Triglyceride Separation by Reverse Phase High Performance Liquid Chromatography[1]

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ABSTRACT AND SUMMARY

Rapid separations of triglycerides by chain length and degree of unsaturation were made by high performance liquid chromatography (HPLC) on a C-18 μ-Bondapak column with acetonitrile-acetone solvent mixtures. For saturated triglycerides, a linear relationship was observed between the carbon number and the log of the retention volume. Each double bond present in the triglyceride decreased the retention volume to approximately that of a saturated triglyceride with two carbon atoms less. Correlations of the fatty acid composition, as determined by gas liquid chromatography (GLC) with the HPLC data, provides much additional insight about triglyceride composition. To calculate triglyceride compositions, an internal standard tripentadecanoin was added to collected fractions before analysis by GLC.

INTRODUCTION

Analysis of the composition of triglyceride oils requires methods of separating their complex mixtures (of triglycerides) into individual components or at least into simpler mixtures containing only a few triglycerides each. Gas chromatography (GC) is not completely satisfactory because it is only able to separate triglycerides by chain length (1). Classically, urea adducts or low-temperature crystallization were used to separate triglycerides by degree of unsaturation (2). Because these procedures do not lead to clear-cut separations, often the investigator is faced with the problem of having to analyze three or four fractions which are nearly as complex as the starting material. The use of Ag^+ ion chromatography, although routinely used for the separation of fatty acid methyl esters, is difficult to apply to complicated natural fat mixtures. The use of countercurrent distribution (3) and reverse phase partition chromatography (4) has been reported for the separation of triglyceride mixtures. The chief disadvantage of these techniques is that they are tedious and time consuming. Recently, several papers have appeared showing rapid separations of fatty acid esters by high performance liquid chromatography (HPLC) with reverse phase packings containing octadecyl silane groups chemically bonded to a permanent stationary support (5-7). Columns of high efficiency have become available with developments in the technology of packing columns with micro-particulate material of uniform size. The good separations obtained with fatty ester derivatives suggested the applicability of these columns to analysis of triglycerides. We recently applied HPLC, on reverse phase columns, to analysis of the liquid wax esters found in jojoba oil (8). During the course of that work we found that HPLC was applicable also to analysis of natural triglyceride mixtures.

EXPERIMENTAL PROCEDURES

Chromatograms were obtained with a Waters Associates ALC-201 liquid chromatograph with a 2 ft x 1/4 in. OD stainless steel C_{18} μ-Bondapak column. Samples were injected in $CHCl_3$ solution by means of a U6K septumless

