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

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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

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spraying the plates with 2,7-dichlorofluorescein (0.1% in ethanol) and examining under ultraviolet light. Each band was marked, scraped off the plate and extracted with 5% methanol in ethyl ether. After evaporation of the solvents, the recovered triglycerides were either transbutylated for fatty acid analysis or were further separated by preparative GLC.

Results and Discussion

The triglyceride sample selected for detailed analysis was the most volatile 2.5% distillate of butter oil (R-1) containing triglycerides with carbon numbers ranging from C_{24} to C_{40} (5). Extensive purification of this distillate on columns of silicic acid resulted in noticeable losses in the proportion of the C_{24} peak and suggested that components other than triglycerides had been present in earlier preparations of this material. On silver nitrate TLC, the purified distillate gave two major bands corresponding to saturated and monounsaturated triglycerides. A third faint band in the approximate area of diunsaturated glycerides failed to provide sufficient material for further analyses.

The GLC elution patterns of the refined distillate and the two fractions recovered from TLC are shown in Figure 1. The total distillate (A) contained major peaks for triglycerides of carbon numbers C_{26} to C_{38}. The saturated triglyceride band obtained by TLC contained only the C_{26} to C_{34} triglycerides, while the monounsaturated triglyceride band possessed peaks corresponding to all of the original triglycerides. Apparently, the C_{36} to C_{38} triglycerides were largely made up of glycerides containing only one unsaturated fatty acid per molecule.

Table I gives the molar fatty acid composition for the original distillate and the saturated and unsaturated triglyceride bands obtained by TLC. The small amounts of the unsaturated fatty acids found in the saturated glyceride band indicated an effective removal of the unsaturated triglycerides. The unsaturated glyceride band was contaminated with saturated glycerides, as it contained only 18.78 mole % of

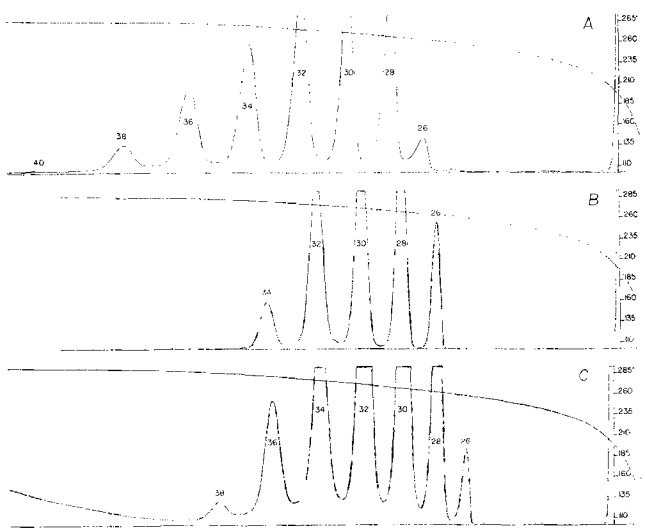

FIG. 1. GLC elution patterns recorded for butteroil distillate R-1 (A) and its saturated (B) and monounsaturated (C) components recovered from $AgNO_3$-TLC. The numbers identifying the triglyceride peaks refer to the number of C-atoms in the acyl groups. Column conditions: 2 ft x ¼ in. O.D. stainless steel tube; 5% SE-30 on siliconized Chromosorb W (60–80 mesh); N_2 200 ml/min. Other conditions as given in the text. Temperature gradient as given in the figure.

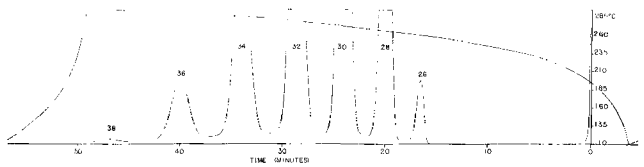

Fig. 2. Preparative GLC separation of butteroil distillate R-1. Peak identity and column conditions as indicated in Figure 1. Temperature gradient as given in the figure.

total unsaturated fatty acid. In order to have one unsaturated fatty acid per glyceride molecule the total unsaturated fatty acid content should have been about 33%. Apparently the migration of the more polar short chain saturated triglycerides was retarded to about the same extent as that of the monounsaturated triglycerides on the silver nitrate treated TLC plate. Corresponding decreases were noted in the proportions of the C_{26} and the C_{28} peaks in the GLC patterns.

