Synthesis of 100-gram Quantities of Highly Purified Mixed Acid Triglycerides[1]

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Abstract

Monopalmitin, mono-olein, and monostearin have been synthesized in quantities of up to 100 grams with recoveries ranging from 80%–85%. The monoglycerides were reacted with a 0.5 molar excess of pyridine and fatty acid chloride to produce 1, 3,-diglycerides. The recoveries ranged from 60%–70% and, in addition, considerable amounts of triglyceride were obtained from this reaction. Partial glycerides were converted to 100-gram batches of triglyceride in a similar manner with recoveries ranging from 80%–90%.

Purification of the partial glycerides was achieved by crystallization. In addition to this technique, certain triglycerides were purified by elution through a column of alumina. Purity of the glycerides, as determined by TLC, GLC, and pancreatic lipolysis, was estimated to be at least 99%.

Introduction

THE AVAILABILITY OF PURE SYNTHETIC GLYCERIDES other than simple triglycerides has been limited on account of the difficulty of preparation, lack of adequate characterization techniques, and the relatively small number of workers involved in this field. However, in recent years, interest in this area of lipid chemistry has grown steadily, largely because of new instruments and such techniques as gas-liquid chromatography (GLC) and thin-layer chromatography (TLC). Paralleling this growth has been the increasing need for large quantities of pure glycerides as model compounds in structural studies of natural fats and as substrates for investigations of lipase specificity.

The preparation of pure glycerides on a large scale is a difficult task. Usually where purity is high, quantities have been limited to small amounts. Now with the aid of improved methods of synthesis, crystallization techniques, and chromatography, final yields of at least 100 grams of pure triglyceride can be realized. This paper describes the preparation of such glycerides by using modifications of existing methods. The procedures herein are drawn from reports in the current literature and our own experience in this area. Several excellent reviews (1–3) have been published, to which the reader may refer for a more general discussion on this topic. It is hoped that others will be able to duplicate this work and, as a result, the availability of pure glycerides will no longer be a limiting factor in lipid research.

Experimental Section

Materials and Methods

Ethanol (Rossville Gold Shield USP alcohol) was obtained from Commercial Solvents Corporation, Terre Haute, Ind. The other solvents including ethyl ether were the quality of Baker analyzed reagent or Fisher certified reagent. Acetone, benzene, chloroform, and petroleum ether (30C–60C) were distilled prior to use. The chloroform was washed with water to remove alcohol, dried with anhydrous calcium chloride, distilled, then used within a period of 72 hr. Petroleum ether was collected in two fractions, 35C–45C and 45C–60C. The low boiling fraction was used as a solvent in TLC work, and the higher boiling fraction was employed in the purification procedures.

Boric acid, 2-methoxyethanol, p-toluenesulfonic acid, pyridine, and thionyl chloride were Fisher certified reagents. Glycerol used in this study was Fisher USP quality. Anhydrous ethyl ether was Mallinckrodt analytical reagent grade, and anhydrous sodium acetate was Merck reagent quality. The pyridine was refluxed and distilled over barium oxide and stored over anhydrous calcium sulfate. Oxalyl chloride (Eastman red label) and thionyl chloride were used as received. Alumina (grade F-20, mesh 80–200) was purchased from Alcoa Chemicals and activated by heating for 12 hr at 260C (4).

The palmitic acid was obtained by saponification of the methyl palmitate furnished by the Eastern Utilization Research and Development Division of the USDA, Philadelphia, Pa. Stearic acid of 95% purity (Emersol 6353) was a gift from Emery Industries Inc., Los Angeles, Calif. It was crystallized three times from a 15-fold excess of acetone at 22C to a purity of 99.5%. Oleic acid was obtained from the Hormel Institute. The purity of all the acids used in this study was 99% or greater by GLC and TLC.

The GLC instrument employed was a Barber-Colman Model 5000, equipped with a flame ionization detector. Glycerides and acids were converted to esters by acid-catalyzed methanolysis and analyzed on 10-ft × ¼-in. stainless steel columns, packed with 18% DEGS on Anakrom ABS (70–80 mesh). A Disc integrator attached to the recorder was used for quantitation. Sensitivity of both GLC and TLC detection was ascertained by using standard mixtures prepared in the laboratory from Hormel (99+%) and USDA (99.8%) fatty acids. One per cent or less of contaminating material could be detected under the conditions employed.

