glycerides and the disappearance of others. Nevertheless, a general pattern of glyceride formation is followed regardless of ecological conditions of plant growth and regardless of the composition of the fatty acid mixture which is available for triglyceride biosynthesis.

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Analysis of Triglycerides by Consecutive Chromatographic Techniques. II. Ucuhuba Kernel Fat[1,2]

T. W. CULP, R. D. HARLOW, CARTER LITCHFIELD and RAYMOND REISER,
Department of Biochemistry & Nutrition, Texas Agricultural Experiment Station, College Station, Texas

Abstract

The triglycerides from ucuhuba kernel fat (*Virola surinamensis*) were analyzed using thin-layer adsorption chromatography (TLC) followed by gas-liquid chromatography (GLC). The triglycerides were first separated into three fractions containing 0, 1, and 2 or more double bonds per molecule on silica gel TLC plates impregnated with $AgNO_3$. The total triglycerides and each individual TLC fraction were then analyzed by GLC for the molecular weights of their component triglycerides and for their fatty acid composition. Quantitation of the TLC fractions was achieved by GLC analysis of their fatty acids using an added internal standard and confirmed by solving simultaneous equations derived from GLC analysis of their triglycerides and fatty acids.

Application of these combined chromatographic techniques separated the ucuhuba kernel fat into 23 triglyceride components. Trimyristin and laurodimyristin comprised over half the total triglycerides, which was expected since the fat contained 20.0 mole % lauric and 71.3% myristic acids.

Introduction

THE INTRODUCTION of new analytical techniques for the investigation of glyceride structure has added great impetus towards a better understanding of the glyceride composition of natural fats. These methods include the widely applied silver-ion method of de Vries for separating triglycerides into fractions differing only in unsaturation (1), GLC separation of triglycerides according to molecular weight (2), and numerous other analytical techniques (3-5).

The complexity of naturally occurring fats and the lack of refinement in our present analytical methods do not, however, permit the use of a single analytical technique for determining triglyceride composition. Combinations of these techniques have likewise failed to provide comprehensive analyses, but do produce experimental data which permit detailed estimation of triglyceride compositions. Such an application of combined analytical techniques for determining triglyceride composition is illustrated in the recent work of Blank et al. (6) who applied Ag^+ TLC in conjunction with lipase hydrolysis to demonstrate the triglyceride composition of synthetic mixtures and of several natural fats. A similar integration of Ag^+ TLC, and GLC, was employed by this laboratory in characterizing the triglyceride composition of *Cuphea llavia* seed fat (7).

Ucuhuba (*Virola surinamensis*) is a tree of the nutmeg family which grows along the swampy coast of northern Brazil and the Guianas. The fruit has a spherical hull which encloses a seed 12–14 mm in diameter. Fatty acid analyses of the seed fat by several investigators (8–13) have shown that myristic and lauric acids comprise 66.6–73.4 and 5.0–20.8 wt %, respectively. Small amounts of additional fatty acids including decanoic, palmitic, oleic and linoleic were also found. Employing a systematic crystallization of the glycerides from acetone, Atherton and Meara (8) estimated that six triglycerides were present in the ucuhuba kernel fat. Trimyristin (MMM) and laurodimyristin (LaMM) were found to comprise 42.6 and 30.7 mole %, respectively, with the remaining composition (26.7%) consisting of OLaM (12.1%), LaMP (10.3%), OMM (3.1%), and LaLaM (1.2%). (See Table IV for explanation of abbreviations).

The current investigation employed the integration of Ag^+ adsorption chromatography with GLC in characterizing the triglycerides of ucuhuba (*V. surinamensis*) kernel fat. The triglycerides were separated by Ag^+ TLC according to the number of double bonds per molecule, with subsequent GLC analysis of the fractionated triglycerides.

Procedures

Materials

Ucuhuba (*V. surinamensis*) seeds were obtained through the courtesy of Mr. H. Bloom, American Consul, Belem, Para, Brazil. The fruits were gathered in 1963 from trees in the Brazilian state of Para in the lower Amazon basin. The seeds were sorted to remove any diseased or damaged ones and stored in a refrigerator for future analysis.