To determine the triglyceride composition of this molecular distillate, glyceride peaks of uniform molecular weight were collected by means of preparative GLC. Figure 2 illustrates a section of identical repeat sequences of preparative separations obtained with the total distillate. All fractions except that representing the C_{38} triglycerides were collected separately. The amounts of material collected averaged 1 to 5 mg of each peak per 20–30 injections and represented better than 95% recovery of injected material, as determined by weighing and the use of internal standards. Figure 3 shows the elution patterns obtained on rechromatography of the collected peaks. No peaks other than those anticipated on the basis of the known composition of the distillate were found in any of the collections.

Table II gives the fatty acid compositions of the individual triglyceride peaks collected from the total distillate. With the exception of C_{26}, which does not contain any 18:0 or 18:1 fatty acids, all the glyceride groups contain all the fatty acid types found in the whole distillate. Although the proportions of the individual acids varied and the saturated triglyceride groups could be shown to be free of unsaturated fatty acids, all fatty acid chain lengths were represented in each peak. As a result, each triglyceride peak could contain all or most of the glyceride types shown in

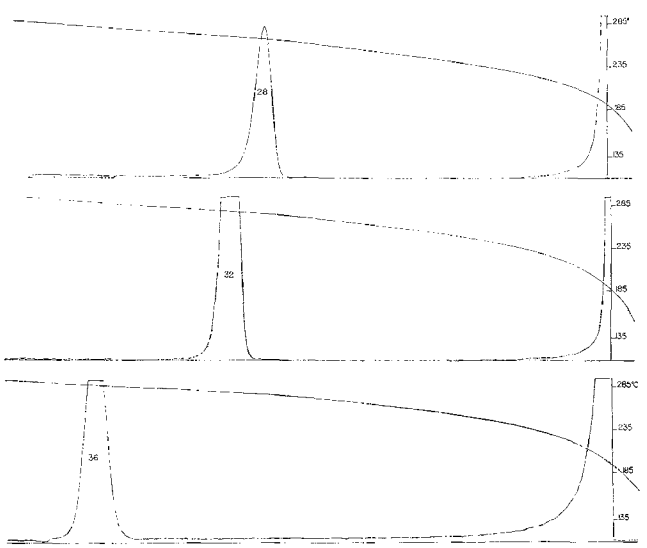

Fig. 3. Rechromatography of individual triglyceride peaks isolated by preparative GLC. Peak identity and general column conditions as given in Figure 1. Temperature gradient as given in the figure.