Extent of reaction and purity of glycerides were estimated on TLC plates coated to a thickness of 0.25 mm with neutral silica gel G or silica gel G-boric acid (95/5, w/w) (5). All glycerides were checked for polar contaminants on the neutral TLC plates by using a petroleum ether-ethyl ether-acetic acid (74/25/1, v/v/v) solvent system and for nonpolar contaminants in a petroleum ether-ethyl ether (94/6, v/v) system. In addition, monoglyceride and diglyceride purity was estimated on the silica gel G-boric acid plates in chloroform-acetone-acetic acid-methanol (72.5/25/0.5/2, v/v/v/v) and chloroform-acetone (96/4, v/v) solvent systems respectively. All TLC plates used to monitor glyceride purity were visualized by spraying with bromothymol blue indicator, followed by exposure to ammonia vapors (6).

Purity of the monoglyceride was also determined by oxidation with periodic acid (7). Gas-liquid chromatography was employed to estimate the ratio of

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fatty acids (as methyl esters) in the glycerides. Lipolysis by pancreatic lipase was used to check the position of the fatty acids on the triglyceride (8). After synthesis all glycerides and fatty acid chlorides were stored at −7C and −25C respectively.

In order to illustrate the synthesis procedures, the preparation of racemic glyceryl 1-palmitate 2-oleate 3-stearate (POS) is described.

Stearoyl and Oleoyl Chloride. The saturated and unsaturated fatty acid chlorides were prepared by using thionyl chloride and oxalyl chloride respectively. The method followed was essentially that of Mattson and Volpenhein (1). Stearic acid (100 g) and thionyl chloride (1.4 molar excess) were mixed together in a 500-ml round bottom flask. A condenser (air-cooled), equipped with a drying tube containing anhydrous calcium sulfate, was then fitted to the flask. The mixture was allowed to react at room temperature for five days, after which it was heated on a steam bath for one hour under vacuum of a water aspirator. Petroleum ether (2 liters) was added, and the stearoyl chloride was washed three times with 200-ml portions of ice water, immediately dried with anhydrous sodium sulfate, and filtered. The solvent was removed by evaporation under vacuum at 35C or less. Oleoyl chloride was prepared in a similar fashion by reacting 100 g of oleic acid with a 0.8 molar excess of oxalyl chloride for three days at room temperature.

1-Monopalmitin. The monoglyceride was prepared by Anfinsen and Perkins' modification (9) of Hartman's procedure (10) except that water was removed with a Dean-Stark trap. Glycerol (0.72 moles) and acetone (1.0 molar excess) were added to a 2-liter, 2-necked, round-bottom flask containing 500 ml of benzene, 2.0 g of p-toluenesulfonic acid, and a few boiling chips. A Dean-Stark water trap (25 ml) with a stopcock on the bottom was placed in one neck and a glass stopper in the other neck. Attached to the trap was a Friedrichs condenser (water-cooled), equipped with a drying tube containing anhydrous calcium sulfate. The mixture was refluxed for 10 hr, after which time the synthesis of isopropylidene glycerol was complete, as indicated by the cessation of water droplets falling through the benzene in the upper part of the trap.

Palmitic acid (0.24 moles) was added, and the mixture was refluxed for 15 hr. Completion of the reaction was indicated as described above. The acid catalyst and excess isopropylidene glycerol were removed by shaking the mixture with anhydrous sodium acetate (2.0 g), washing four times with 150-ml portions of distilled water, and drying with anhydrous sodium sulfate. The benzene was evaporated under vacuum.

The cleavage of the isopropylidene glycerol palmitate was achieved by dissolving the ester in 420 ml of 2-methoxyethanol, adding 140 g of boric acid, and heating on a steam bath for 1.5 hr. This procedure was carried out in a 2-liter, round-bottom flask, equipped with a condenser (air-cooled), containing a drying tube with anhydrous calcium sulfate. The mixture was then taken up in ethyl ether (1.5 liters), washed four times with one-liter portions of distilled water, and dried with anhydrous sodium sulfate. The volume of solvent was reduced to 400 ml by evaporation under reduced pressure at 30C or less. Petroleum ether (600 ml) was added, and the monopalmitin was crystallized from this mixture at 22C, filtered, and washed with 500 ml of petroleum ether at room temperature.