[1] Presented at the AOCS Meeting in Houston, Texas, 1965.
[2] Supported in part by grants from the National Institutes of Health (AM-06011) and the Corn Products Institute of Nutrition.

TABLE I

Fatty Acid Composition of Ucuhuba Kernel Fat Triglycerides (Mole %)

Fatty acids	Total triglycerides	TLC fractions [a]		
		0	1	2+
8:0	tr	0.1	....	....
10:0	0.9	0.9	0.5	0.7
12:0	20.0	20.5	13.1	14.2
14:0	71.3	75.0	51.2	28.0
14:1	0.7	....	8.1	2.8
16:0	3.5	3.4	3.5	5.0
16:1	0.3	....	3.0	2.1
18:0	0.5	0.1	0.3	0.9
18:1	2.4	....	20.3	30.4
18:2	0.4	....	....	15.9
Unknown			tr	tr

[a] TLC fractions separated according to the number of double bonds per triglyceride molecule.

Methods

Gas-Liquid Chromatography of Fatty Acids. Fatty acid composition was determined by GLC analysis of the methyl esters. An Aerograph Hy Fi A-600-B gas chromatograph equipped with a 6 ft by 1/8 in. column containing 20% (w/w) diethylene glycol succinate polyester coated on acid-washed 60/80 mesh Chromosorb W was used as previously described (7). All fatty acid compositions were reported in mole percent.

Gas-Liquid Chromatography of Triglycerides. GLC analysis of the total triglycerides and each triglyceride fraction was achieved using the general methods of Litchfield et al. (14). The gas chromatograph (F & M 400) was equipped with a hydrogen flame detector and automatic temperature programing. The 24 in. × 3.0 mm I.D. glass column contained 3.0% JXR on 100/120 mesh Gas-Chrom Q and was automatically programmed from 170C–305C at 4C/min with a nitrogen gas flow of 100 ml/min. The peaks, which correspond to triglycerides with identical carbon numbers, were identified by comparing retention times with those of known compounds. Quantitative calibration factors were determined by using a known composition mixture of trioctanoin, tridecanoin, trilaurin, trimyristin, tripalmitin and tristearin (Applied Science Laboratories, State College, Pa.), and all peak areas were corrected accordingly. All identical molecular weight triglycerides were assumed to have the same calibration factor. Peak areas were determined by triangulation and results were reported as mole % of total triglycerides.

Thin-Layer Chromatography. Separation of lipid classes was carried out on 1.0 mm thick Silica Gel G TLC plates developed with a 2:1 $CHCl_3$- benzene (v/v) solvent mixture. Bands were identified by comparison with the R_f values of known materials.

Silver-ion chromatoplates were prepared according to the method described by Barrett et al. (15) involving the use of Silica Gel G and 12.5% (w/v) aqueous $AgNO_3$. The plates were developed by ascending chromatography using a 0.1% ethanol in chloroform (v/v) mixture. Before use, the chloroform was distilled and passed through a column of alumina to remove the ethanol which is added as a preservative. A specific amount of ethanol was then added to the distilled chloroform to achieve the desired solvent polarity.

To remove any possible lipid contaminants, all TLC plates were predeveloped with distilled diethyl ether before applying the sample.

TABLE II

Triglyceride Composition of Ucuhuba Kernel Fat (Mole %)

Carbon numbers	Total triglycerides	TLC fractions [a]		
		0	1	2+
36	0.9	0.8	....	....
38	5.6	5.7	tr	....
40	39.2	39.2	0.3	tr
42	41.0	38.5	2.1	tr
44	7.5	4.4	2.6	0.2
46	3.6	0.6	2.8	0.5
48	1.2	....	0.6	0.6
50	0.8	....	0.1	0.7
52	0.2	....	tr	0.2
54	tr	....	....	tr
56		....	....	tr
Totals	100.0	89.2	8.5	2.3

[a] TLC fractions separated according to the number of double bonds per triglyceride molecule.

