METHODS

Analysis of Triglyceride Species by High-Performance Liquid Chromatography Via a Flame Ionization Detector

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ABSTRACT

The analysis of triglyceride species by high performance liquid chromatography (HPLC) with a flame ionization detector (FID) and reversed-phase chromatography using chemically bonded octadecyl silane (ODS) Zorbax columns and gradient or isocratic solvent elution with methylene chloride/acetonitrile is described. Triglycerides containing acyl groups of critical pairs, *trans* and positional isomers, as well as mixtures of even and odd chain lengths are separated. Identification of triglycerides is made on the basis of retention times compared with equivalent and theoretical carbon numbers, and comparison with chromatograms of reference triglyceride mixtures. The methodology is demonstrated by fractionizing the triglycerides of olive oil under different chromatographic conditions using single and coupled conventional 250 × 4.6 mm columns and a short 80 × 6.2 mm column for fast separations.

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INTRODUCTION

The application of reversed-phase liquid chromatography to glyceride species was demonstrated by Nickell and Privett (1) with the separation of triglycerides differing by one methylene group, one double bond or a *trans* unsaturated acyl group by a gravity flow procedure in which fractions were collected for analysis by gas liquid chromatography (GLC). When continuous detectors of the refractive index type became available for liquid chromatography, Pei et al. (2) developed a fast, simple procedure for the separation of simple saturated triglycerides by modern high performance liquid chromatography (HPLC) using a reversed-phase system of chemically bonded octadecyl silane (ODS)-silica as the stationary phase.

In an extension of this technique, Plattner and colleagues (3,4) demonstrated the separation of triglyceride species on the basis of chain length and unsaturation and obtained baseline separation of critical pairs of triglycerides containing oleic and palmitic acid by the addition of silver nitrate to the solvent system. These investigators suggested the separation of triolein and tripalmitin as a guide for determining column efficiency and estimated that ODS columns of ca. 15,000 plates should be adequate for the separation of the triglyceride species of these acids other than isomers, which require enzymatic techniques for an analysis. El-Hamdy and Perkins (5) and Perkins et al. (6) obtained similar types of separations directly with ODS columns. They developed the concept of theoretical carbon number (TCN) to define species separation of unsaturated triglycerides. The TCN is a very useful concept for the identification of unsaturated triglyceride species and assists in identifying triglycerides in this work.

In the studies by El-Hamdy and Perkins (5), standard 5 μ ODS columns of ca. 250 × 4.6 mm were used. These columns and the solvent systems employed were geared to an elution time of ca. 30 min. Recently Dong and DiCesare (7) demonstrated that similar separations could be made in about one-half the time with shorter (100 × 4.6 mm) columns packed with 3 μ ODS particles. In the abovementioned studies, a refractive index or UV detector with isocratic elution was used. In earlier work (8-12), we demonstrated the separation of the lipid classes by HPLC using gradient elution systems with a flame ionization detector (FID). In the present study, the separation of reference mixtures of triglycerides by HPLC using reversed-phase chromatography with ODS columns and gradient or isocratic elution is demonstrated and applied to olive oil under different chromatographic conditions.

MATERIALS AND METHODS

Reference Triglyceride Mixtures

The following reference mixtures of highly purified >99% triglycerides were obtained from Nu-Chek Prep, Inc., Elysian, MN.

HPLC #G-1. This mixture consists of the following pure glycerol triesters in the ratio 3:2:1 (even glyceride carbon #:odd glyceride carbon

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#:unsaturated glyceride): tricaprylin (24:0), trinonanoin (27:0), tricaprin (30:0), triundecanoin (33:0), trilaurin (36:0), tritridecanoin (39:0), trimyristin (42:0), tripentadecanoin (45:0), tripalmitin (48:0), tripalmitolein (43:3Δ9), trihepatadecanoin (51:0), tristearin (54:0), triolein (54:3Δ9), trilinolein (54:6Δ9,12) and trilinolenin (54:9Δ9,12,15).

HPLC #G-3. A mixture of triolein (54:3Δ9) and tripetroselinin (54:3Δ6) of equal weight.

HPLC Interesterified Test Mixtures. These mixtures consist of the following triglyceride molecular species.

Mixture A: $P_3 - P_2O_1 - P_1O_2 - O_3$ where P = palmitate and O = oleate.