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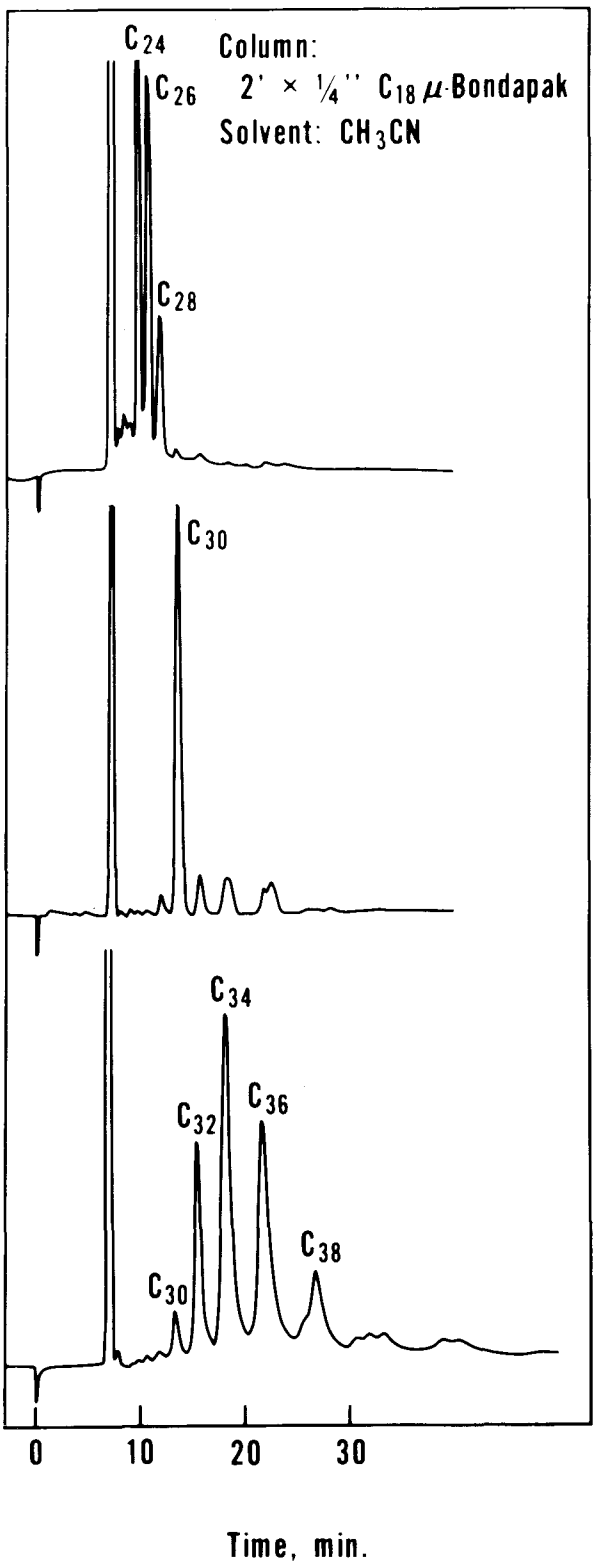

FIG. 1. HPLC of three different *Cuphea* seed oils on a 2 ft x 1/4 in. C_{18} μ-Bondapak column eluted with acetonitrile at 1 ml/min.

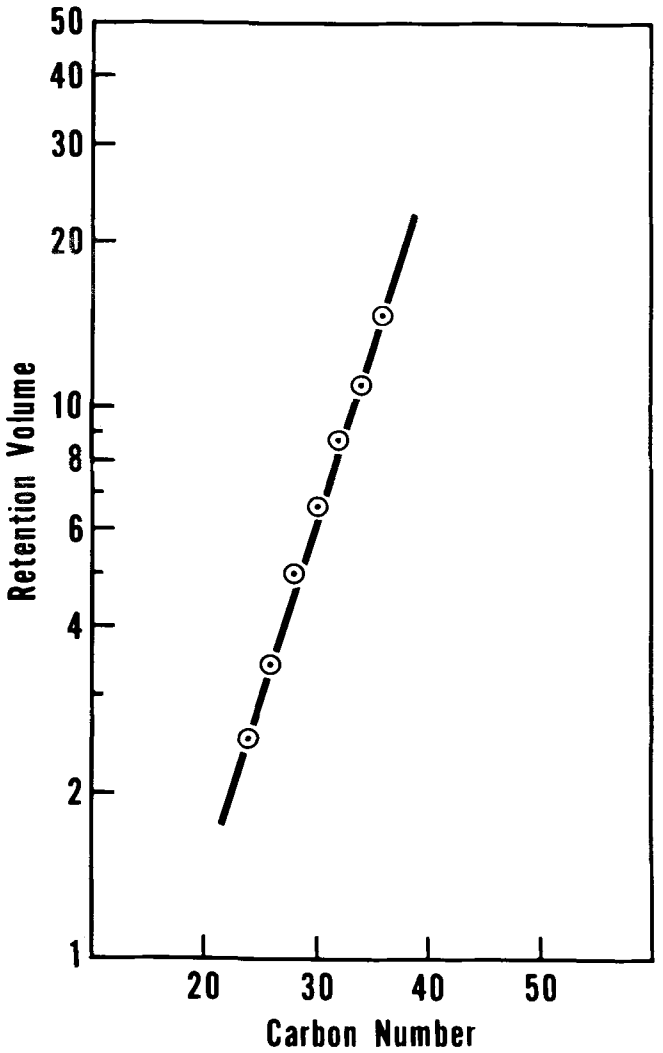

FIG. 2. A plot of retention volume vs. carbon number for the medium chain length saturated triglycerides (C_{24} to C_{38}) from *Cuphea* seed oils analyzed by HPLC on a 2 ft x 1/4 in. C_{18} μ-Bondapak column eluted with acetonitrile at 1 ml/min.