TABLE III

Quantitation of the Triglyceride Fractions of *Ucuhuba* Kernel Fat Separated by Ag^+ Thin-Layer Chromatography (Mole %)

Methods	TLC fractions [a]		
	0	1	2+
Internal standard	88.9	8.5	2.6
Simultaneous equations	89.4	8.5	2.1
Average	89.2	8.5	2.3

[a] TLC fractions separated according to the number of double bonds per triglyceride molecule.

Results

Experimental

Four ucuhuba (*V. surinamensis*) seeds (4 83 g) were homogenized with distilled hexane in a Potter-Elvehjem homogenizer until a fine powder was obtained. The mixture was quantitatively transferred to a filter paper thimble and extracted in a Soxhlet extraction apparatus for 4 hr with 150 ml distilled petroleum ether (bp 30–60C). Evaporation of the solvent yielded 2.84 g fat which represented 58.8% of the seed weight. Approximately 220 mg of extracted fat was applied to 2 Silica Gel G plates which were developed with 2:1 $CHCl_3$-benzene to isolate the triglycerides. The triglyceride band was scraped off the TLC plate into a glass column and eluted off the silicic acid with distilled diethyl ether. Evaporation of the eluate yielded the purified ucuhuba triglycerides.

About 200 mg of the purified triglycerides was applied to two preparative Ag^+ TLC plates and separated into four distinct bands by development with 0.1% ethanol in $CHCl_3$ (Fig. 1). The plates were sprayed with a 0.02% alcoholic solution of 2', 7' - dichlorofluorescein and the bands visualized under ultraviolet light. Since the lower band represented only a very small percentage of the total triglycerides ($< 1\%$), the lower two bands were combined and analyzed as a single fraction. The three triglyceride bands were separately scraped off the plates into small sintered glass funnels containing 2–4 g of activated silicic acid and the triglycerides eluted with diethyl ether. Subsequent identification of the TLC bands, based on fatty acid composition and com-

TABLE IV

"Best Estimate" of Major Triglycerides in Ucuhuba Kernel Fat Based on Experimental Data in Table II and Assumptions Described in Text

Mole %	Probable components [a]	Carbon number: TLC fraction
39.2	LaMM (29.8–39.2), DMP, LaLaP	40:0
38.5	MMM (29.1–38.5), DPP, LaPM	42:0
5.7	LaLaM, DMM, DLaP	38:0
4.4	MMP, LaPP	44:0
2.8	MMO, LaPO, MPPo, MoPP	46:1
2.6	LaMO, MMPo, LaPPo, MMoP	44:1
2.1	MMMo, LaMoP, LaMPo, LaLaO	42:1
4.7	Others	

[a] C=8:0, D=10:0, La=12:0, M=14:0, Mo=14:1, P=16:0 Po=16:1, S=18:0, and O=18:1. Positional isomers not considered.

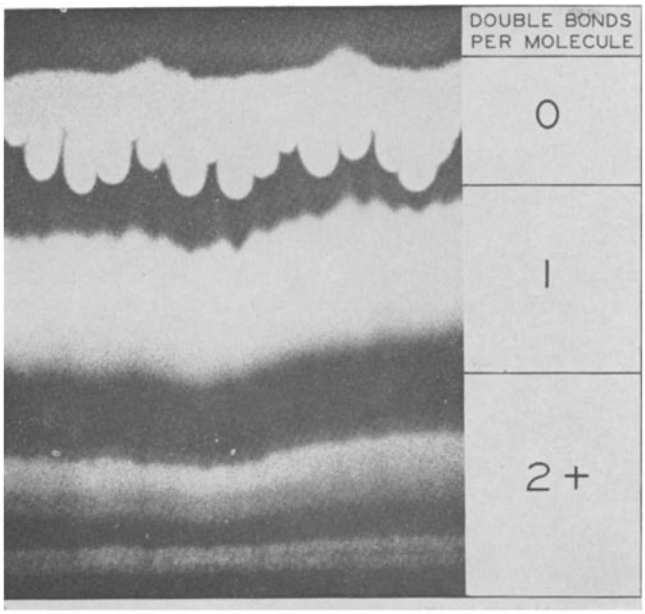

FIG. 1. TLC of ucuhuba kernel triglycerides on 1.0 mm thick Silica Gel G impregnated with $AgNO_3$. The plate was developed using 0.1% ethanol in alcohol-free $CHCl_3$, sprayed with a solution of 2′,7′-dichlorofluorescein, and photographed under U.V. light. Quantitation procedure described in text.

parison of TLC R_f values with those of known triglycerides, characterized the triglyceride fractions as containing 0, 1 and 2 or more double bonds per molecule. An accurately weighed amount of pentadecanoic acid was then added to each fraction.