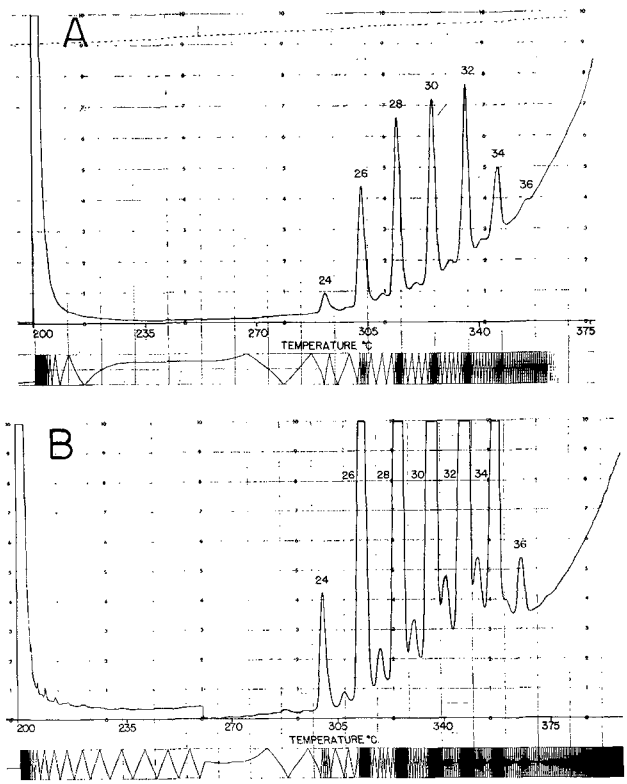

Fig. 4. GLC elution pattern recorded for the saturated triglycerides of butteroil distillate R-1. Peak identity as in Figure 1. Both patterns represent the same material: lower print recorded at 4 times higher sensitivity. Column conditions: 5 ft x ⅛ in. O.D. stainless steel tube; partially stripped 5% SE-30 on siliconized Chromosorb W (60–80 mesh). N_2 100 ml/min. Temperature program as given in figure.

Table III. In deciding the glyceride types no distinction was made between saturated and unsaturated fatty acids. Thus, a fatty acid with 18 carbon atoms may represent either 18:0, 18:1 or both. The complexity of the table was reduced by ignoring the odd carbon number fatty acids.

Table III shows that exact estimates can be obtained only for a few triglyceride types. In C_{26}, for example, exact values can be determined for triglycerides containing 16:0 and 16:1 fatty acids, as well as 18:0 and 18:1 containing triglycerides, if present. In case of C_{28}, only the glycerides containing 18:0 and 18:1 fatty acids can be measured. Nevertheless, on the basis of the mole proportions of fatty acids in the individual peaks, it is possible to arrive at empirical distributions that provide approximate ranges of concentrations for the possible component triglycerides.

Table IV gives an empirical solution for the tri-

TABLE II

Fatty Acid Composition of Individual Triglyceride Peaks in the Total Distillate (Mole %)

Fatty acids	C_{26}	C_{28}	C_{30}	C_{32}	C_{34}	C_{36}
4:0	20.86	16.77	13.30	26.80	28.12	27.01
6:0	13.18	10.72	13.22	4.15	4.49	8.22
8:0	14.43	12.85	12.32	2.65	1.59	1.02
10:0	16.72	17.73	20.22	10.89	3.84	2.05
12:0	9.88	6.94	9.83	13.16	7.19	1.89
14:0	16.51	17.30	10.52	14.44	23.19	15.49
14:1	Trace	Trace	Trace	Trace	Trace	Trace
15:0	—	0.28	0.16	0.35	0.40	0.40
16:0	8.42	13.36	15.56	19.80	24.51	32.66
16:1	Trace	Trace	Trace	Trace	Trace	Trace
17:0	—	Trace	Trace	Trace	Trace	Trace
18:0	Trace	1.32	1.87	3.12	3.80	6.06
18:1	Trace	2.73	3.00	4.70	2.88	5.40

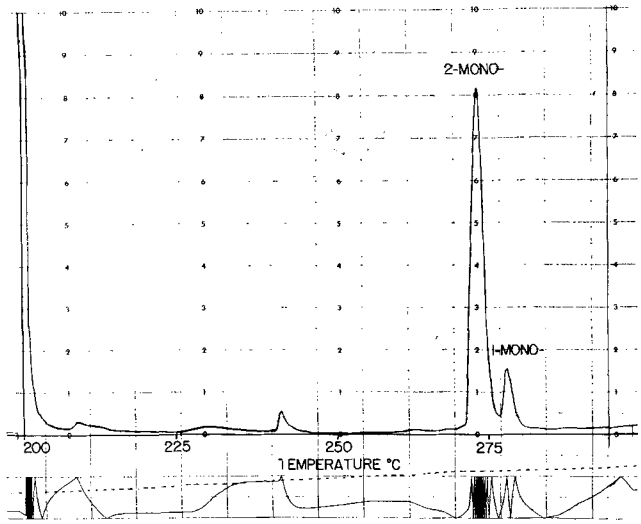

Fig. 5. GLC elution pattern recorded for an isomeric monopalmitin dibutyrate. Column: 8 ft x ⅛ in. O.D. stainless steel tube; 5% SE-30 on acid washed Chromosorb W (60–80 mesh). N_2 100 ml/min. Temperature gradient as given in figure.

