0	0.3	7.7	5.2	1.1	6.9	5.1
18:1(n-9)	47.5	12.4	60.8	69.4	16.1	57.2	73.0	52.8
18:1(n-7)	1.6	0.9	1.8	2.0	1.5	1.2	2.5	0.8
18:2	13.0	2.3	31.3	5.4	0.0	33.6	3.1	26.0
				Tallow				
14:0	3.4	2.9	1.5	5.7	0.2	4.3	2.9	5.5
15:0	0.6	0.6	0.4	0.8	0.4	0.7	0.5	0.4
16:0	29.5	42.0	24.6	21.9	45.7	21.0	25.5	15.5
16:1	1.9	2.1	0.8	2.9	0.4	2.4	1.3	0.3
16-br	1.0	1.6	1.2	0.1	1.6	1.3	0.0	0.7
17:0	1.2	1.2	1.0	1.4	1.5	0.7	1.7	1.4
18:0	26.0	34.4	11.3	32.4	33.1	12.5	31.2	28.3
18:1	33.1	13.6	55.0	30.8	16.6	52.0	33.7	45.2
18:1t	1.8	1.3	0.3	3.8	0.2	1.4	2.7	0.8
18:2	1.5	0.2	3.9	0.3	0.4	3.7	0.4	1.9
				Rat adipose tissue				
14:0	2.5	3.5	−0.8	4.8	1.6	1.1	2.9	2.7
16:0	32.4	52.0	15.9	29.2	54.3	13.5	31.6	17.4
16:1	7.0	8.4	2.0	10.5	3.5	6.8	5.7	8.3
18:0	3.4	5.6	1.5	3.0	6.3	0.7	3.8	0.5
18:1(n-9)	23.3	13.6	35.4	21.0	22.7	26.3	30.1	25.4
18:1(n-7)	3.3	5.2	0.3	4.3	4.7	0.9	3.8	1.3
18:2(n-6)	26.7	11.2	44.9	24.1	6.8	49.3	19.7	42.1
18:3(n-3)	1.5	0.7	0.8	3.0	0.1	1.4	2.5	2.2

*a = 3 × TG − 2 × 2,3-DG; b = 3 × TG − (a + c); c = 3 × TG − 2 × 1,2-DG; d = 2 × 1,2-DG − e; e = via pancreatic lipase hydrolysis; f = 2 × 2,3-DG − e; g = 3 × TG − 2 × 1,3-DG.

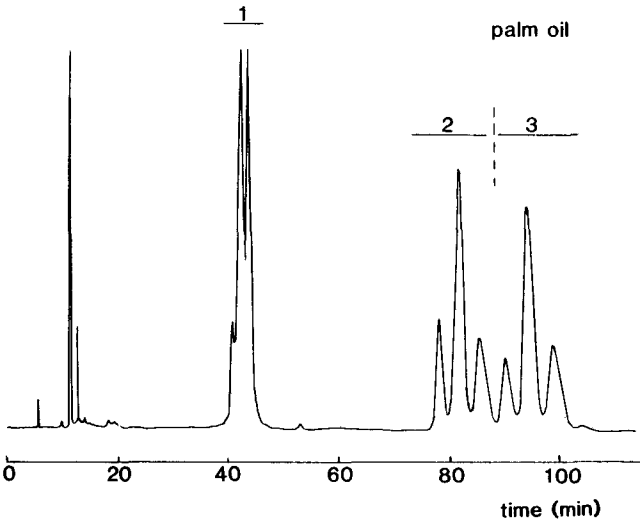


FIG. 2. HPLC resolution of (*S*)-(+)-1-(1-naphthyl)ethyl urethane derivatives of diacyl-*sn*-glycerols prepared from palm oil. The mobile phase was isooctane with 0.33% 1-propanol (containing 2% water). Other conditions were as in the Experimental section. The numbers 1, 2 and 3 above peaks refer to the 1,3-, 1,2- and 2,3-*sn*-diacylglycerol derivatives, respectively.