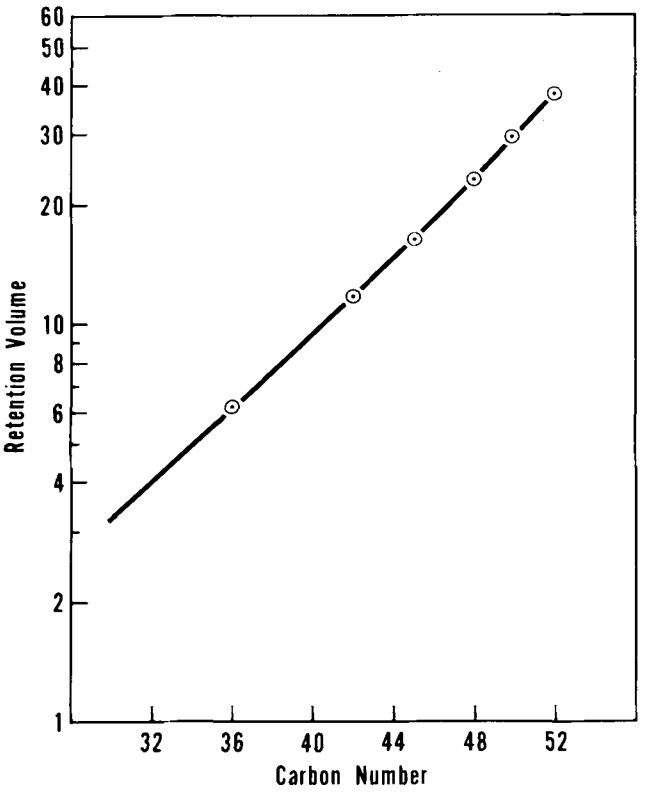

FIG. 3. A plot of retention volume vs. carbon number for saturated triglycerides (C_{36}-C_{52}) analyzed by HPLC on a 2 ft x 1/4 in. C_{18} μ-Bondapak eluted with acetonitrile-acetone (2:1, v/v) at 1 ml/min.

loop injector. The Waters differential refractometer was used as a detector. Samples were run isocratically using mixtures of methanol-water or acetonitrile-acetone. A flow rate of 1-2 ml/min was used, and the solvent system was chosen such that all compounds were eluted within 20 column volumes (ca. 60 ml). All retention volumes were corrected by subtracting the column void volume.

GLC analyses of triglycerides were made with a 3 ft x 1/8 in. stainless steel column operated isothermally at 320 C. Methyl esters were made from triglycerides by adding 0.4N sodium methoxide in methanol in a disposable 5-dram vial and heating to 50 C for 10-15 min. GLC analyses of the methyl esters were made on a 10 ft x 1/4 in. glass column packed with 5% LAC-2-R446 and on a 6 ft x 1/4 in. glass column packed with 5% Apiezon L.

Tripentadecanoin was added to samples as an internal standard in GLC of the triglycerides and esters.

