Preparation of Highly Purified Fatty Acids Via Liquid-Liquid Partition Chromatography[1,2]

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Abstract

The application of reversed-phase, liquid-liquid partition chromatography to the preparation of highly purified methyl esters of fatty acids is described. The parameters of fractionation of methyl esters by this method are demonstrated with model mixtures of these compounds. Model mixtures are also used to demonstrate the use of adsorption chromatography on columns of silicic acid, impregnated with silver nitrate, in conjunction with liquid-liquid partition chromatography to eliminate fractional distillation in the preparation of polyunsaturated methyl esters.

The formation of artefacts of 4, 5 and 6 double bond methyl esters during fractional distillation and their fractionation is described.

The use of liquid-liquid partition chromatography for preparative purposes on a large laboratory scale is demonstrated by the preparation of pure methyl linolenate from linseed oil esters. Methyl arachidonate, eicosapentaenoate, docosapentaenoate and docosahexaenoate are also prepared in high purity.

Introduction

THE PREPARATION of highly purified fatty acids is generally carried out in two stages. In the first stage concentrates representing a compromise between yield and purity are prepared by gross methods of fractionation: urea adduct formation, low temperature crystallization, fractional distillation, and a combination of these and other methods. The use of these techniques are well documented (2,5,19,17,22,23). Final purification of the concentrates is usually carried out on a much smaller scale by more refined methods. Countercurrent distribution (1,4,16,26,27) and adsorption chromatography (9,10,15,21,32) are the most commonly used techniques of this type. Liquid-liquid partition (3,6,7,13,14,18,20,24,25,31), displacement (11,12), and gas-liquid chromatography (6,18,24,27) have been used for the isolation of fatty acids, but these methods have not been generally adopted for the preparation of highly purified fatty acids.

Liquid-liquid partition chromatography is well suited for the preparation of fatty acids, especially polyunsaturated, because it does not involve a harsh treatment of the sample. Furthermore, it can be carried out with a high degree of resolution and has been employed as an analytical method for the quantitative analysis of saturated fatty acids (14,28,29).

The general application of a reversed-phase system of liquid-liquid partition chromatography for the preparation of methyl esters of high purity is described in this paper. Methyl linolenate, arachidonate, eicosapentaenoate, docosapentaenoate, and docosahexaenoate are prepared in high purity.

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Experimental

Materials and methods. Highly purified methyl laurate, myristate, palmitate, stearate, arachidate, oleate, palmitoleate, 11-eicosenoate, erucate, and linolenate were obtained from The Hormel Foundation.

Determination of structure was carried out by reductive ozonolysis as previously described by the authors (19).

Iodine values (I.V.) were determined by the Wijs method. Diene conjugation was determined with a Beckman Model DU Spectrophotometer at 233 mμ using 95% ethanol as the solvent.

Methyl esters are analyzed by gas-liquid chromatography (GLC) with a Research Specialties instrument using a strontium 90 detector and a $4' \times 1/4''$ column packed with 15% diethylene glycol succinate polyester on chromosorb W at 175C.

Liquid-liquid partition chromatography was carried out by the reversed-phase system previously described by the authors for the purification of concentrates of methyl arachidonate (20). The solvents in this method consist of acetonitrile-methanol (85:15 V/V) as the mobile phase and heptane as the stationary phase. The heptane phase is supported on 100 mesh celite made nonwetting to polar solvents by treatment with dimethyl silane as described by Howard and Martin (14).

Studies on two columns, 2.5×120 cm., and 10×150 cm. are reported in this paper. These columns contained 120 g and 3300 g of support, the flow rate of each was 0.2 and 5 ml per min, and the free volume of each was approximately 100 and 5000 ml respectively. The columns were maintained at 20C by circulating water through jackets around them.

The first step in the procedure is to pass heptane, saturated with the acetonitrile-methanol phase, through the column. When the heptane starts to emerge from the bottom of the column, acetonitrile-methanol (85:15 V/V) saturated with heptane is passed through the column and as soon as it starts to emerge, the sample is placed on the top of the column. When the sample has perculated a few centimeters below the top of the column, the mobile phase is started through the column again. After an amount of solvent representing the free volume of the column passes through the column, the eluate is collected in a fraction collector. The fraction collector is placed in an atmosphere of CO_2 in an insulated cabinet at −20C for the collection of methyl esters of polyunsaturated fatty acids. The contents of each tube is analyzed by thin-layer chromatography (TLC), semi-quantitatively by densitometry of the charred spots as follows.

