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Fractionation of Triglycerides by Reversed-Phase Partition Chromatography

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Summary

The separation of triglycerides by reversed-phase partition chromatography in columns is described. Heptane on nonwetting Celite is used as the stationary phase and acetonitrile-methanol as the mobile phase. Mixtures of triglycerides differing by only one methylene group or one double bond are well resolved. The method may be used for the separation of triglycerides containing *cis* and *trans* unsaturated fatty acids that cannot be separated by either gas-liquid or argentation chromatography.

For the complete analysis of the composition of natural fats procedures are required that permit a quantitative fractionation of triglycerides into simple mixtures. Triglycerides may be efficiently fractionated by degree of unsaturation by argentation chromatography (*1–4*). Triglycerides may also be fractionated by carbon number, as well as degree of unsaturation, by countercurrent distribution, as demonstrated by Scholfield, Dutton, and co-workers (*5–11*), but separation of molecular species differing by only one methylene group appears to be beyond the practical limitations of this technique. Triglycerides differing by a single methylene group may be separated partially by gas-liquid chromatography (GLC), but quantitative separations and recoveries, especially triglycerides of high molecular weight and unsaturation, need improvement (*12–16*).

Although fractionation by liquid-liquid partition generally is most efficient in a chromatographic system, little effort has been devoted to the application of this technique to triglycerides. The

fractionation of triglycerides by reversed-phase partition chromatography on paper has been studied by Mangold et al. (*17*) and Kaufmann and Makus (*18*), but the low capacity of this technique limits its usefulness. Anker and Sonanini (*19*) have described the reverse-phase partition of triglycerides by thin-layer chromatography, but this technique needs further development to permit the separation of triglycerides differing by one methylene group. Using a reversed-phase system consisting of acetone-water as the mobile phase and heptane as the stationary phase, Black and Hammond (*20*) calculated that a difference of four methylene groups or two double bonds was required for the separation of triglycerides. Hirsch (*21*) obtained separations of triglycerides of the same order of efficiency with "factice" columns which served as a stationary phase for a mobile phase of aqueous acetone.

Previous work by the authors (*22,23*) demonstrated that excellent separation of methyl esters differing in chain length and/or unsaturation may be obtained with a reversed-phase system consisting of heptane on nonwetting Celite as the stationary phase and acetonitrile-methanol as the mobile phase. Described here is the application of this system to the fractionation of mixtures of triglycerides differing by one methylene group or one double bond.

EXPERIMENTAL

Materials

Highly purified (>99%) preparations of trinonanoin, tridecanoin, tripentadecanoin, tripalmitin, tripalmitolein, triolein, and trilinoelaidin were purchased from The Hormel Institute Lipid Preparation Laboratory, Austin, Minnesota.

Text mixtures of triglycerides differing by one methylene group or one double bond were prepared by interesterification of trinonanoin with tridecanoin (I), tripentadecanoin with tripalmitin (II), tripalmitin with tripalmitolein (III), and triolein with trilinoelaidin (IV). The first mixture (I) gave a mixture of triglycerides with carbon numbers of 27, 28, 29, and 30; the second (II), 45, 46, 47, and 48; the components in the third mixture (III) all have a carbon number of 48, but they differ by having zero, one, two, and three double bonds per molecule. The components of the fourth mixture all have the carbon number 54, but differ in the total number and the numbers of *cis* and *trans* double bonds. This mixture

consists of triolein (three *cis* double bonds); trilinoelaidin (six *trans* double bonds); monooleo-dilinoelaidin (one *cis* and four *trans* double bonds); and monolinoelaidin-diolein (two *cis* double bonds and two *trans* double bonds).

The above test mixtures were prepared by heating a 1:1 molar mixture of the triglycerides with 0.2% sodium methoxide for 4 hours at 100°C under an atmosphere of nitrogen. The catalyst was extracted with 50% aqueous methanol from a petroleum ether–ethyl ether (1:1) solution of the reaction products. The crude triglyceride mixtures were purified by preparative thin-layer chromatography (TLC) on plates coated with Silica Gel G (A. G. Merck, Darmstadt, Germany) which was extracted with ethyl ether to remove organic contaminants.

The test mixtures of triglycerides differing by one methylene group (I and II) were analyzed by GLC. As an example, the separation of mixture II is illustrated in Fig. 1. Since the components with carbon numbers of 46 and 47 exist in two isomeric forms, twice as much of these components are formed.