Glyceryl 1-Palmitate 3-Stearate. The diglyceride was synthesized by using a modified direct esterification procedure (11). Monopalmitin (0.203 moles) was added to a 2-liter, round-bottom flask containing 900 ml of chloroform and 0.305 moles of pyridine. Stearoyl chloride (0.305 moles) was then slowly added with shaking and cooling of the flask in ice water. After the reaction had proceeded for 24 hr at room temperature, the solvent was removed by evaporation under vacuum at 35C or less. The diglyceride was crystallized four times from one liter of petroleum ether −95% ethanol (8/2, v/v) at 22C. Prior to each crystallization the funnel and wash solution (500 ml petroleum ether) were stored at 12C until ready for use at room temperature.

Glyceryl 1-Palmitate 2-Oleate 3-Stearate. The partial glyceride was converted to the triglyceride by reaction with a 50% molar excess of pyridine and acid chloride (1). The diglyceride (0.138 moles), 700 ml of chloroform, and 0.207 moles of pyridine were mixed together in a 2-liter, round-bottom flask, and oleoyl chloride (0.207 moles) was added as described above. The reaction proceeded for 72 hr at room temperature, after which time the solvent was evaporated under reduced pressure at 35C or less. The triglyceride was crystallized two times at 12C from 1.2 liters of 95% ethanol-acetone (9/1, v/v). Before each crystallization the funnel and wash solution (500 ml of the 9/1 mixture) were stored at 12C. The estimated purity of the triglyceride at this point was 95% by TLC; the contaminants were diglyceride and fatty acid.

The triglyceride was further purified by passage through a column of alumina (4). The impure glyceride (106.8 g) was added to a 3-cm diameter column (81.6 g alumina) in 100 ml of petroleum ether-anhydrous ethyl ether (9/1, v/v), at 22C. The triglyceride (100.4 g) was recovered from the column by elution with 400 ml of the 9/1 mixture at room temperature.

Results and Discussion

1-Monoglycerides. In the synthesis of isopropylidene glycerol and its fatty acid ester the progress of the reaction is followed by the formation of water as indicated by the amount collected in the trap. The total reaction time is usually 25 hr. However amounts of water relatively considerable are often present in the reagents. To insure completion of both reactions the cessation of water formation is used as the indicator. For example, in the previously described synthesis of 1-monopalmitin, the theoretical amounts of water for isopropylidene glycerol and palmitate ester were 13.0 ml and 4.3 ml respectively, but the actual amounts collected were 15.0 and 6.0 ml. For this reason, the extent of reaction is more accurately measured by the TLC method of Anfinsen and Perkins (9).

Using this procedure, we have not been able to prepare large amounts of isopropylidene glycerol or its ester in the time periods indicated in the literature (9,10). This is partially attributed to the sizable quantities of materials used in the synthesis. Nevertheless this method has been successfully employed to synthesize monopalmitin, monostearin, and monoolein in quantities of up to 100 g. The recoveries range from 80%–85% and are similar to those previously reported (9,10). Recovery of the saturated monoglyceride is usually higher than that of the unsaturated glyceride (Table I).

The monoglycerides are purified by removal of the 2-isomer (estimated as 5%–10% by TLC) through crystallization at the appropriate temperature (Table II). Based on the 88–12 equilibrium mixture of 1- and 2-monoglyceride, the recovery of product is excellent. The purity of the 1-monoglycerides is estimated to be at least 99%.

1,3-Diglycerides. The ratio of fatty acid chloride to monoglyceride may be varied, depending on the nature of the component fatty acids and the quantity of product desired. The amount of unreacted monoglyceride and that of di- and triglyceride formed is a function of this ratio. Usually it is easier to purify diglyceride in the presence of triglyceride than in the presence of monoglyceride. In addition, the triglyceride may be recovered and purified to a greater extent than the monoglyceride.