A 10 ml sample of each tube or appropriate dilution thereof is spotted by means of a micro-syringe on a $2'' \times 8''$ plate coated with Silica Gel G (according to Stahl, Brinkmann Instrument, Inc.). The spots (usually 25 are put on each plate) are charred by heating the plates at 180C for 30 min after spraying them with chromic-sulfuric acid. The maximum density of each spot is determined with a densitom-

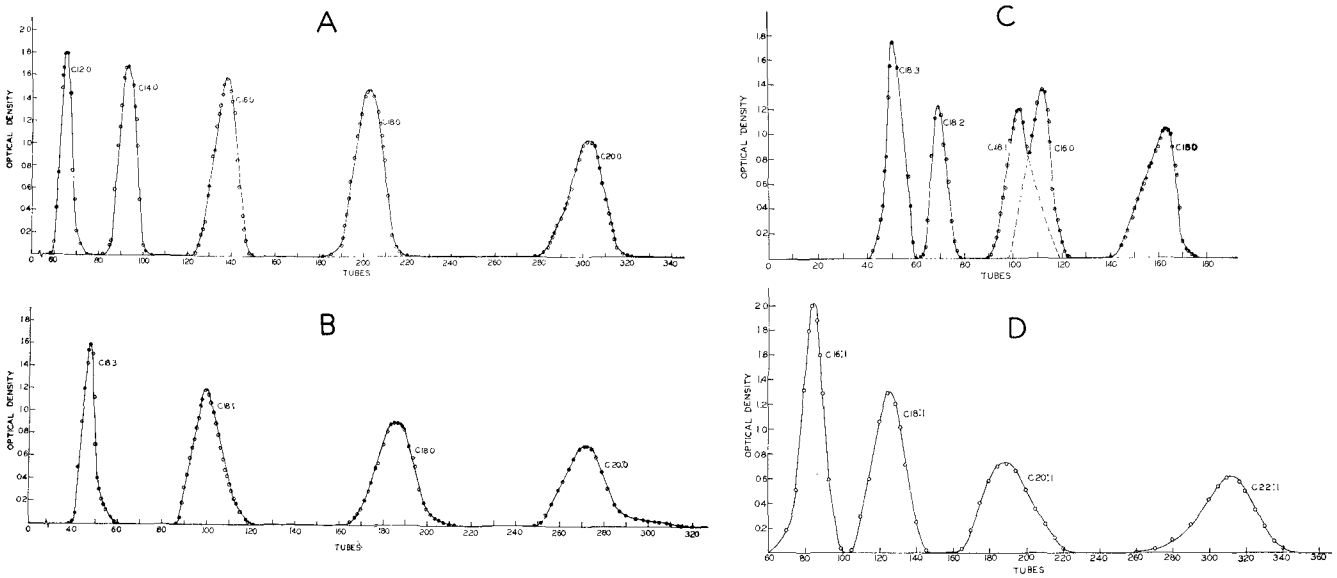

FIG. 1. Separation of 2 g samples of equimolar model mixtures of methyl esters by reversed-phase liquid-liquid chromatography using acetonitrile-methanol (85:15 V/V) as the mobile phase and heptane as the stationary phase on a 2.5 × 120 cm column of nonwetting celite.

eter (Photovolt Corp., New York) equipped with a semiautomatic stage and a 1 × 10 mm slit. Each spot gives a peak; the peak height is taken as the maximum density and expressed in terms of optical density. As little as 3 mg of material in a tube can be detected without concentrating the volume of the solution by this method. Tubes containing mixtures of esters were analyzed by GLC. The contents of the tubes from the large column were usually analyzed gravimetrically after a qualitative analysis by TLC as well as by GLC. The composition of tubes containing or suspected of containing mixtures of polyunsaturated methyl esters was also analyzed by reductive ozonolysis (19). This method is very sensitive for the detection of mixtures of polyunsaturated methyl esters.