To show that the interesterification reaction had gone to completion, the test mixture prepared from tripalmitin and tripalmitolein was analyzed by argentation-TLC (*6*). Because of the difficulty of

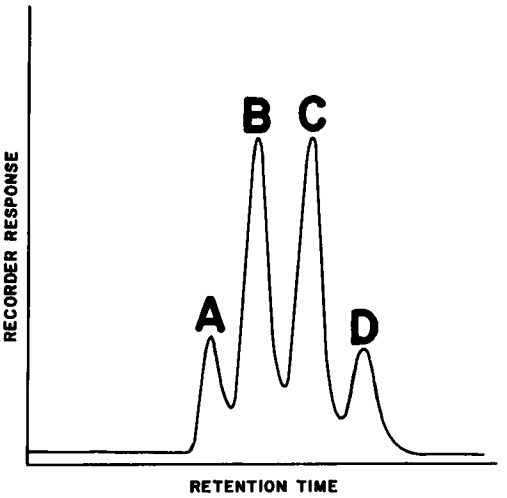

FIG. 1. GLC analysis of the products of the interesterification of tripalmitin and tripentadecanoin. A, tripentadecanoin; B, monopalmitodipentadecanoin; C, monopentadecanodipalmitin; D, tripalmitin.

separating triolein from trilinoelaidin, it was not possible to analyze the product of the reaction of these two triglycerides by this method. Furthermore, since all components of this mixture had the same carbon number, it was also not possible to analyze it by GLC. Evidence that interesterification had occurred to give the expected mixture of triglycerides was obtained by the reversed-phase partition method described herein.

Analytical Methods

Analysis of Methyl Esters. The composition and quantification of some triglyceride fractions could only be determined by an analysis of their constituent acids. The triglycerides were converted to methyl esters in a 15-ml conical centrifuge tube under an atmosphere of nitrogen. The solvent was evaporated from each fraction (collected from the column) in a stream of nitrogen; then approximately 1 ml of 6% absolute methanol containing 6% by weight of HCl was added to the tube and it was stoppered. Under these conditions interesterification was complete in about 12 hours and the general practice was to allow the tubes to stand overnight prior to analysis. Methanol and HCl were removed by evaporation in a stream of nitrogen, leaving the methyl esters which were then dissolved in 100 μl of carbon disulfide and analyzed by GLC. The amount of a triglyceride fraction was determined from the GLC analysis by means of an internal standard. Identification of the triglycerides was made on the basis of the ratios of the fatty acids. In the case of the randomized mixture of tripalmitin and tripalmitolein, identification was also made by argentation-TLC.

Gas-liquid chromatography of the methyl esters was carried out with an Aerograph Model Hi-Fi 600D instrument equipped with a hydrogen flame detector and a 6-ft by $\frac{1}{4}$-in. coiled aluminum column packed with 8% ethylene glycol succinate polyester (EGSSX) on Gas-Chrom P (Applied Science Laboratory, State College, Pa). Helium was used as the carrier gas at a flow rate of 75 ml/min and the column was operated at a temperature of 200°C.

Gas-liquid chromatography of triglycerides was carried out with the same instrument using a 2-ft by $\frac{1}{8}$-in. coiled aluminum column packed with 3% JXR on Gas-Chrom P (Applied Science Laboratories, State College, Pa.). The column was generally operated isothermally at a temperature best suited for the particular triglyceride

being analyzed. A temperature of 200°C was used for trinonanoin; tripalmitin and the longer-chain triglycerides were analyzed at 320°C at a gas flow of 100 ml/min.

Reversed-Phase Partition Chromatography

Solvents. All solvents were freshly distilled in an all-glass apparatus prior to use. Heptane was distilled over KOH pellets; methanol was distilled over a mixture of KOH pellets and zinc dust; technical-grade acetonitrile was purified by refluxing it for several hours over phosphorus pentoxide and distillation.

Preparation of Support. Nonwetting Celite was used as the support for the stationary phase. It was prepared by treatment of Celite 545 (Johns-Manville) with dichlorodimethylsilane as described by Howard and Martin (*24*). After the Celite was made nonwetting, it was washed by stirring it on a steam bath with concentrated HCl and distilled water; then it was washed in turn with warm methanol, acetonitrile, chloroform, benzene, and finally with heptane. The solvent and suspended fines that did not immediately settle out were decanted after each washing. Washings with HCl and distilled water were continued until the supernatants were colorless and treatment with organic solvents was continued until there was no detectable residue in the washings. The washed product was then screened and the fraction that passed through a 200-mesh screen was used as the stationary support.