Two methods of quantitation were employed for determining the amount of triglyceride in each of the three fractions. The first method involved GLC analysis of the fatty acids using an internal standard (pentadecanoic acid) as described by Gunstone et al. (16) and Blank et al. (6). The second method (see calculations below for a detailed description) utilized simultaneous equations derived from the GLC data on the fatty acids and triglycerides of the respective fractions. The values obtained by the two methods were in close agreement (Table III).

The fatty acid composition of the total ucuhuba triglycerides was determined by GLC and is reported in Table I. Myristic and lauric acids comprised 91.3 mole % of the total fatty acids. Although additional saturated fatty acids were found (decanoic, palmitic, and stearic), they occurred only as minor components. Small amounts of several unsaturated fatty acids were found including 2.4% oleic and 0.4% linoleic acid.

The fatty acid composition of the individual TLC fractions (Table I) indicated a quantitative separation. This was expected since no visible overlapping of the fractions had occurred on the plate (Fig. 1). The 0 double bond triglycerides contained only saturated fatty acids, while the 1 double bond fraction consisted of approximately 33 mole % monoenoic fatty acids. Analysis of the 2+ double bond fraction revealed slightly over two double bonds per triglyceride molecule. Homogeneity of the Ag^+ TLC separation was further substantiated by the fact that linoleic acid occurred only in the 2+ triglyceride fraction.

The total ucuhuba triglycerides and each TLC fraction were separated according to molecular weight using GLC on a JXR column (Fig. 2). The total triglycerides (Table II) ranged in size from carbon number 36 through 54 with those of carbon numbers 40 and 42 constituting 80.2 mole % of the total. The large concentrations observed for these two molecular weight triglycerides was not surprising since lauric and myristic acids composed 91.3 mole % of the total triglycerides and would be expected to be present as trimyristin (MMM) and laurodimyristin (LaMM) with carbon numbers 42 and 40, respectively. Triglycerides with carbon numbers greater than 48 occurred only in small amounts. GLC of the individual triglyceride fractions showed a progressive increase in triglyceride molecular weights with increasing unsaturation (Fig. 2). This particular distribution of triglycerides would, of course, seem a likely possibility in view of the higher molecular weight fatty acids which appear in the 1 and 2+ double bond fractions (Table I). The 1 double bond fraction, as shown by the chromatogram (Fig. 2), contained predominantly triglycerides of carbon numbers 42, 44 and 46. Since the 2+ double bond fraction contained carbon numbers 50, 52 and 54, undoubtedly some di-C_{18}-triglycerides are present. The unexpected appearance of C_{56} triglyceride (trace) in our 2+ fraction could be explained by assuming the unidentified compound in Table I to be arachidic acid.

Calculations

The calculations for quantitating TLC triglyceride fractions using pentadecanoic acid as an internal standard have been described by Blank et al. (6).

A second method of quantitation which entailed the construction of simultaneous equations derived from the triglyceride and fatty acid GLC data was employed for comparison. For example, since 18:2 occurs only in the 2+ double bond triglyceride fraction (15.9 mole %) and represents 0.4 mole % of the total fatty acids (Table I), the following equation can be written:

$$15.9\left(\frac{Z}{100}\right) = 0.4$$

$$\therefore Z = \frac{\text{mole \% of 2+ double bond triglycerides}}{} = 2.5\%$$

Similarly, 18:1 occurs only in the 1 and 2+ double bond fractions where it represents 20.3 and 30.4 mole %,respectively. Thus, we know that:

$$20.3\left(\frac{Y}{100}\right) + 30.4\left(\frac{Z}{100}\right) = 2.4$$

$$\therefore Y = \frac{\text{bond triglycerides}}{\text{mole \% of 1 double}} = 8.1\%$$