After proceeding for 4 hr, the reaction is checked by TLC to estimate the amount of unreacted monoglyceride. If needed, small amounts of additional pyridine and chloride are added until essentially all the monoglyceride is reacted. If the reaction is complete in 4 hr, the mixture is often allowed to stand an additional 20 hr without any noticeable difference in yield or purity of product. We have found that a 24-hr reaction with monoglyceride and a 0.5 molar excess of pyridine and acid chloride gives maximum yield of 1,3-diglyceride. Based on the monoglyceride, the recoveries range from 60%–70% and are in agreement with those of Hartman (11).

This procedure also allows recovery and purification of triglyceride. For example in the synthesis of glyceryl 1-palmitate 3-stearate previously described, 19.3 g of PSS were recovered. This result was achieved by evaporating the diglyceride filtrate and crystallizing four times from 400 ml of 95% ethanol-acetone (5/5, v/v) at 30C.

Purification of the diglycerides is obtained by direct crystallization at the specified temperatures (Table II). This technique eliminates any time-consuming washing or column procedures (11,12). In addition, it allows the separation of large quantities of 1,3-diglyceride from other partial glycerides, pyridine hydrochloride, fatty acid, triglyceride, and ester. (The latter compound is formed by reaction of some of the excess acid chloride and ethanol during crystallization. Purification is facilitated by this reaction as the ester is considerably easier to remove than the acid.) For example, the 68% recovery of glyceryl 1-palmitate 3-stearate, as shown in Table I, represents a 82.4-g yield. On the basis of diglyceride and triglyceride obtained, the recovery of products is 78%. The purity of the 1,3-diglycerides is estimated to be 99% or greater.

Triglycerides. Diacid triglycerides of the type listed in Table I are prepared by a single acylation step in which the monoglycerides are reacted with excess fatty acid chloride. The preparation of triacid triglycerides usually requires the synthesis of the appropriate 1,3-diglyceride, which is then converted to the triglyceride in a similar manner. In both cases the reaction is monitored by TLC and, if necessary, small amounts of additional pyridine and chloride are added to ensure complete conversion of the partial glyceride. Maximum yields of triglyceride are obtained by using a 50% molar excess of pyridine and acid chloride for complete acylation (1). Although the reaction is usually complete in three days, the mixture is sometimes permitted to stand an additional four days without affecting the yield or quality of the product.

TABLE I

Recoveries of Partial Glycerides and 100-gram Quantities of Triglycerides

Triglyceride[a]	1-Monoglyceride[b] (%)	1,3-Diglyceride[c] (%)	Triglyceride[d] (%)	Over-all (%)
PSS	84.7(P)	.....	80.0	67.8
SPP	84.5(S)	.....	91.0	76.9
POS	84.6(P)	68.0	84.5	48.6
OPP	80.0(O)	.....	81.0	64.8

[a] P = palmitate, S = stearate, O = oleate.
[b] Based on fatty acid.
[c] Based on 1-monoglyceride.
[d] Based on 1-monoglyceride or 1,3-diglyceride.

Based on the partial glycerides, the recoveries usually range from 80%–90% and occasionally exceed 95%. Recoveries for several 100-g batches of triglycerides and their intermediates are shown in Table I. The over-all recovery of the diacid triglycerides is considerably higher than that of the triacid glyceride. This is attributed to the extra step required to prepare the 1,3-diglyceride.

The separation of triglyceride from partial glyceride, fatty acid, and ester is achieved by direct crystallization at the indicated temperature (Table II). The presence and position of oleate on the glyceride greatly influences this temperature. In the case where oleate occupies one of the primary positions on the triglyceride (SSU and SUU), it is sometimes necessary to initiate the procedure by cooling the solution to −25C prior to storage and crystallization at 0C. Also it has been found that cooling the funnel and wash solution to a temperature of 10–20 degrees below that of crystallization will aid in preventing losses of glyceride.

In addition to the crystallization procedure, final purification of certain triglycerides may be obtained through use of a column of alumina. In order to use this technique efficiently, the glyceride must be soluble at room temperature in the elution mixture of petroleum ether-anhydrous ethyl ether (usually 9/1, v/v). Because of this limitation, trisaturated triglycerides of the type listed in Table I are purified by crystallization alone.