glyceride type distribution in the mixed glyceride group of carbon number 34. This distribution was derived by using a pegboard with colored washers. Similar types of distributions can be derived for other triglyceride groups of uniform molecular weight, and restricted concentration ranges assigned. An exact solution by means of simultaneous equations is impossible with the limited information available. It is possible that the triglyceride types occurring in the mixed glyceride groups of uniform molecular weight could be deduced on the basis of some enzymic specificity, solubility effects or even molecular configuration. No clear cut rules for arriving at suitable fatty acid complements in this way have yet been established.

In order to obtain additional experimental data for assigning glyceride structures, attemps were made to resolve the triglyceride mixtures further by more efficient GLC. The first efforts were directed at obtaining a complete resolution between the odd and even carbon number triglycerides. Since only 15:0 and 17:0 fatty acids occurred in readily detectable quantities, it was felt that either a separate or a joint estimate of the proportion of the odd carbon number glyceride peaks would simplify the overall pattern of fatty acid distribution. Figure 4 shows the resolution obtained for the R-1 distillate on a 5 ft long 5% SE-30 column. The proportions of the odd carbon number peaks in the upper print roughly correspond to the content of odd carbon number fatty acids in the distillate. The lower print represents a recording made with this

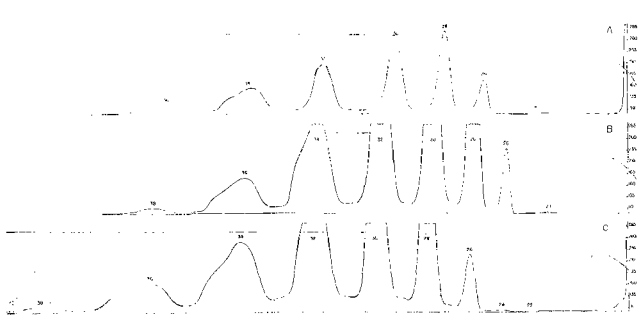

Fig. 6. GLC elution patterns recorded for butteroil distillate R-1 under low increment temperature programming. Temperature gradient as given in figure. Peak identity and general column conditions as indicated in Figure 1.