Since myristic acid occurs in all three fractions (Table I) and constitutes 71.3 mole % of the total triglycerides, we can write the equation:

$$75.0\left(\frac{X}{100}\right) + 51.1\left(\frac{Y}{100}\right) + 28.0\left(\frac{Z}{100}\right) = 71.3$$

$$\therefore X = \frac{\text{mole \% of 0 double bond triglycerides}}{} = 88.6\%$$

Because of the experimental error involved in obtaining the GLC data, the values obtained for X, Y, and Z varied somewhat depending on which simultaneous equations were solved. Therefore, more precise results were obtained by using the above approximations for X, Y, and Z and making a trial and error solution of the entire series of possible simultaneous equations.

For example, the equation derived from the GLC data on C_{42} triglycerides (Table II) was:

$$\left(\frac{38.5}{0.892}\right)\left(\frac{X}{100}\right) + \left(\frac{2.1}{0.085}\right)\left(\frac{Y}{100}\right) = 41.0$$

Substituting X = 89.4, and Y = 8.5 gave a better solution to this equation than the original calculated values of X = 88.6, Y = 8.1. All simultaneous equations derived from the fatty acid and triglyceride GLC data were similarly analyzed until the overall average values for X, Y, and Z were obtained.

Discussion

The integration of Ag^+ TLC and GLC has separated the triglycerides of ucuhuba kernel fat into 23 different components (Table II). It is apparent that each of these components may contain more than one triglyceride, since the analytical methods employed resulted in a separation based solely on unsaturation and molecular weight. For example, since the 0 double bond fraction contains 75.0% 14:0, the most likely C_{42} triglyceride would appear to be trimyristin. However, the fatty acid composition indicates that other C_{42} saturated triglycerides such as DPP and LaPM could also be present. Similarly, MMO appears to be the most likely C_{46} triglyceride of the 1 double bond fraction, but LaPO, MPPo, and MoPP must also be considered as possible constituents.

Therefore, the experimental data were not sufficient to establish unequivocally the individual triglycerides present. However, the probable triglycerides in each of the 7 major components separated were estimated by: (a) calculating all possible triglycerides which had the correct carbon number, amount of unsaturation, and fatty acid composition for that component, and (b) eliminating all triglycerides containing fatty acids constituting $< 0.5\%$ of the TLC fraction in which the component occurred. The latter assumption introduced very little error, since such minor fatty acids make a very small contribution to the overall triglyceride composition. This "best estimate" of the major triglycerides in ucuhuba kernel fat is shown in Table IV.

While precise amounts of the proposed triglycerides of ucuhuba kernel fat cannot be exactly determined, it is, however, possible to calculate a range for LaMM and MMM. The saturated C_{40} triglycerides which might possibly be present are CMS, CPP, DLaS, DMP, LaLaP, and LaMM. Since the 0 double bond fraction contains 0.1% 18:0, the maximum possible amount of CMS + DLaS in the total triglycerides is 3(0.1) (0.892) = 0.3%. And since the 0 double bond fraction contains 3.4% 16:0, then the maximum possible amount of CPP + DMP + LaLaP present must be 3(3.4) (0.892) = 9.1%. Therefore, the minimum possible amount of LaMM present is given by (39.2 − 0.3 − 9.1) = 29.8% and the maximum is 39.2%. Similarly, the possible saturated C_{42} triglycerides are MMM, CPS, DMS, DPP, LaLaS, and LaMP. The maximum amounts of CPS + DMS + LaLaS and DPP + LaMP in the total triglyceride are 0.3% and 9.1%, respectively. Therefore, the minimum possible amount of MMM is (38.5 − 0.3 − 9.1) = 29.1%, whereas the maximum is 38.5%. Similar ranges could not be calculated for the other individual triglycerides as they occur only in very small amounts.