Adsorption chromatography was performed on a silicic acid impregnated with silver nitrate according to the method of deVries (30) with a column (2.5 × 150 cm) scaled up for 2 g samples. The sample was fractionated by stepwise elution with various ratios of diethyl ether-petroleum ether (b.p. 35–60C) beginning with the petroleum ether and ending with the diethyl ether. The eluate was collected in 20 ml fractions and analyzed by TLC and GLC as well as gravimetrically.

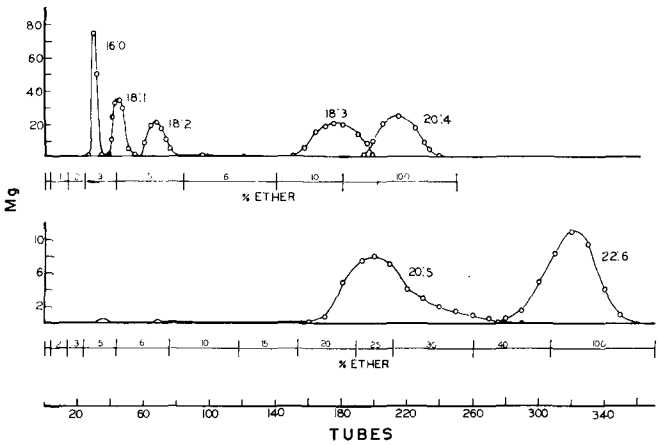

FIG. 2. Separation of equimolar model mixtures of methyl esters by adsorption chromatography on a 2.5 × 120 cm column of silicic acid impregnated with silver nitrate according to the method of deVries (30).

Results and Discussion

Separation of model mixtures. The separation of several equimolar mixtures of 2 g of methyl esters on the small column by liquid-liquid partition chromatography is shown in Figure 1. Saturated methyl esters are separated on the basis of their chain length (Fig. 1, A). Normal unsaturated methyl esters, containing the same number of double bonds, are also separated on the basis of the length of the carbon chain by this method (Fig. 1, D).

The degree of unsaturation is a factor in the separation of methyl esters, as illustrated by the results in Figure 1, C. Methyl palmitate was included in the mixture in Figure 1, C to demonstrate the prob-

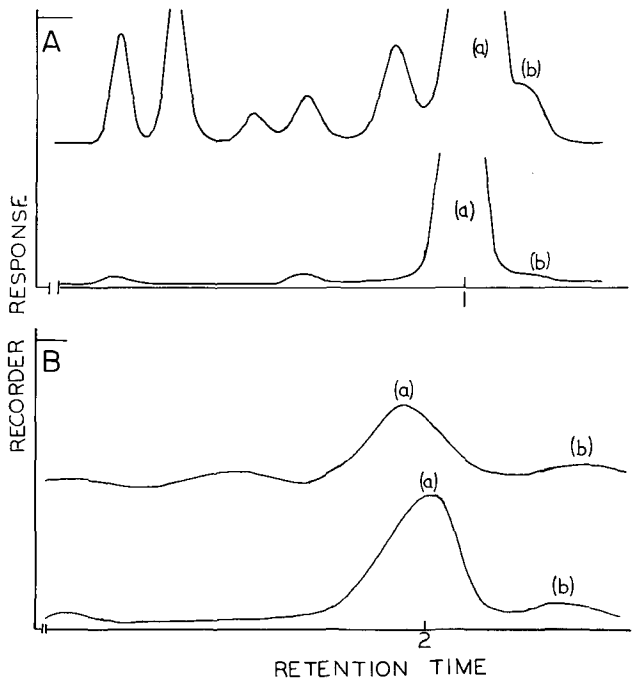

FIG. 3. GLC analyses of fractions collected as urea adducts at 0C and—15C of distilled methyl esters. A, menhaden oil C-20 unsaturated methyl esters. B, tuna oil C-22 unsaturated methyl esters, A (a) and (b) are the natural isomer and artefact, respectively, of methyl eicosapentaenoate. B (a) and (b) are the natural isomer and artefact of methyl docosahexaenoate, respectively.