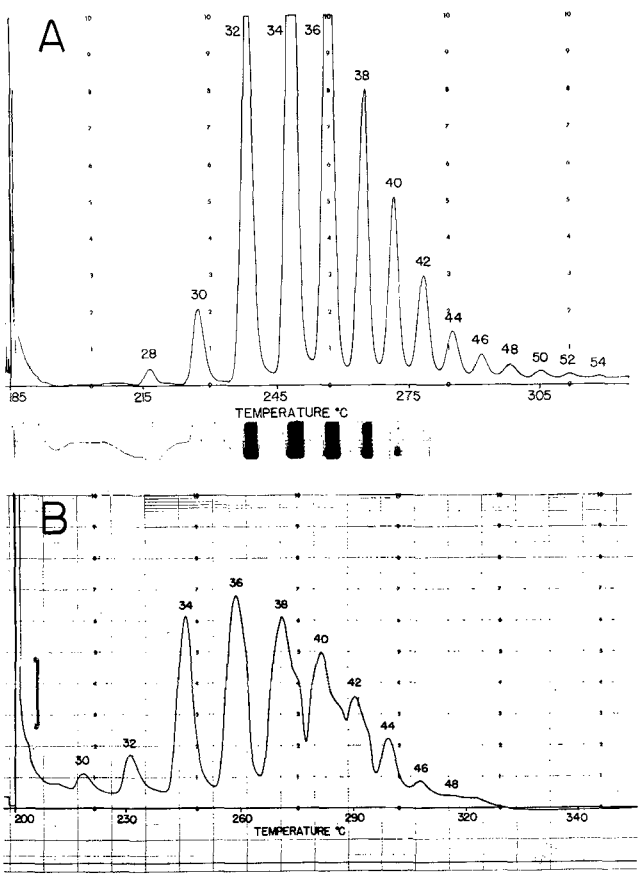

Fig. 7. GLC elution patterns recorded for coconut oil during optimum operation (upper print) and after nearly complete stripping of the liquid phase (lower print). Column conditions: 18 in. x ⅛ in. O.D. stainless steel tube; 2.25% SE-30 on acid washed Chromosorb W (60–80 mesh). N_2 150 ml/min. The chromatogram shown in the lower print was obtained after stripping the liquid phase overnight at 400C to 450C under nitrogen flow. Peak identity as given in Figure 1. Temperature gradient as given in figure.

material at a higher sensitivity. It shows much better peak definition and clearly demonstrates that odd carbon number glycerides occur in butterfat and that they can be resolved from glycerides of even carbon number by means of GLC. The carbon number distribution indicates that each of the odd carbon number acids occurs only once per triglyceride molecule and preferentially in combination with the more numerous even carbon number acids. Determination of the glyceride type composition of the odd carbon number triglycerides still requires collection of pure peaks which can be accomplished only by repeated preparative GLC.

An experimental basis for a GLC resolution of triglycerides beyond their carbon numbers was suggested by the report of Huebner (9) that the 1-monoglyceride diacetates are eluted later than the diacetates of the 2-monoglycerides. This observation has been confirmed by Wood et al. (10) who demonstrated that trimethylsilyl ethers of the 2-monoglycerides are eluted ahead of the trimethylsilyl ethers of 1-monoglycerides. Similar differences in retention times between the 1- and 2-isomers had been earlier demonstrated by McInnes et al. (11) who analyzed the monoglyceride allyl esters and isopropylidine derivatives. In the present series of experiments, it has been demonstrated that the isomeric monoglycerides can also be resolved as their benzylidine derivatives or dibutyrates. Figure 5 shows the resolution of isomeric

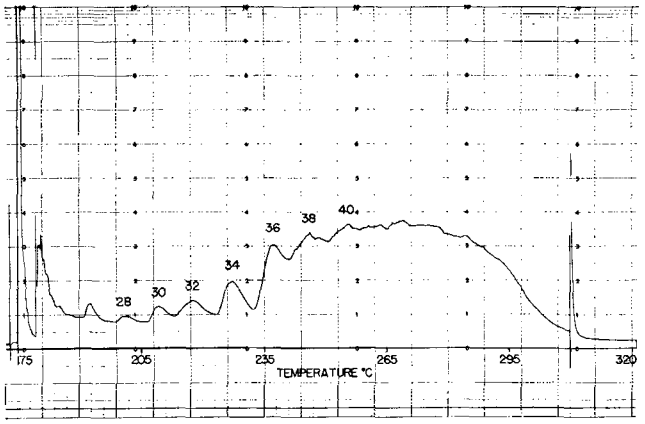

Fig. 8. GLC elution pattern recorded for coconut oil after complete stripping of liquid phase. Original column conditions as in Figure 7. Complete stripping was indicated by the inability to recover any liquid phase from the support by extraction with toluene.

monopalmitins as the dibutyrates on an 8 ft long 5% SE-30 column. Attempts to extend this type of separation to the dicaproates of isomeric monopalmitins were unsuccessful although occasional shouldering was observed under isothermal conditions or during programming using low temperature increments. Since the separations are apparently based on differences in the shape of the triglyceride molecules, the column efficiency was a more important factor in the resolution than the type of stationary phase employed. With an increase in the molecular weight of the isomeric monoglyceride derivatives, the differences in the shape of the molecules must have been minimized resulting in nearly identical elution times for both compounds.