Comparison of the present results on ucuhuba kernel fat triglycerides with those previously described by Atherton and Meara (8) shows only fair agreement. Atherton et al. reported 1.2% C_{38}, 30.7% C_{40}, and 52.9% C_{42} saturated triglycerides, where we found 5.7, 39.2, and 38.5%, respectively. Where Atherton has reported 12.1% C_{44}, and 3.1% C_{46} triglycerides containing one double bond per molecule, we have found 2.6% and 2.8%, respectively. The variations between Atherton's values and those presented here may be due to the slightly different fatty acid compositions and to the assumptions that Atherton had to make to interpret his fractional crystallization data. However, these discrepancies are not surprising since the separation produced by Ag^+ TLC and GLC allow a more accurate analysis of the triglycerides than the fractional crystallization technique.

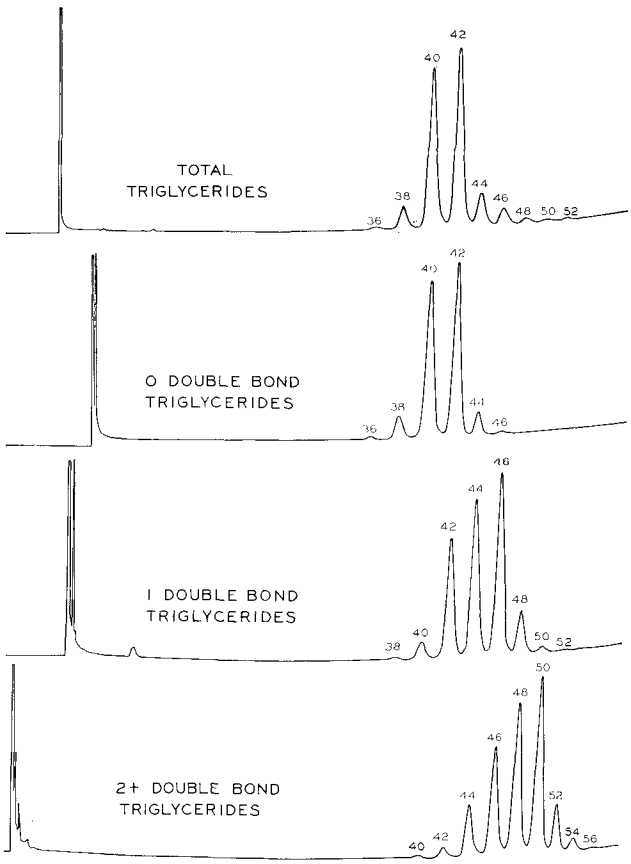

FIG. 2. Gas chromatograms of total ucuhuba kernel triglycerides and 0, 1, and 2+ double bond fractions separated by silver-ion TLC. Peaks labeled by carbon number. GLC conditions: 24 in. × 3.0 mm I.D. glass column packed with 3.0% JXR on Gas Chrom Q; 100 ml/min nitrogen carrier gas; column temperature programmed 170–305C at 4C/min.

Statistical calculations have indicated that the ucuhuba fat does not have a random distribution of fatty acids among the triglycerides. Such a random distribution would produce 10.0% C_{38}, 31.1% C_{40}, 39.5% C_{42}, and 6.5% C_{44} saturated triglycerides, whereas the experimental values found were 5.7%, 39.2%, 38.5%, and 4.4%, respectively. The amount of monounsaturated fatty acids indicated by the fatty acid composition of the 2+ double bond fraction is also considerably higher than would be expected with a random distribution.

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Gas Chromatographic Resolution of Butteroil and Synthetic Triglycerides Beyond Their Carbon Numbers

A. KUKSIS[1] and W. C. BRECKENRIDGE,[1]
Department of Biochemistry, Queen's University, Kingston, Ontario, Canada

Abstract

Preparative gas chromatography was used to isolate the C_{26} to C_{38} triglycerides of butteroil in groups of uniform molecular weight. Determination of fatty acids allowed a preliminary assignment of triglyceride structure and an estimation of the relative proportions of the various glyceride types. A basis for a further gas chromatographic resolution of saturated triglycerides of identical molecular weight but differing in composition and positional distribution of fatty acids was recognized in the easy separation of various derivatives of 1- and 2-monoglycerides. The study indicated that it might be practical to separate positional isomers of those triglycerides which contain fatty acids of both short and long chain lengths. Other promising routes to increased glyceride resolution were suggested by experiments using chromatographic conditions approaching gas-solid systems.