RESULTS AND DISCUSSION

HPLC chromatograms from the seed oils of three *Cuphea* species with acetonitrile as the elution solvent are presented in Figure 1. The three *Cuphea* species, *C. painteri* rich in C_8 acids, *C. llevea* rich in C_{10} acids, and *C. carthagenesis* rich in C_{12} acids (9) contained saturated triglycerides from C_{24} to C_{38}. A plot of carbon number vs. log elution volume (Fig. 2) shows a linear relationship similar to that found for methyl esters in HPLC reported by Scholfield (7) and triglycerides in reverse phase partition chromatography reported by Nickell and Privett (4). The linear relationship between carbon number and log retention volume for saturated C_{42}, C_{48}, C_{50}, and C_{52} triglycerides was observed when acetonitrile-acetone (2:1, v/v) is used as the eluting solvent (Fig. 3). Figure 4 shows the linear relationship observed between the number of double bonds vs. log retention volume for C_{54} triglycerides with between one and eight double bonds. Adding double bonds to the triglycerides decreases the retention volume, each double bond being almost exactly equivalent to a reduction of two methylene units in the molecule when acetonitrile is used in the solvent. So far, no separation has been achieved between tripalmitin and triolein or triglycerides with mixtures of palmitoyl and oleoyl groups by means of acetonitrile-acetone solvent systems. This observation is analogous to our experiences with methyl palmitate and methyl oleate which also do not separate on C_{18} μ-Bondapak columns when eluted with acetonitrile. However, if methanol instead of acetonitrile is used as a solvent, methyl palmitate elutes slightly before methyl oleate. Likewise, methanol-containing solvents give partial separation between tripalmitin and triolein. With acetonitrile solvent systems, there is baseline separation of triglycerides differing by only one double bond or by two carbon atoms. With methanol solvent systems there is further separation of triglycerides within each class, and the chromatogram becomes complex and difficult to interpret. Therefore, methanol-containing solvents were not investigated further.

Figures 5 and 6 show typical HPLC curves for some common seed oils. Coconut oil contains predominantly shorter chain (C_8, C_{10}, C_{12}, and C_{14}) saturated acyl groups with about 8% palmitoyl and 2% oleoyl and 4% linoleoyl groups. The eight major peaks represent saturated triglycerides with C_{30}, C_{32}, C_{34}, C_{36}, C_{38}, C_{40}, C_{42}, and

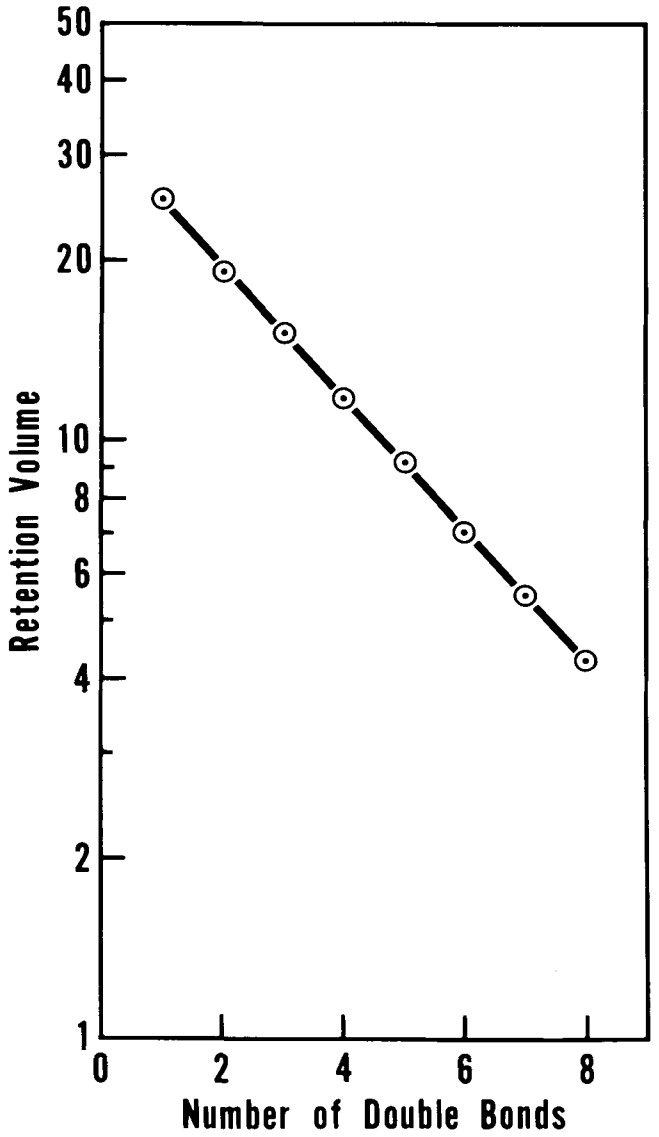

FIG. 4. A plot of retention volume vs. number of double bonds for C_{54} triglycerides with one to eight double bonds analyzed by HPLC on a 2 ft x 1/4 in. C_{18} μ-Bondapak column elutes with acetonitrile-acetone (2:1, v/v) at 1 ml/min.