Shouldering effects, similar to those noted for the monoglyceride dihexanoates, or dibutyrates on shorter columns, were also observed with certain mixed triglyceride peaks of the buteroil distillates. Figure 6 shows three runs with distillate R-1. Except for small discrepancies in the amounts of the material injected, the chromatograms differ only in the rates of temperature programming. In all runs large shoulders are seen for peaks with carbon numbers 34 and 36. On the basis of the fatty acid composition (Table II) of these peaks, the major triglyceride types are 18,14,4 and 16,16,4 for C_{36}, and 18,12,4 and 16,14,4 for C_{34}. If the major shoulders are to be attributed to differences in the molecular shape of the predominant triglycerides, they must be resulting from the association of the butyric acid residue with the 1 and the 2 positions of the glyceride molecule. In the past it had been suggested (12) that the short chain fatty acid residues are exclusively associated with the 1 or 3 positions of the butterfat triglycerides. On the basis of further studies of the mechanics of enzymic hydrolysis of short chain triglycerides of milk fat, Boudreau and de Man (13) have claimed that significant

TABLE III
Triglyceride Types of Uniform Molecular Weight[a]

C_{26}	C_{28}	C_{30}	C_{32}	C_{34}	C_{36}	C_{38}
		18,6,6	16,10,6	14,14,6	18,10,8	16,14,8
18,4,4	18,6,4	18,8,4	18,10,4	18,12,4	18,14,4	18,16,4
16,6,4	16,8,4	16,10,4	16,12,4	16,14,4	16,16,4	18,14,6
14,8,4	14,10,4	14,12,4	14,14,4	18,10,6	18,12,6	16,16,6
12,10,4	12,12,4	16,8,6	18,8,6	16,12,6	16,14,6	18,12,8
14,6,6	14,8,6	14,10,6	14,12,6	18,8,8	16,12,8	18,10,10
10,8,8	12,10,6	12,12,6	16,8,8	16,10,8	14,14,8	16,12,10
10,10,6	12,8,8	14,8,8	14,10,8	14,12,8	16,10,10	14,14,10
12,8,6	10,10,8	12,10,8	12,12,8	14,10,10	14,12,10	14,12,12
	16,6,6	10,10,10	12,10,10	12,12,10	12,12,12	

[a] Based on fatty acid composition given in Table II. No differentiation is made between saturated and unsaturated fatty acids of same carbon number. Odd carbon number fatty acids have been ignored.

TABLE IV
An Empirical Triglyceride Type Distribution for C_{34}

Possible triglyceride types[a]	Approximate concentrations of different triglyceride types (mole %)
18,12,4	18
16,14,4	66
18,10,6	6
16,12,6	3
14,14,6	3
18,8,8	0
16,10,8	6
14,12,8	0
14,10,10	0
12,12,10	0

[a] Based on the fatty acid composition given for peak 34 in Table II. Odd carbon number fatty acids were ignored.

amounts of short chain fatty acids could be associated with the 2-position. Rechromatography of these peaks (34 and 36) or runs with the total distillate on more efficient columns have failed to give complete separation and have resulted in further shouldering and supposedly additional resolution. Attempts are presently made to collect the triglycerides from these shoulders by means of perparative GLC in order to determine if alternative explanations can be found to account for this phenomenon.

Another observation that has this far remained unexplored for the resolution of butterfat triglycerides beyond their carbon numbers was made on aged or nearly completely stripped SE-30 columns. Figure 7 shows the elution patterns recorded for coconut oil on a 2 ft column containing 2.25% SE-30 on Chromosorb W, before (A) and after (B) complete stripping. After stripping, several peaks (36,38,40,42) are seen to yield shoulders, while others, preceding and following these, are eluted as symmetrical curves. Differences apparently exist in the polarity between triglycerides of identical molecular weight and these effects can lead to separation of triglycerides within a carbon number under GLC conditions approximating gas-solid systems (stripped columns). As the column continues to age, the triglyceride peaks are further split up resulting in the formation of an ill-defined elution pattern (Fig. 8). Adsorption effects are clearly obvious and there is indication that part of the triglyceride chromatographed, serves as a stationary phase for the rest of the volatile material.

The separation effects noted for the mixed triglycerides of short and long chain fatty acids are unlikely to be observed with long or medium chain length triglycerides. Although additional resolution would be desirable also in this range of saturated glycerides, the column efficiencies will have to be greatly increased or some basis other than molecular shape found for their separation.

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