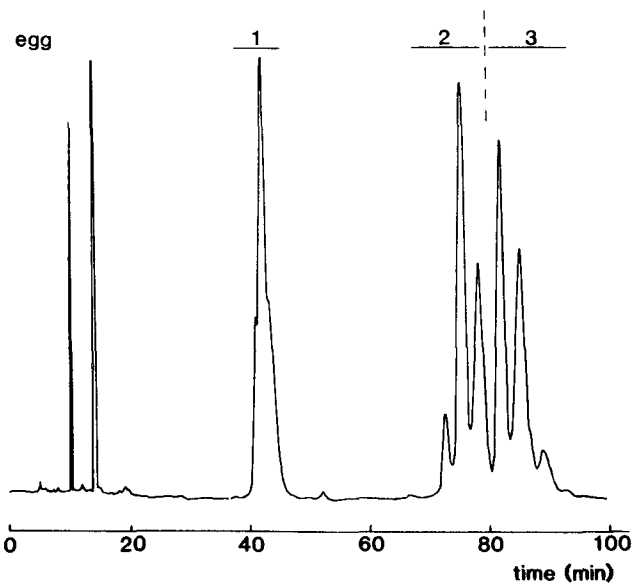


FIG. 3. HPLC resolution of (*S*)-(+)-1-(1-naphthyl)ethyl urethane derivatives of diacyl-*sn*-glycerols prepared from egg triacylglycerols. The experimental conditions were as in Figure 2. The numbers 1, 2 and 3 above peaks refer to the 1,3-, 1,2- and 2,3-*sn*-diacylglycerol derivatives, respectively.

the remarkable difference in the relative distributions of the two isomers of the 16:1 fatty acid.

The two calculation methods gave comparable results for tallow and these also agreed with an earlier analysis of "beef" fat (17) and with comparable data for sheep

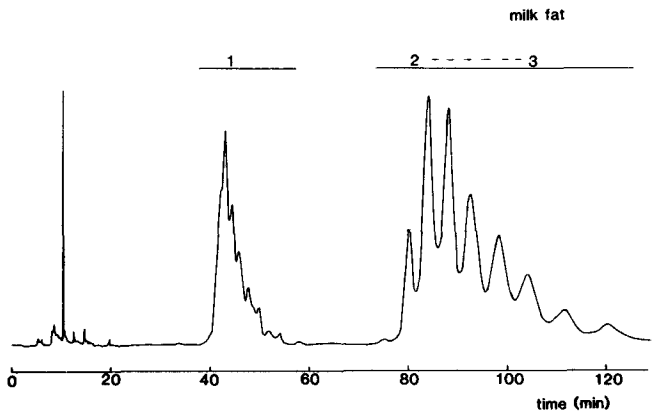


FIG. 4. HPLC resolution of (*S*)-(+)-1-(1-naphthyl)ethyl urethane derivatives of diacyl-*sn*-glycerols prepared from milk fat. The experimental conditions were as in Figure 2. The numbers 1, 2 and 3 above peaks refer to the 1,3-, 1,2- and 2,3-*sn*-diacylglycerol derivatives, respectively.

adipose tissue (18). Similarly, good agreement was obtained in the two sets of results here and with previous analyses of rat adipose tissue (17,19). With the latter, the remarkable difference in the relative distributions of the two 18:1 fatty acid isomers appears to be a novel finding. Only the data for myristic acid appears dubious, probably because of carry-over of molecular species enriched in this component from the 1,2-*sn*-diacylglycerol group of peaks into the 2,3-group. As before, the results for position *sn*-2 obtained by analysis of the contaminated 1,3-di-acylglycerols were appreciably in error.

The stereospecific analysis procedure described here did not appear to be suitable for natural triacylglycerol samples containing a wide range of different fatty acids, and this was confirmed by applying it to milk fat, coconut oil, palm kernel oil and a fish oil. The nature of the separation of the diastereomeric diacylglycerol urethane derivatives prepared from milk fat is illustrated in Figure 4. The 1,2- and 2,3-*sn*-diacylglycerol derivatives were separated into a number of distinct peaks but there is certainly overlap of different diastereomers. Similarly, up to 10 distinct peaks were obtained with coconut and palm kernel oils for molecular species, but with some co-elution of diastereomeric groups. No sensible pattern was discernable with the fish oil. As noted earlier (3), HPLC linked to mass spectrometry would appear to be the only method likely to succeed with such samples, especially if both the (*S*)- and (*R*)-1-(1-naphthyl)ethyl urethane derivatives were examined.