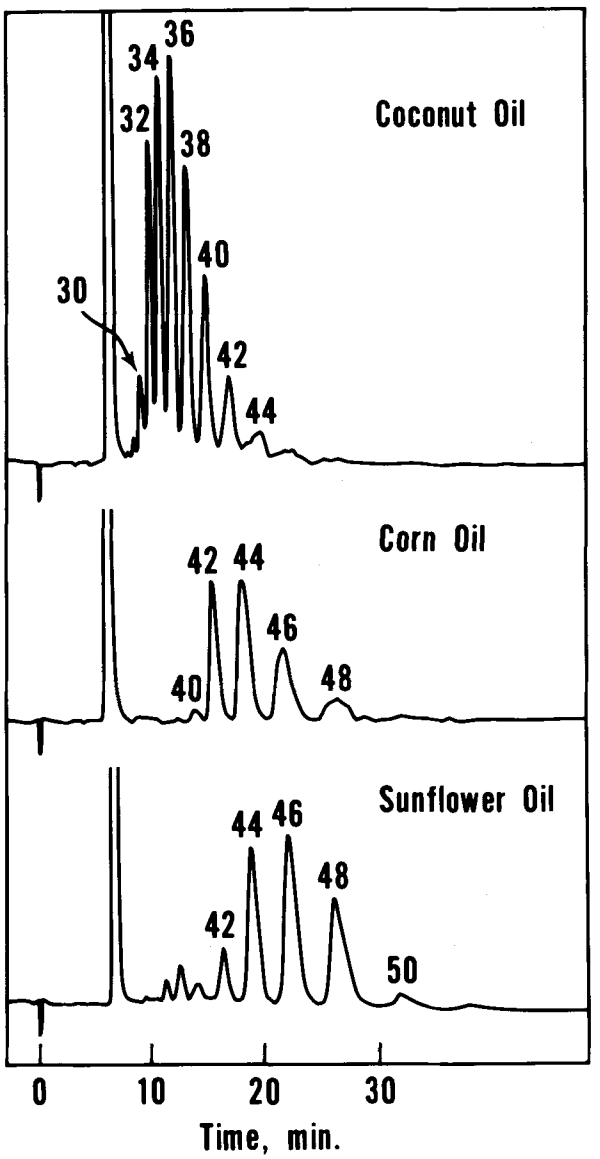

FIG. 5. HPLC of three common seed oils.

C_{44}. Triglycerides containing the oleoyl and linoleoyl acyl groups behave as if they were triglycerides two and four carbon atoms smaller. Therefore, some of the peaks can contain mixtures of smaller saturated triglycerides and larger unsaturated ones. Extending the concept of equivalent chain length (10) to HPLC is convenient to help identify the possible components of a HPLC peak of given retention volume. Saturated triglycerides are assigned equivalent carbon numbers (ECN) equal to their carbon number. Each double bond present in the triglyceride molecule reduces the ECN by two. The remaining seed oils in Figures 5 and 6 have mainly C_{54} and C_{52} triglycerides containing from two to nine double bonds. The relative degree of unsaturation in the seed oil is evident from the ECNs of the major peaks.

When hydroxy or epoxy functional groups were present in the triglyceride, they decreased the retention volume dramatically. Figure 7 shows HPLC of *Vernonia anthelmintica* and *Euphorbia lagascae* seed oils. Both seed oils contain about 60% vernolic acid (12,13 epoxy-*cis*-9-octadecanoate). Most of the vernoyl acyl groups are specifically found as trivernolin in *Vernonia anthelmintica* (11) while they are more randomly distributed in *Euphorbia lagascae* (12). This is evident from the HPLC of these seed oils. Since the trivernolin peak elutes quite rapidly compared to normal triglycerides, it can easily be quantitated in an oil. This subject will be discussed in a subsequent publication.