lem of the separation of critical pairs. It is apparent from the results in Figure 1, A, C, and D that separations by this system of liquid-liquid partition chromatography occur on the basis of differences in polarity of the compounds. Since lengthening of the chain decreases the polarity and an increase in the number of double bonds increases the polarity, these factors compensate each other in some pairs of methyl esters (methyl oleate and methyl palmitate [Fig. 1, C]) and their separation is difficult by this method. An analogous situation exists in gas-liquid chromatography as may be demonstrated by the model mixture shown in Figure 1, B. If methyl oleate is well separated from methyl stearate in a gas-liquid partition system, methyl linolenate usually overlaps methyl arachidate. Thus a combination of conditions is usually required for a complete separation of a natural mixture by GLC. Since the reversed-phase system described here functions like a nonpolar phase in GLC and yet fractionates unsaturated methyl esters, it may be used very effectively in conjunction with GLC for the isolation and fractionation of methyl esters as illustrated by the results in Figure 1, B.

Liquid-liquid partition chromatography also combines very well with selective adsorption on silicic acid impregnated with silver nitrate for the preparation of pure methyl esters. Fractionation by the latter occurs chiefly on the basis of the number of double bonds in the molecule using petroleum ether-diethyl ether (Fig. 2).

Critical pairs which can't be resolved by liquid-liquid partition may be separated by this adsorption system (Fig. 2), since they differ in the number of double bonds they contain. Thus, through the use of a combination of these techniques, fractional distillation which frequently causes the formation of artefacts, particularly in the preparation of polyunsaturated methyl esters, may be eliminated.

Preparation of polyunsaturated methyl esters. The more common polyunsaturated methyl esters can be prepared in greater than 90% purity in fair yields by a combination of urea adduct formation, low temperature fractional crystallization and fractional distillation. Examples of the preparation of concentrates of several polyunsaturated methyl esters by a combination of gross methods of fractionation as previously described by the authors (20) is presented in Table I.

Special precautions must be taken to eliminate artefacts produced by the action of heat in 20 and 22 methyl esters containing 4, 5, and 6 double bonds. Distillation, as well as other processes which involve treatment at high temperature, should be avoided or at least used only when absolutely necessary. The duration as well as the temperature of heating during distillation are important factors in the formation of artefacts. Alteration of polyunsaturated methyl esters containing 4, 5, and 6 double bonds occurs in prolonged alembic distillations even when carried out at the highest possible vacuum and in all-glass equipment.