Packing and Operation of Column. Most separations were carried out using a column, 125 × 2.5 cm, filled with 150 g of stationary phase. The mobile solvent, which consisted of 85 parts of acetonitrile to 15 parts of methanol (v/v), was equilibrated with heptane in a large separatory funnel at room temperature. The solid support was packed into the column as a slurry of heptane saturated with acetonitrile-methanol. Uniform packing of the stationary phase was obtained by pouring the slurry into the column, a small amount at a time, and then tapping the column from the side while applying a slight pressure of nitrogen from the top. The bottom of the column was plugged with glass wool, and 1 to 2 inches of glass beads were placed on the top of the column to keep the support together. The column was kept at 12°C by means of a jacket of circulatory water. To cool the solvent before it entered the column, the water jacket was allowed to extend about 6 inches above the packing. After

approximately 200 ml of the stationary phase had passed through the column, the acetonitrile-methanol phase was added to the top. The column was operated by gravity or free flow with a head of about 1 liter of mobile phase in a separatory funnel connected by a 24/40 ₸ ground-glass fitting to the top of the column. Best separation efficiency was achieved by packing the column very firmly instead of restricting the flow by means of a stopcock.

After all the excess stationary phase had emerged and the equilibrium was established between the phases, the sample, dissolved in about 1 ml of mobile phase, was placed directly on the top of the column. Studies with standard mixtures were carried out on samples of approximately 15 mg. The amounts of the individual components, therefore, consisted of between 2 and 4 mg. In order to place the sample directly on the top of the column, the solvent above the packing was temporarily removed. The eluant was collected in fractions of 6 ml by means of an automatic fraction collector. The amount and composition of each fraction is determined by GLC after evaporation of the solvent in a stream of nitrogen and redissolving it in 100 μl of carbon disulfide or an appropriate volume to give a suitable concentration for an analysis. A semiquantitative analysis was made on the basis of the peak area relative to that obtained on a standard of known concentration with those samples that lent themselves to a direct GLC analysis. The components of the randomized mixture of tripalmitin and tripalmitolein and that of triolein and trilinoelaidin have the same carbon number. Thus identification, as well as the amount of each of the components in the fractionation of these mixtures, were determined by GLC of the corresponding methyl esters.

RESULTS AND DISCUSSION

Figure 2 shows the fractionation of the two standard mixtures (I and II) containing triglycerides differing by one double bond or one carbon atom. It is evident that a complete separation of the components of these mixtures was achieved. In our system, as in all other partition systems, there is an opposite effect of an increase in the number of methylene groups and increase in unsaturation on the retention volume. With triglycerides, one double bond is equivalent to a reduction of about 2.3 methylene groups in the molecule. Thus overlapping of some triglycerides can be expected.

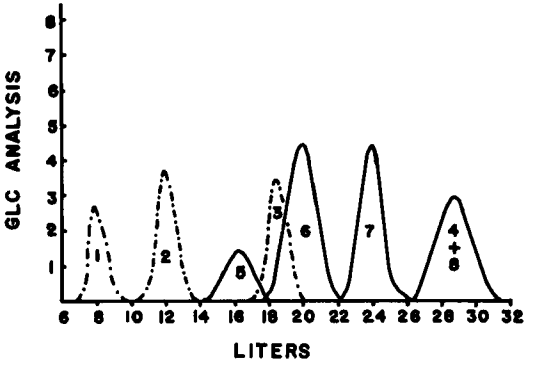

FIG. 2. Fractionation of triglycerides by reversed-phase partition chromatography. 1, tripalmitolein; 2, monopalmitodipalmitolein; 3, monopalmitoleodipalmitin; 4 and 8, tripalmitin; 5, tripentadecanoin; 6, monopalmitodipentadecanoin; 7, monopentadecenodipalmitin.

The capacity of the column for complete separation of triglycerides differing by one methylene group was about 4 mg per single component. Separations of this degree of difficulty are not encountered in most natural fats, and thus in practice much larger samples may be used.