Figure 8 shows the HPLC of castor oil and *Linum macronatum* seed oil. The castor oil contains ~85% ricinoleic (12 hydroxy-*cis*-9-octadecanoate) acyl groups which are present mainly as triricinolein while the *Linum* seed oil contains about 18% ricinoleic acyl groups found in monoricinoloyl triglycerides with longer retention volumes eluting slightly before the normal triglycerides in the oil. When triple bonds and cyclopropenes are present in an oil, they have about the same effect as two double bonds.

One problem in the use of HPLC for triglyceride analysis is our inability to successfully chromatograph tristearin or longer chain fully saturated triglycerides. Retention volumes can be decreased by adding acetone to the solvent system, but tristearin apparently is not soluble enough in the acetonitrile-acetone mixtures to be moved down the column. Also, tristearin precipitated in our loop injector where the chloroform solution we injected came into contact with the eluting solvents. Some peak tailing was observed for the other triglycerides examined when the chloroform solutions were too concentrated (>~50%

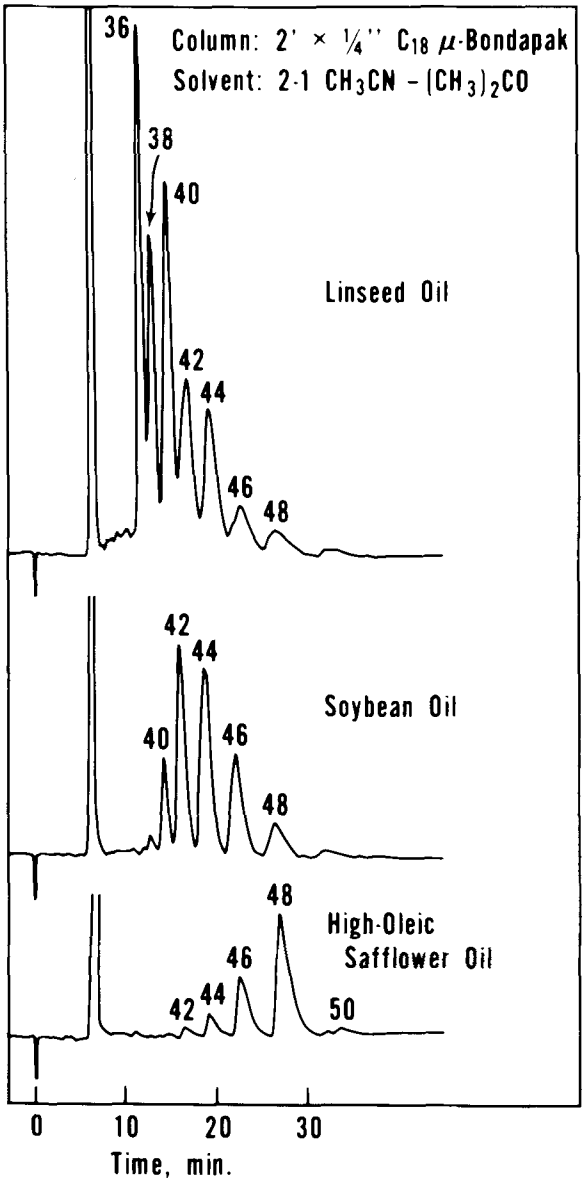

FIG. 6. HPLC of three common seed oils.