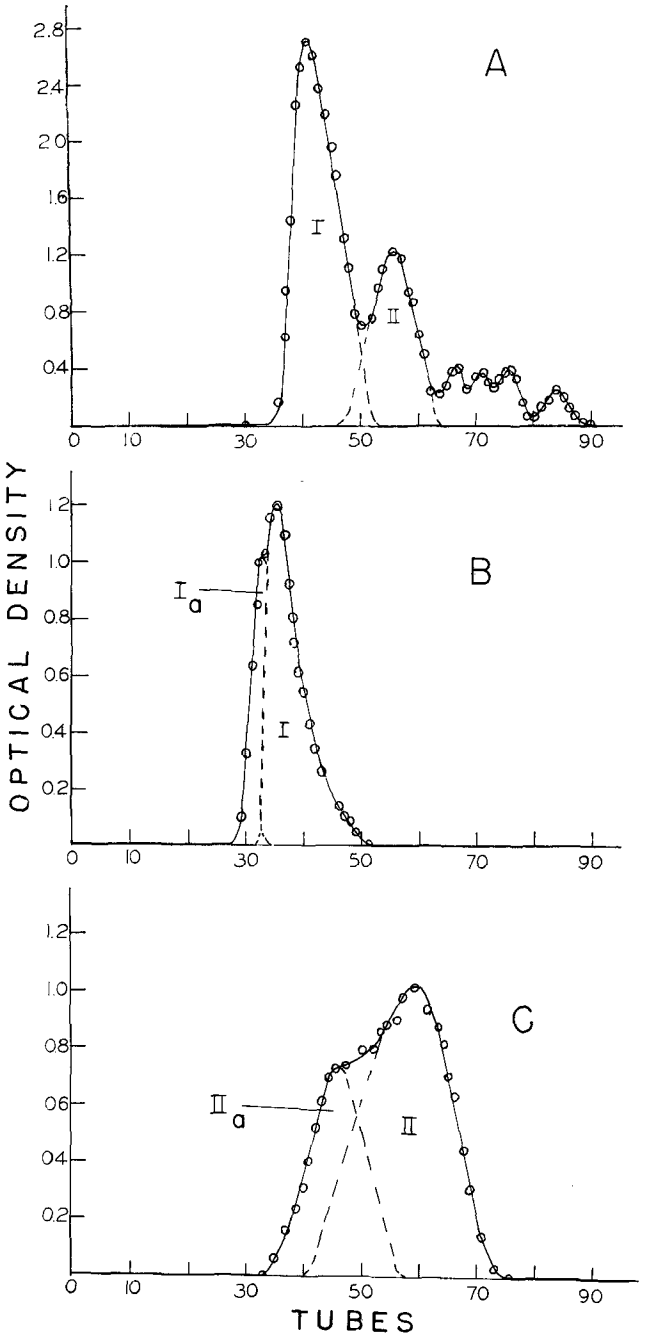

FIG. 4. Fractionation of the 22 carbon chain unsaturated methyl esters of egg oil by reversed-phase, liquid-liquid chromatography using acetonitrile-methanol (85:15 V/V) as the mobile phase and heptane as the stationary phase on a 2.5 × 120 cm column of nonwetting celite. A, I = docosahexaenoate, II = docosapentaenoate; B, I = docosahexaenoate, Ia = artefact; C, II = docosapentaenoate, IIa = artefact.

Fractional distillation may be eliminated entirely for the preparation of concentrates of methyl docosahexaenoate of >90% from tuna oil by the propitious use of low temperature fractional crystallization and urea adduct formation. When fractional

TABLE I

Methyl Ester Concentrates

Preparation	Source	Yield	Iodine value		Purity GLC	Impurities GLC
			Found	Theory		
		%			%	
Linolenate	Linseed oil	20	240.0	260.0	84.6	18:1, 18:2
Arachidonate	Pork liver fat	3	314.2	318.8	94.8	20:3, 20:5
Eicosapentaenoate	Menhaden oil	4	395.0	401.0	96.2	20:4
Docosapentaenoate	Egg lipid	0.8–1	252.5	368.4	30.0	22:6
Docosahexaenoate	Tuna oil	3	435.5	444.7	95.0	22:5

TABLE II

Purified Methyl Esters

Preparation	Iodine value		Refractive index (20C)		Position of double bonds	Diene conjugation (k233)
	Found	Theory	Found	Calculated		
Linolenate	260.0	260.4	1.4715	1.4710	Δ9,12,15	0.24
Arachidonate	318.1	318.8	1.4805	1.4803	Δ5,8,11,14	0.16
Eicosapentaenoate	401.5	401.0	1.4896	1.4898	Δ5,8,11,14,17	0.84
Docosapentaenoate	368.0	368.4	1.4901	1.4883	Δ4,7,10,13,16	0.34
Docosahexaenoate	444.8	444.6	1.4980	1.4974	Δ4,7,10,13,16,19	0.90

distillation is carried out with a spinning band column on a small amount of concentrate (<50 g) and, as fast as permitted to obtain a satisfactory fractionation, the 20 carbon chain polyunsaturated esters can be obtained in yields of about 70%, free of artefacts.

The presence of artefacts in distillates of polyunsaturated methyl esters may be detected by GLC with a polyester column (Fig. 3). The artefacts have a slightly longer retention time than the corresponding unaltered esters. Proof that A (b) and B (b) are artefacts of methyl eicosapentaenoate and docosahexaenoate respectively is readily obtained by further analysis of these fractions after repeated distillations. Since artefacts are formed during fractional distillation, distillates of polyunsaturated methyl esters are always further fractionated by urea adduct formation. Some artefacts (polymers, for example) do not form adducts with urea while others seem to form adducts more easily than the unaltered ester. Figure 3 shows that, in general, distilled artefacts are concentrated more in the fraction of urea adducts collected at 0C than at −15C. The non-urea adduct forming material, of course, remains in the final filtrate. Thus, fractions collected as adducts at −15C generally contain the purest concentrates of polyunsaturated methyl esters.