One criterion of a good partition system is its stability. A good test of stability is the reproducibility obtained on successive fractionations of the same sample. The system described here appeared to be stable indefinitely, and the column was only regenerated to remove slow-moving compounds that might interfere with a new analysis. Essentially complete reproducibility could be obtained on the fractionation of the standard mixtures shown in Fig. 2 carried out months apart. The reproducibility of the system in any one column operated under standardized conditions is so good that the relationships between carbon number and retention volume (Fig. 3) may be used as a guide in an analysis. In practice, the number of tubes that must be analyzed can usually be greatly reduced through the use of the relationship shown in Fig. 3 together with a GLC analysis of the original mixture to determine the carbon numbers of the species of the sample.

The results in Fig. 4 show the fractionation of a mixture of triglycerides that cannot be resolved by either argentation-TLC or GLC. The mixture cannot be fractionated by GLC because all com-

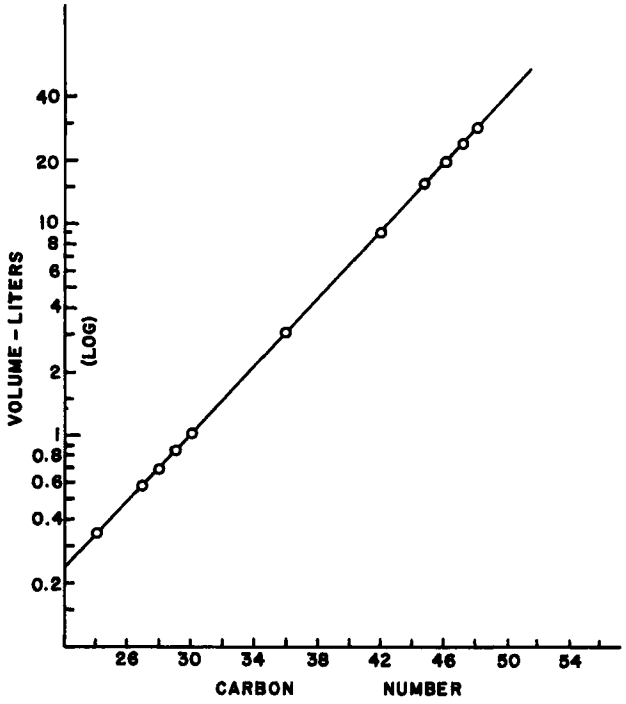

FIG. 3. Relationship of the volume of eluant vs. carbon number in the reversed-phase partition chromatography of triglycerides.

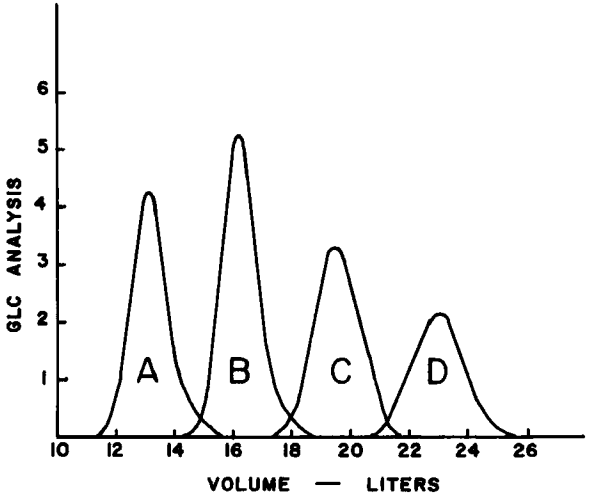

FIG. 4. Fractionation of a randomized mixture of trilinoelaidin and triolein by reversed-phase partition chromatography. A, trilinoelaidin; B, monooleodilinoelaidin; C, monolinoelaidin-diolein; D, triolein.

ponents of the mixture have the same carbon number; it cannot be fractionated by argentation-TLC because two *trans* double bonds are very nearly equivalent to one *cis* double bond, insofar as the polarity of their silver complexes is concerned. That separation of the components had been achieved in the mixture was indicated by the peaks on the chromatogram. Identification of the material in each peak was possible by a GLC analysis of the corresponding methyl esters. For example, monooleo-dilinoelaidin may be characterized because a GLC analysis will reveal a ratio of 2 parts octadecadienoic acid to 1 part oleic acid. Trilinolein was not present in this sample but, if it were, it would have been separated completely because it has a lower retention volume than trilinoelaidin.