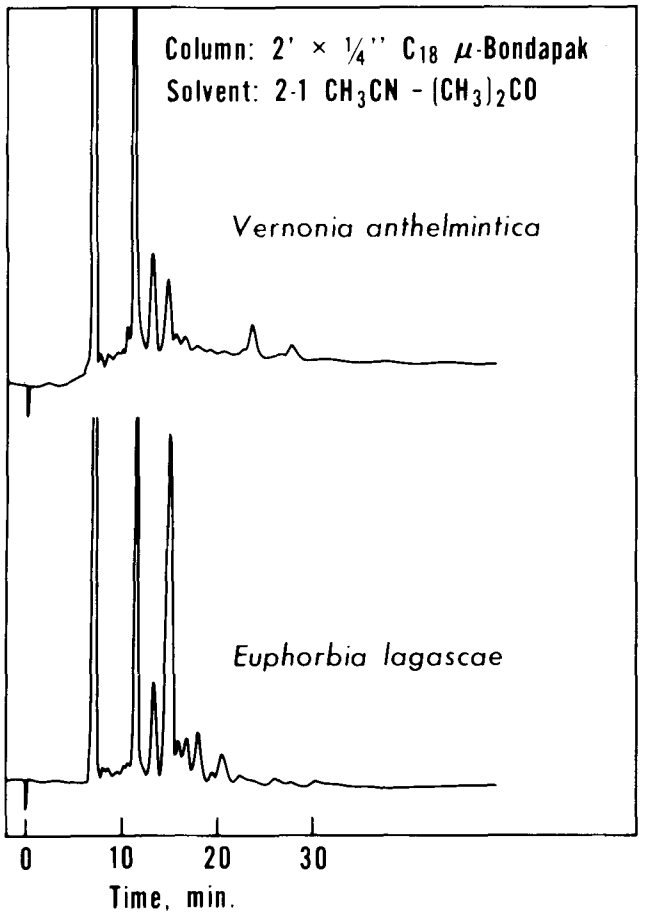

FIG. 7. HPLC of two high epoxy seed oils.

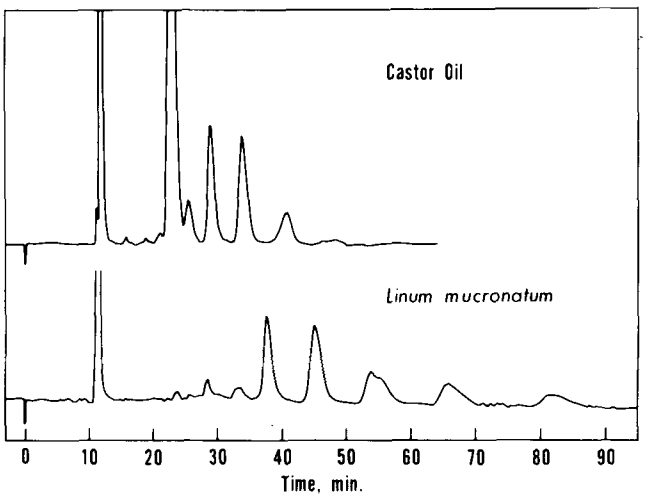

FIG. 8. HPLC of two seed oils which contain ricinoloyl acyl groups.

sample in $CHCl_3$). This was attributed to solubility and viscosity problems in the injector loop.

To utilize HPLC for the examination of natural triglyceride mixtures, one must recognize that each peak can be due to a mixture of many different triglycerides, and the HPLC chromatogram alone is often not enough to characterize the composition of a sample completely. If the acid composition of the sample is known, HPLC can be used to infer the probable triglyceride composition. More importantly, the sample size necessary for good refractive index response is relatively large, and it is practical to collect the components represented by the HPLC peaks, transesterify them, and analyze the esters by GLC, thereby providing more information about the triglyceride composition of each fraction.

Figure 9 shows the HPLC chromatogram obtained from 2 mg (in 5 μl $CHCl_3$) of soybean oil. The seven peaks represent C_{54} triglycerides with from eight to two total double bonds, C_{52} triglycerides with six to one total double bonds, and C_{50} triglycerides with two to zero total double bonds. Since the peaks represent several triglycerides with different refractive index responses, the weight represented by each peak was calculated by an internal standard procedure. Fractions represented by the peaks were collected and 50 μg of tripentadecoin was added to each fraction. GLC analyses were made for the triglycerides and the methyl esters from transesterification. The area of the $C_{15.0}$ ester was used to calculate the weight % for each fraction. Exact compositions of the triglycerides could be worked out for the first three fractions. However, fractions 4-7 had some triglycerides which could contain two (or three) palmitoyl and/or oleoyl groups per molecule, and the necessary independent equations are not available to solve for all the possible triglycerides in these fractions. For example, fraction 4 contains C_{54} triglycerides with five