The small amounts of impurities including artefacts may be removed from the concentrates of polyunsaturated methyl esters (Table I) by liquid-liquid partition chromatography as described above. The fractionation of the 22 carbon chain fraction of egg lipid which consists of about 60% methyl docosahexaenoate and 30% docosapentaenoate is shown in Figure 4, A. These separations were carried out on 2 g samples in the 120 g column.

In this fractionation the artefacts are concentrated in the first tubes in each peak on rechromatography of the combined tubes of the two major components (Figure 4, B and C). The structures of the artefacts separated from these compounds are being determined.

Analyses of a number of methyl esters prepared by a combination of gross methods of fractionation and liquid-liquid partition chromatography are summarized in Table II.

Preparation of methyl linolenate. Liquid-liquid partition chromatography, as described above, is well suited for the preparation of pure methyl linolenate directly from linseed oil esters. The preparation of

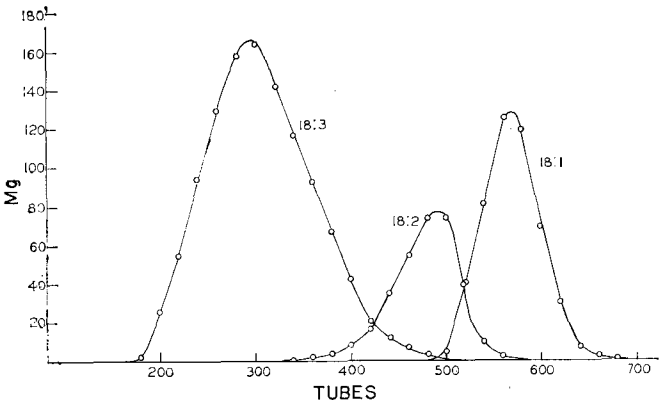

FIG. 5. Fractionation of 50 g of fractionally distilled linseed oil methyl esters by reversed-phase, liquid-liquid chromatography using acetonitrile-methanol (80:15 V/V) as the mobile phase and heptane as the stationary phase on a 10 × 150 cm column of nonwetting celite.