A fractionation by the present system requires a prolonged period, especially if long-chain saturated triglycerides are involved. In an effort to speed up the fractionation, a number of parameters of the process were examined. Experiments with mobile phases of different compositions of acetonitrile and methanol were carried out but did not provide a solution to the problem. A mobile phase of 100% acetonitrile gave very broad peaks and was less efficient than methanol-acetonitrile mixtures. A mixture of methanol-acetonitrile, 1:1, tended to hasten fractionation but decreased separation efficiency. Increasing flow rate decreased the retention volume but also resulted in a loss of efficiency. Higher temperatures of operation also resulted in a speedup of the fractionation, but above 20°C there was a marked decrease in separation efficiency.

Other parameters of the system that are under investigation as a means of accelerating the fractionation process are the dimensions of the column. Decreasing column diameter generally gives an increase in separation efficiency but is accompanied by a decrease in capacity. Since the diameter of the present column appeared to be large relative to its length, as judged by its capacity, columns of smaller diameter may be more optimal, in terms of the relationship between separation efficiency and capacity. Separation efficiency may be increased by increasing the length of the column, but obviously a change in this dimension of the present system would slow the fractionation. Thus experiments are being carried out on the use of columns of smaller diameter to determine if a change in this dimension will accelerate the fractionation process without loss of efficiency or capacity.

Results of the fractionation of a mixture of tridecanoin and tri-

nonanoin on a column of 100 × 1.0 cm is compared with that of a partially randomized mixture of these triglycerides on the large column in Fig. 5. This column contained only 35 g of support and retained 60 ml of stationary phase compared to the large column, which contained 150 g of support and 215 ml of stationary phase. The small column was operated at the same flow rate and temperature as the large column. Thus they were roughly equivalent except in regard to diameter. The results in Fig. 5 show that, although the smaller column was slightly less efficient than the large column, the fractionation was much faster. The amount of sample applied to the small column was 2 mg, 1 mg of each component; thus, as expected, it does not have as high a capacity as the large column but it appears to be more efficient in this respect, since it contained much less support material and accordingly less stationary phase.

Since the system described here is able to fractionate triglycerides containing mixtures of geometric isomers of fatty acids, it should have a valuable application in the analysis of the molecular species of the triglycerides of partially hydrogenated fats. At present these fats have resisted analysis by the usual argentation-TLC and GLC techniques because of the inability of these techniques

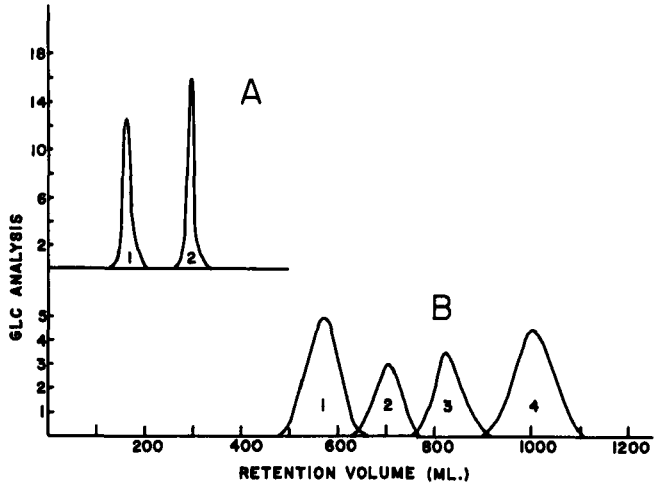

FIG. 5. Fractionation of trinonanoin and tridecanoin by reversed-phase partition using columns of different dimensions. (A) 100 × 1.0 cm column, 1, trinonanoin; 2, tridecanoin. (B) 125 × 2.5 cm column, 1, trinonanoin; 2, monodecanodinonanoin; 3, mononononanoin-didecanoin; 4, tridecanoin.

to separate various mixtures of triglycerides containing geometric isomers of monoenoic and dienoic fatty acids. Triglycerides containing positional isomers of fatty acids, of course, cannot be separated by liquid-liquid partition chromatography; triglyceride species with compositions of this type can be analyzed only insofar as they might be separated into selected fractions by carbon number or degree of unsaturation.

Virtually a complete analysis of the composition of natural fats is within reach with the development of enzymatic techniques for the determination of the distribution of fatty acids in the 1 and 3 positions of the triglyceride molecules (25,26,11). Since only about 1 mg of a triglyceride is required for the determination of the positional distribution of fatty acids between the 1, 2, and 3 positions of the molecule, it appears that the technique described here can be incorporated into a general procedure for the analysis of the molecular species of triglycerides in natural fats.

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