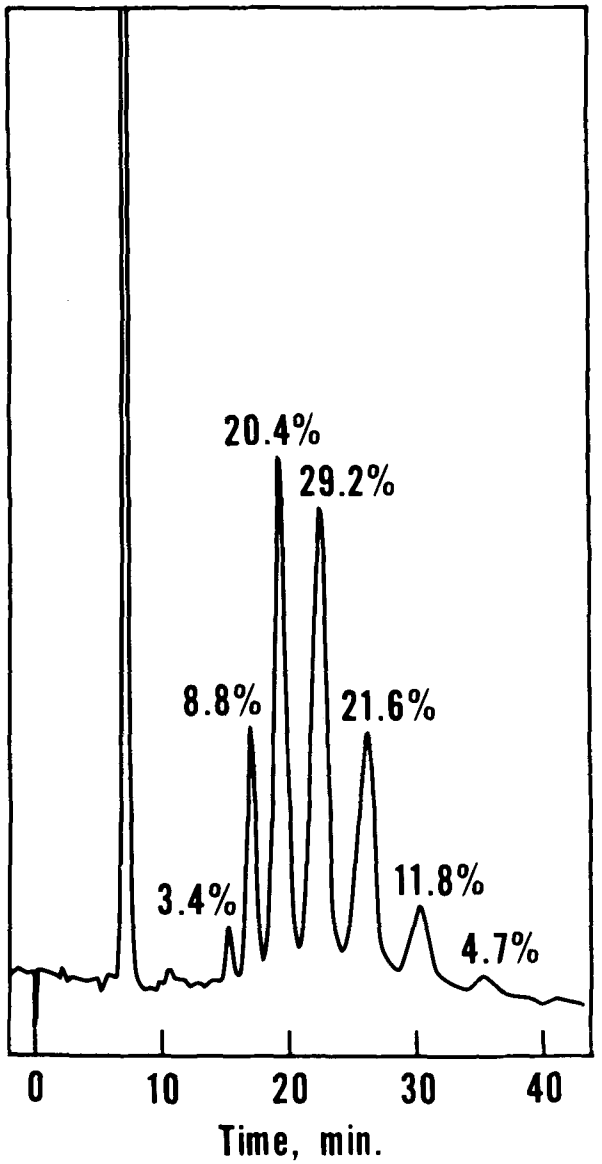

FIG. 9. HPLC of soybean oil on a 2 ft x 1/4 in. C_{18} μ-Bondapak column eluted with acetonitrile-acetone (2:1, v/v). Peaks were detected with a differential refractometer at 8X. Quantitation of fractions was determined by GLC of collected material after addition of tripentadecanoin for an internal standard.

double bonds (OLL, OOLn, and SLLn)[2] and C_{52} triglycerides with four double bonds (PLL and POLn). No C_{50} triglycerides (PPLn) were detected by GLC of the fraction. Therefore, from GC analysis of the esters, the following equations can represent fraction triglycerides:

$$3 \times C16.0 = PLL + POLn \quad (I)$$
$$3 \times C18.0 = SLLn \quad (II)$$
$$3 \times C18.1 = OLL + 2OOLn + POLn \quad (III)$$
$$3 \times C18.2 = 2OLL + 2PLL \quad (IV)$$
$$3 \times C18.3 = POLn + OOLn + SLLn \quad (V)$$

These are five equations in five unknowns; however, they are not all independent equations. The concentration of SLLn is obtained from equation II and can be dropped from equation V. The remaining four equations are not solvable because equation V is now also contained in equation III. Thus, the total concentration of PLL and POLn (equation I) could be obtained as well as the sum of OLL and OOLn (equations V-III), of PLL and OLL (equation IV), and of POLn and OOLn (equation V), but one cannot quantitate them individually. By separating these fractions into C_{50}, C_{52}, and C_{54} triglycerides, complete quantitation of all triglycerides in the oil is possible.

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[2] P = palmitoyl, S = stearoyl, O = oleyol, L = linoyl, Ln = linoenoyl.