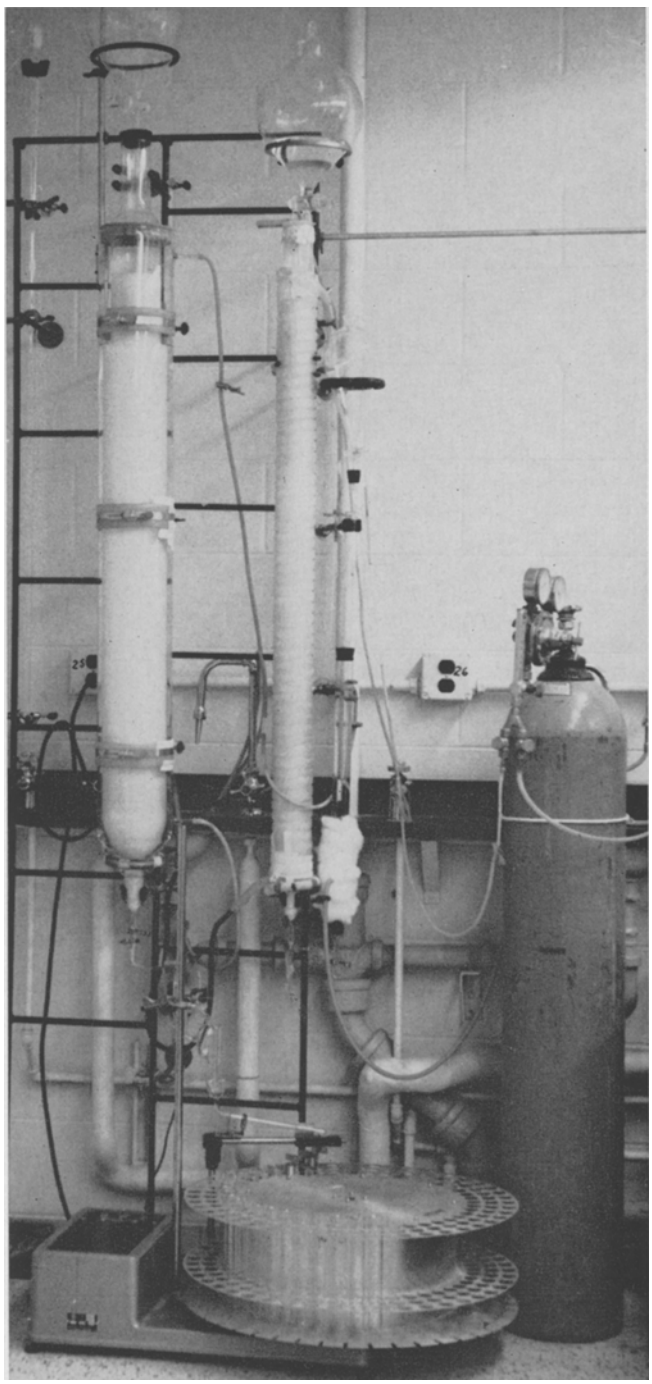

FIG. 6. Photograph of preparative columns for liquid-liquid partition chromatography. Column A (on the left) is 10 × 150 cm and contains 3300 g of support. Column B (on the right) is 15.5 × 125 cm and contains 1000 g of support.

methyl linolenate from 50 g of fractionally distilled linseed esters on a large 10 × 150 cm column is illustrated in Figure 5. There was some overlapping of the components in this experiment. Nevertheless, 83% of the methyl linolenate was separated in pure form.

Figure 6 shows a photograph of two preparative columns for liquid-liquid partition chromatography. The results in Figure 5 were obtained on the larger column. It is the column described above containing 3300 g of supporting material. The other column in Figure 6 which is cooled by circulating water through the rubber tubing wrapped around it contains 1000 g of nonwetting celite. Ten g of esters can be fractionated on this column with the same efficiency as 2 g on the small column as described above. This column was only used in the stepwise development of conditions for the larger column. It is expected that further refinements in conditions will give improved efficiency and permit the fractionation of larger samples as well as the use of larger columns than that shown in Figure 6.

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Glycidyl Esters. IV.[1] Hydration of Glycidyl Stearate

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Abstract

The acid-catalyzed conversion of glycidyl stearate to monostearin has been investigated. By use of an essentially nonaqueous reaction medium and a two-step procedure, high yields of monostearin were obtained at 15–20C in brief reaction periods, while avoiding ester hydrolysis. Distearin was found to be a by-product. The influence of solvent, reaction temperature, and type of acid catalyst upon the relative amounts of monostearin and distearin produced were studied. A possible reaction path leading to the formation of distearin is proposed.

Introduction

DURING THE COURSE of our investigations into the synthesis and reactions of glycidyl esters of fat-derived acids, a practical method of converting these esters to the corresponding glycerides was desired. We chose to concentrate our efforts upon studying the hydration of a model compound, namely glycidyl stearate. Beyond general statements to the effect that such hydrations are feasible (1,2), we could find little evidence that this problem has been studied previously in any detail. On the other hand, there is considerable wealth of published information concerning the hydration of oxirane compounds which do not contain ester functions. Much of this work has been summarized capably and evaluated critically by Parker and Isaacs (3).

The commonly used method of converting epoxides to vicinal glycols, i.e., prolonged reflux in an aqueous acid medium, was unsuitable to the accomplishment of our purposes, both because glycidyl stearate is quite insoluble in hot water and because the hot aqueous acid would tend to hydrolyze the ester function which we wished to preserve. Since acid catalyzed hydration of epoxides and hydrolysis of esters are competing reactions which proceed along rather analogous reaction paths, it was necessary to find conditions which would favor hydration over hydrolysis.

The desired degree of selectivity was achieved by performing the reaction in a two-step sequence. Glycidyl stearate was dissolved in a nonaqueous, but water-miscible solvent, and sufficient acid to protonate the oxirane function was added at room temperature or below. Water was then added, and, after sufficient time was allowed for hydration, the reaction was stopped by pouring the mixture into benzene. In most runs a period of 5–10 min for each step was sufficient to effect a complete disappearance of epoxide, usually without formation of detectable amounts of stearic acid.

Nishiyama et al. (4) reported the hydration of esters of epoxidized fatty acids both in heterogeneous and in homogeneous (acetone) media. In a certain general way our work parallels and complements

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