Before addition to the column, the triglyceride is usually crystallized to about 90% purity or greater as estimated by TLC. In addition, this procedure must remove ester which is normally eluted with the triglyceride. Alcohol, chloroform, or water will adversely affect the separation and must also be removed before column treatment. The details of this column have been presented in a recent publication by Jensen et al. (4).

The oleate triglycerides listed in Table I were purified by the column procedure with excellent results. For example, in the synthesis of POS, the 100.4 g of triglyceride obtained from the column represents a 94% recovery based on the weight of the impure glyceride. The corresponding value for the OPP is 97%.

Both the alumina column method and crystallization give similar results in the recovery of triglyceride

TABLE II

Crystallization Temperature (C) Employed in the Purification of Glycerides

Glyceride[a]	S	SUS[c]	SSU	SUU	USU	US	U
MG[b]	22	....	....	....	....	....	−25
DG[b]	22	....	....	....	....	−25	−25
TG[b]	22	12	0	0	−25	....	−25

[a] MG = 1-monoglyceride, DG = 1,3-diglyceride, TG = triglyceride.
[b] Solvent systems (10–15-fold excess): MG = petroleum ether—ethyl ether (6:4, v/v); DG = petroleum ether—95% ethanol (8:2, v/v); TG = 95% ethanol—acetone (9:1, v/v).
[c] S = saturated (palmitate and stearate); U = unsaturated (oleate).

TABLE III

Fatty Acid Composition of 100-gram Quantities of Triglycerides and Some of the Lipolysis Products Derived by Pancreatic Lipase Digestion

Triglyceride[a]	Fraction	Fatty acid (M%) P	S	O
PSS	Intact TG[b]	33.3	66.7	.....
	FFA	51.0	49.0	.....
	MG	1.0	99.0	.....
SPP	Intact TG	67.0	33.0	.....
	FFA	52.0	48.0	.....
	MG	>99.0	.....	.....
POS	Intact TG	33.8	33.1	33.1
	FFA	50.0	50.0	.....
	MG	....	.....	>99.0
OPP	Intact TG	66.7	.....	33.3
	FFA	50.0	.....	50.0
	MG	99.0	.....	1.0

[a] P = palmitate, S = stearate, O = oleate.
[b] TG = triglyceride, FFA = free fatty acid, MG = 2-monoglyceride.

(Table I). The column procedure normally takes the place of the final crystallization. Where large quantities of glycerides are concerned, the main advantages of the column are speed and ease of operation.

The purity of the triglycerides was determined by TLC, GLC, and pancreatic lipolysis. The results of the positional analysis for a few 100-g batches are listed in Table III. On the basis of the methods employed, the purity is certainly close to 99%.

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REFERENCES

1. Mattson, F. H., and R. A. Volpenhein, J. Lipid Res. *3*, 281–296 (1962).
2. Hartman, L., Chem. Revs. *58*, 845–867 (1958).
3. Malkin, T., and T. H. Bevan, "Progress in the Chemistry of Fats and Other Lipids," edited by R. T. Holman, W. O. Lundberg, and T. Malkin, Vol. 4, Pergamon Press, New York, 1957, p. 64.
4. Jensen, R. G., T. A. Marks, J. Sampugna, J. G. Quinn, and D. L. Carpenter, Lipids *1*, 451–452 (1966).
5. Thomas, A. E. III, J. E. Scharoun and Helma Ralston, JAOCS *42*, 789–792 (1965).
6. Mangold, H. K., JAOCS *38*, 708–727 (1961).
7. Jensen, R. G., and M. E. Morgan, J. Dairy Sci. *42*, 232–239 (1959).
8. Jensen, R. G., J. Sampugna and R. L. Pereira, Biochim. Biophys. Acta *84*, 481–483 (1964).
9. Anfinsen, J. R., and E. G. Perkins, JAOCS *41*, 779–780 (1964).
10. Hartman, L., Chem. and Ind. (London) 711–712 (1960).
11. Hartman, L., J. Chem. Soc. 3572–3575 (1957).
12. Quinlin, P., and H. J. Weiser Jr. JAOCS *35*, 325–327 (1958).

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