Introduction

FRACTIONATION OF HIGH molecular weight triglycerides by gas chromatography (GLC) (1) and thin-layer chromatography (TLC) on silver nitrate impregnated silica gel (2) has resulted in considerable progress in the elucidation of the structure of natural fats and oils. The integration of TLC and GLC has provided natural triglyceride groups simple enough for meaningful enzymatic positional analyses and for the first time has severely restricted the domain of statistical speculation. Unfortunately, the combined TLC-GLC system is of little value for the study of those fats that contain significant amounts of several different saturated fatty acids (3). The following report suggests an experimental basis for a further resolution of certain groups of triglycerides of uniform molecular weight by means of GLC.

Experimental

Materials and Methods

Monoglyceride isopropylidines, benzylidines, diacetates and dibutyrates were synthesized in the laboratory as described by Mattson and Volpenhein (4). The 1-palmito-2,3-dibutyrate was a gift from R. G. Jensen. The molecular distillates of butteroil were those previously described (5). The homogeneity of the triglyceride fractions was determined by silicic acid TLC (6).

Fatty acid composition of triglycerides was determined by GLC of butyl esters (7). The esters were prepared by heating 1–10 mg of triglyceride with 2 ml of 10% H_2SO_4 in n-butanol at 100C in sealed glass ampules. The butyl esters were analyzed in an Aerograph Hy-Fi instrument equipped with an F and M Proportional Temperature Programmer. A 5 ft x ⅛ in. O.D. stainless steel column packed with 5% (w/w) SE-30 on Chromosorb W (60–80 mesh) was programmed from 75C–275C.

Preparative GLC of triglycerides was carried out in an Aerograph Autoprep 700 equipped with a stream splitter (split ratio 1:4.5) and a hydrogen flame ionization detector (8). A 2 ft x ¼ in. O.D. aluminum column packed with 5% (w/w) SE-30 on siliconized Chromosorb W (60–80 mesh) was programmed manually from 190C–325C. Preparative runs were made with the total distillate and the saturated and monounsaturated triglyceride groups isolated by TLC. The collected peaks were checked for cross-contamination by analytical GLC and for oxidation and hydrolytic degradation by TLC.

Analytical GLC of triglycerides was performed in an Aerograph Hy-Fi (Model 600) equipped with an F and M Proportional Temperature Programmer. Stainless steel tubes ⅛ in. O.D. and 1.5, 3, 5 and 8 ft in length were packed with 5% SE-30 on siliconized Chromosorb W (60–80 mesh) or 6% DEGS on Gas Chrom P (80–100 mesh). The silicone columns were conditioned without flow at 350C overnight, and were temperature programmed. The DEGS columns were conditioned with flow at 240C overnight and were operated isothermally at 200C or 230C.

Glass inserts were used in the metal vaporizing blocks of all instruments.

TLC of triglycerides was performed on 20 x 20 cm chromatoplates coated with silicic acid impregnated with silver nitrate as described by Litchfield et al. (6). The bands of separated triglycerides were located by

TABLE I

Fatty Acid Composition of Bands Obtained From Silver Nitrate TLC of the R-1 Distillate (Molar %)

Fatty acid carbon No.	R-1 distillate	Saturated band	Unsaturated band
4:0	19.57	17.48	22.91
6:0	11.57	12.30	7.56
8:0	6.34	7.34	6.91
10:0	9.75	11.83	10.89
10:1	0.95	—	—
12:0	6.43	9.69	7.43
12:1	Trace	—	—
13:0	Trace	Trace	—
14:0	13.84	17.17	11.48
14:1	1.09	—	1.49
15:0	1.00	1.51	0.84
16:0	19.68	19.20	10.58
16:1	0.90	—	2.67
17:0	Trace	0.34	0.59
18:0	2.39	2.88	2.04
18:1	6.49	0.24	14.62

[1] Present address: Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada.