Stereospecific analysis of triglycerides: an alternative method

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ABSTRACT A new method for the stereospecific analysis of triglycerides is demonstrated on a corn oil which had been analyzed by an earlier method. The triglyceride (1,2,3-triacyl L-glycerol) was partially degraded by means of methyl magnesium bromide. The 1,3-diglyceride was then isolated, and converted into L-2-phosphatidyl phenol, from which phospholipase A cleaved the fatty acids from position 1. The remaining lysophosphatide was analyzed to yield the fatty acids at position 3 of the original triglyceride. The fatty acid composition of position 2 was determined after hydrolysis of the corn oil with pancreatic lipase. This method has the advantage over the one reported previously of yielding direct analyses of the fatty acids in each of the three positions.

KEY WORDS stereospecific analysis · triglycerides · 1,3-diglyceride · L-2-phosphatidyl phenol · fatty acid distribution · corn oil · lipolysis · phospholipase A

THE STEREOSPECIFIC ANALYSIS of a triglyceride, i.e., the determination of the fatty acid composition in positions 1, 2, and 3 of 1,2,3-triacyl L-glycerol, can be accomplished (1) (broken arrows in Fig. 1) by degrading the triglyceride (I) by means of pancreatic lipase (EC 3.1.-1.3) to a racemic α,β-diglyceride (III) and converting this to a phospholipid (VI) which is then resolved with the aid of the stereospecific enzyme phospholipase A (EC 3.1.1.4).

This procedure has two disadvantages. First, it cannot be applied to marine oils that contain the acids 20:5 or 22:6 because pancreatic lipase will not produce representative diglycerides from these fats (2). Second, the fatty acids of position 3 cannot be isolated and directly analyzed; their composition has to be calculated by difference, and this makes the determination of minor components in position 3 rather inaccurate and that of trace components impossible (2). The first problem has been solved by the preparation of representative α,β-diglycerides by means of a chemical procedure which dispenses with the lipase (3). The second problem is solved by the following method (solid arrows in Fig. 1). A triglyceride (I) is degraded to its 1,3-diglyceride (IV), and this is converted to L-2-phosphatidyl phenol (VII; Ph–P is the phenyl phosphate group), and then use is made of the discovery of de Haas and van Deenen (4) that phospholipase A will liberate the fatty acid (XI) from position 1 of an L-2-phosphatide. Fatty acid 3 can then be isolated from the lysophosphatide (XII).

MATERIALS AND METHODS

The corn oil that had been analyzed previously by our first method (broken arrows in Fig. 1) (5) was subjected to the new method of analysis.

Adjunctive procedures, gas–liquid chromatography and calculations, have been described in previous papers (1, 2).

PROCEDURE AND RESULTS

2 ml of 3 M CH_3MgBr in ether was added to a vigorously stirred solution of 1 g of fat in 50 ml of anhydrous ether. 30 seconds after the start of the experiment 1 ml of acetic acid and, 30 seconds later, 10 ml of water were added. Stirring was continued for 2 min. The ether layer was then recovered, washed successively with 10 ml of water, 10 ml of 2% aqueous $NaHCO_3$, and 10 ml of water, and then dried with Na_2SO_4.

The lipids in portions of 100 mg were separated on thin-layer plates, 20 × 20 cm, prepared from 25 g of Silica Gel G and 40 ml of 3% aqueous boric acid solution, activated for 30 min at 110°C. The first developing solvent was ether–petroleum ether (bp 30–60°C) 8:92; the

Fatty acids are designated by number of carbon atoms: number of double bonds.

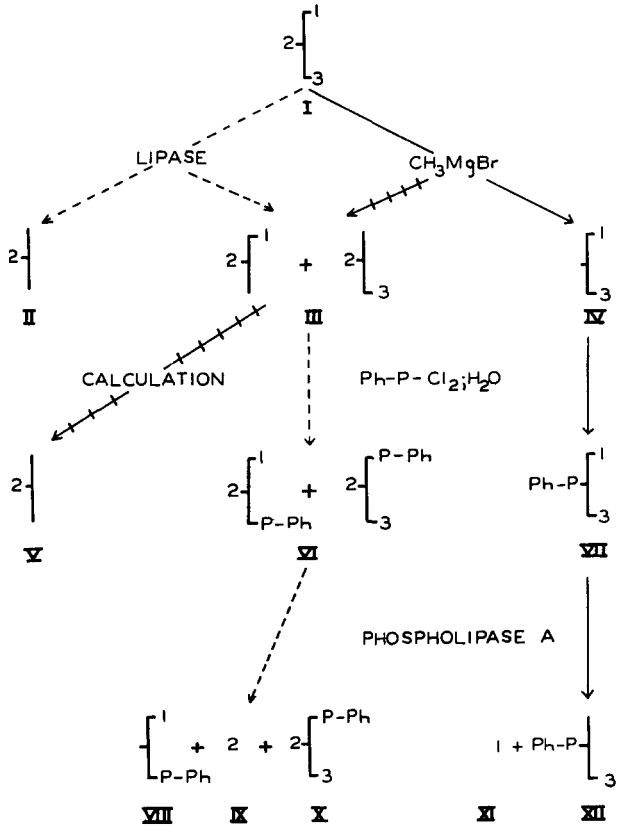

FIG. 1. Stereospecific analysis of a triglyceride according to method 1 (— — →) and method 2 (——→), and possible calculation of the fatty acid composition at position 2 (—+—+→).

second, ether–petroleum ether 40:60. The 1,3-diglyceride, which runs between the α,β-diglyceride and the tertiary fatty alcohol, was located by means of dichlorofluorescein, eluted with ether–petroleum ether 1:1, and purified by rechromatography with ether–petroleum ether 1:1. From 1 g of corn oil, 60 mg of 1,3-diglyceride was obtained.

The isolated 1,3-diglyceride (55 mg dissolved in 1 ml of ether) was added to a solution of 0.25 ml of phenyl dichlorophosphate (Aldrich Chemical Co., Milwaukee, Wis.) in 1 ml of pyridine and 1 ml of ether. After 90 min at room temperature, 5 ml of pyridine and then several drops of water were added, the reaction mixture being cooled with water. Then 25 ml of water, 30 ml of methanol, 30 ml of chloroform, and 1 ml of triethylamine were added, the mixture was shaken, and the chloroform layer was recovered and evaporated below 40°C under reduced pressure and finally under high vacuum. The L-2-phosphatidyl phenol had been formed in good yield; on thin-layer chromatography only traces of diglyceride were found as a contaminant.

Ether (2 ml) was added to the phospholipid, followed by 15 ml of 0.1 M aqueous triethylammonium bicarbonate of pH 7.5 (triethylamine in water saturated with CO_2), 1 drop of 0.1 M $CaCl_2$, and 1 mg of snake venom (*Crotalus atrox*). After the reaction mixture had been shaken overnight under nitrogen, 20 ml of isobutanol was added (to prevent foaming) and the mixture was evaporated below 40°C under reduced pressure and finally under high vacuum. The lipids (fatty acid plus lysophosphatide) were dissolved in 1 ml of chloroform–methanol 1:1 plus 1 drop of acetic acid, and applied (20 mg per plate) to thin-layer plates, 10 × 20 cm, containing 10 g of silicic acid (Mallinckrodt) and 0.4 g of $CaSO_4$. The plate was developed with the first solvent, ether–petroleum ether 35:65, dried in the air for 5 min, kept over concentrated aqueous ammonia for 10 min, and then developed in concentrated aqueous ammonia–methanol–ether 2:14:84. The lipids were located with dichlorofluorescein and eluted with chloroform–methanol 1:1. The approximate R_f for the free acid (from position 1) was 0.7, and for the lysophosphatide, 0.05. The band at the location of the original L-2-phosphatide, R_f 0.4, was very faint.

Table 1 gives the results of an analysis of corn oil. The 2-monoglyceride, II, in the second row, was determined by enzymatic lipolysis (6); in the fourth row (V) it was calculated by difference between α,β-diglyceride and triglyceride. In the last two rows, the analyses (appropriately divided) are given for comparison with the 1,3-diglyceride (1 + 3) and the original triglyceride (1 + 2 + 3). In the latter addition the analysis of II (fatty acid 2 determined by lipolysis) is used rather than the calculated analysis of V (see Discussion).

In Table 2 the results of method 2 can be compared with a previous analysis according to method 1 (4).

DISCUSSION

In a previously reported method of stereospecific analysis (1) the fatty acid composition in position 3 could be calculated but not measured, and large relative errors had to be accepted, especially for minor and trace components (2). In a previous analysis of the corn oil, for instance, the accuracy was taken as ±1% (absolute) (3); therefore, position 3 could have contained several times as much palmitoleic acid as stated (Table 2) or none at all; linolenic acid could also have been absent. The present method establishes the presence of both acids at position 3, and their percentage can be considered accurate within the limitations of gas–liquid chromatography.

The composition of fatty acids at position 2 can be calculated (3) from data for α,β-diglyceride and triglyceride (crossed arrows in Fig. 1) or from triglyceride minus 1,3-diglyceride, so that lipase can be omitted from the procedure. However, a direct analysis is preferable, since, for instance, calculation from 4 × α,β-diglyceride minus 3 × triglyceride could result in a sevenfold multi-

TABLE 1 STEREOSPECIFIC ANALYSIS OF CORN OIL ACCORDING TO METHOD 2

Compound		Position	Fatty Acid 16:0	16:1	18:0	18:1	18:2	18:3
			mole %					
Triglyceride	I	1,2,3	11.3	0.5	1.6	27.5	58.1	0.9
2-Monoglyceride	II	2	1.8	0.1	0.2	25.8	71.2	0.9
α,β-Diglyceride	III	1,2;2,3	9.4	0.5	1.0	28.1	59.9	1.0
2-Monoglyceride calcd*	V	2	3.7	0.5	(−0.8)	29.9	65.3	1.3
1,3-Diglyceride calcd†	IV	1,3	16.1	0.7	2.3	28.4	51.6	0.9
1,3-Diglyceride found	IV	1,3	14.9	0.6	2.7	28.4	52.0	1.2
Fatty acid 1	XI	1	18.5	0.4	3.5	28.1	48.5	1.0
Lysophosphatide	XII	3	12.6	0.5	2.2	31.0	52.6	1.1
(XI + XII) ÷ 2		1 + 3	15.6	0.5	2.9	29.6	50.6	1.1
(XI + II + XII) ÷ 3		1 + 2 + 3	11.0	0.5	2.0	28.3	57.4	1.0

Roman numerals refer to Fig. 1.
* Calculation: (4 × III) − (3 × I).
† (3 × I − II) ÷ 2.

TABLE 2 RESULTS OF STEREOSPECIFIC ANALYSES OF CORN OIL ACCORDING TO METHOD 1 AND METHOD 2

	Position	Fatty Acid 16:0	16:1	18:0	18:1	18:2	18:3
		moles %					
Method 1*	1	17.9	0.3	3.2	27.5	49.8	1.2
	2	2.3	0.1	0.2	26.5	70.3	0.7
	3	13.5	0.1	2.8	30.6	51.6	1.0
Method 2	1	18.5	0.4	3.5	28.1	48.5	1.0
	2	1.8	0.1	0.2	25.8	71.2	0.9
	3	12.6	0.5	2.2	31.0	52.6	1.1

* Analysis taken from (5).

plication of analytical errors. Fatty acid 2 was therefore determined with the aid of pancreatic lipase.

The success of the analysis is easily ascertained by adding 1 and 3 and comparing the result with the 1,3-diglyceride (Table 1), and by comparing the sum of 1, 3, and 2 (from pancreatic lipolysis) with the triglyceride.

In a paper describing the reduction of lard with methyl magnesium bromide (3), we concluded that the isolated 1,3-diglyceride might contain 6% isomerized α,β-diglyceride. The 1,3-diglyceride from corn oil is probably equally contaminated. This isomerization would influence the results in such fats which show dramatic differences in the compositions at positions 1, 2, and 3. Position 2 in lard, for instance, may contain 70% palmitic acid, of which $^1/_2 \times 6 \times 70$ or 2% would migrate to position 3, which originally contains little or no palmitic acid (7). Lard is, however, an exceptionally difficult fat to analyze: the indirect calculation of the composition at position 3 according to the first method also suffers from the high concentration of palmitic acid in position 2 and in compound IX, and the consequently high errors in the gas–liquid chromatographic analyses. Furthermore, the α,β-diglyceride prepared with the aid of lipase (2) contains about 4% 1,3-diglyceride (unpublished observation) which will follow the path of the second method (Fig. 1) and also distort the results if not removed. However, none of these factors will have much influence on the analysis of fats with less eccentric fatty acid distributions.

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