Most of the natural fats and oils of commercial and scientific interest contain a relatively restricted number of different fatty acids. For these, the procedure for stereospecific analysis described here, involving partial hydrolysis with ethyl magnesium bromide, preparation of (*S*)-1-(1-naphthyl)ethyl urethane derivatives, purification on solid-phase extraction columns, and resolution of the diastereomeric diacylglycerols by HPLC, is entirely satisfactory. Only the 1,2- and 2,3-*sn*-diacylglycerols need be isolated for fatty acid analysis as the composition of

positions *sn*-1, -2 and -3 can be calculated accurately from these data and those of the total triacylglycerols. It does not therefore matter that the 1,3-diacylglycerols do not give reliable results for position *sn*-2. Nor is a separate determination of this position by means of pancreatic lipase hydrolysis necessary. The procedure does not require the use of phospholipases or costly chiral-phase columns and derivatizing agents. Rather, it utilizes chemical reagents and HPLC equipment that are readily available. An analysis can be accomplished comfortably within two working days, and indeed, several samples can be handled simultaneously.

ACKNOWLEDGMENTS

The Karlshamns Foundation of Sweden provided financial assistance for B. Nikolova-Damyanova, and the Foundation for Nutrition Research of Finland supported P. Laakso.

REFERENCES

1. Christie, W.W., *Analysis of Oils and Fats*, edited by R.J. Hamilton, and J.B. Rossell, Elsevier Applied Science Publishers, London, England, 1986, p. 313.
2. Breckenridge, W.C., *Handbook of Lipid Research*, Vol. 1, edited by A. Kuksis, Plenum Press, New York, New York, 1978, p. 197.
3. Laakso, P., and W.W. Christie, *Lipids 25*:349 (1990).
4. Kuksis, A., L. Marai, J.J. Myher and Y. Itabashi, *Proceedings, 15th Scandinavian Symposium on Lipids*, edited by V.K.S. Shukla, and G. Holmer, Rebild Bakker, Denmark, 1989, p. 336.
5. Takagi, T., and Y. Ando, *Lipids 25*:398 (1990).
6. Christie, W.W., and J.H. Moore, *Biochim. Biophys. Acta 176*:445 (1969).
7. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman and R.W. Riemenschneider, *J. Am. Oil Chem. Soc. 41*:693 (1964).
8. Christie, W.W., *J. Lipid Res. 23*:1072 (1982).
9. Christie, W.W., *Gas Chromatography and Lipids*, The Oily Press, Ayr, Scotland, 1989.
10. Yurkowski, M., and H. Brockerhoff, *Biochim. Biophys. Acta 125*:55 (1966).
11. Brockerhoff, H., *J. Lipid Res. 8*:167 (1967).
12. Brockerhoff, H., *Lipids 6*:942 (1971).
13. Brockerhoff, H., and M. Yurkowski, *J. Lipid Res. 7*:62 (1966).
14. Lawson, L.D., and B.G. Hughes, *Lipids 23*:313 (1988).
15. Mattson, F.H., and R.A. Volpenhein, *J. Lipid Res. 4*:392 (1963).
16. Christie, W.W., and J.H. Moore, *Biochim. Biophys. Acta 218*:83 (1970).
17. Brockerhoff, H., R.J. Hoyle and N. Wolmark, *Ibid. 116*:67 (1966).
18. Christie, W.W., and J.H. Moore, *J. Sci. Food Agric. 22*:120 (1971).
19. Henderson, R.J., W.W. Christie and J.H. Moore, *Biochim. Biophys. Acta 574*:8 (1979).

[Received April 19, 1991; accepted July 30, 1991]