Mixture B: $P_3 - P_2Pa_1 - P_1Pa_2 - Pa_3$ where P = palmitate and Pa = palmitoleate.

Mixture C: $La_3 - La_2Ln_1 - La_1Ln_2 - Ln_3$ where La = laurate and Ln = linolenate.

Mixture D: $M_3 - M_2L_1 - M_1L_2 - L_3$ where M = myristate and L = linoleate.

Mixture E: $P_3 - P_2H_1 - P_1H_2 - H_3$ where P = palmitate and H = heptadecanoate.

Mixture F: $P_3 - P_2O_1 - P_1O_2 - O_3 - P_2L_1 - POL - O_2L_1 - P_1L_2 - O_1L_2 - L_3$ where P = palmitate, O = oleate and L = linoleate.

Mixture G: $O_3 - O_2E_1 - O_1E_2 - E_3$ where O = oleate (18:1 Δ9-*cis*) and E = elaidate (18:1 Δ9-*trans*).

Pure olive oil was obtained from Dr. Eduardo Vioque of the Institute de La Grasa Sus Derivados (C.S.I.C.), Sevilla, Spain.

HPLC

HPLC was carried out with a Spectra Physics Model 3500 B liquid chromatograph equipped with a FID of our own design (13). Three ODS Zorbax columns, obtained from E. I. Dupont De Nemours and Company, were used in this work. Columns I and II were 250 x 4.6 mm and varied in their porosity and carbon content, the first having ca. 15% and the second ca. 6%. The third column (III) was a Golden Series Zorbax ODS column, 60 x 6.2 mm with a 3 μ particle size packing. Peak areas were automatically recorded as previously described (11).

Methyl esters were prepared by interesterification with methanol using the method of Christie (14). A Hewlett Packard Model 5840A gas chromatograph equipped with a 12′ x 0.125″ o.d. column of 10% Silar 10 C (Applied Science, State College, PA) on 100-200 mesh Gas Chrom Q was used. The column temperature was programmed from 200 to 225 C at 2 C/min with a helium flow rate of 10 ml/min.

Solvents

Methylene chloride was a reagent grade purchased by the University of Minnesota from local suppliers and was purified by a preliminary distillation followed by shaking it in a separatory funnel with concentrated sulfuric acid several times, then with dilute sodium carbonate and finally with water. The washed solvent was dried over calcium chloride and redistilled in an all-glass still.

Acetonitrile was a reagent grade obtained from Fisher Scientific Co., Fairlawn, NJ. It was fractionally distilled through a 2-meter Hyper-Cal Podbielniak column at a reflux ratio of 20:1, or mixed with phosphorous pentoxide and, after several days, distilled in an all-glass still at ca. 10 C under reduced pressure. Spectral-grade purity of acetonitrile was generally not required, but the above procedure was performed to remove any nonvolatile contaminants and to ensure a uniform solvent from batch to batch.

The chromatography was carried out with a linear gradient elution program starting with various concentrations, generally 20% by vol, of methylene chloride in acetonitrile, in which the concentration of methylene chloride was increased until all the components were eluted, or in an isocratic solvent system of different concentrations of methylene chloride in acetonitrile.

RESULTS

Chromatograms of the separation of reference mixture HPLC #G-1 of pure triglycerides by gradient and isocratic solvent systems are shown in Figures 1A and 1B, respectively. The analysis in Figure 1A was obtained with ODS Zorbax column I and elution with a linear gradient solvent system of acetonitrile and methylene chloride. The lowest chain length species of the saturates were eluted first with the highest concentration of acetonitrile. With the addition of more methylene chloride, the longer chain length saturated species were eluted. The unsaturated triglyceride species were eluted in reverse order depending on their degree of unsaturation, i.e. the more unsaturated species were eluted faster than those of lower unsaturation. Figure 1A also shows that the triglycerides of this mixture are separated with baseline efficiency using a gradient solvent system. The peaks are sharp and little band spreading occurs over the entire range of the mixture. The gradient and flow rate, as well as the column, were selected for their general applicability as well as for high resolution. Triglyceride species less polar than 54:0 can also be analyzed by allowing the gradient to go to a higher concentration of methylene chloride. This mixture also contains several

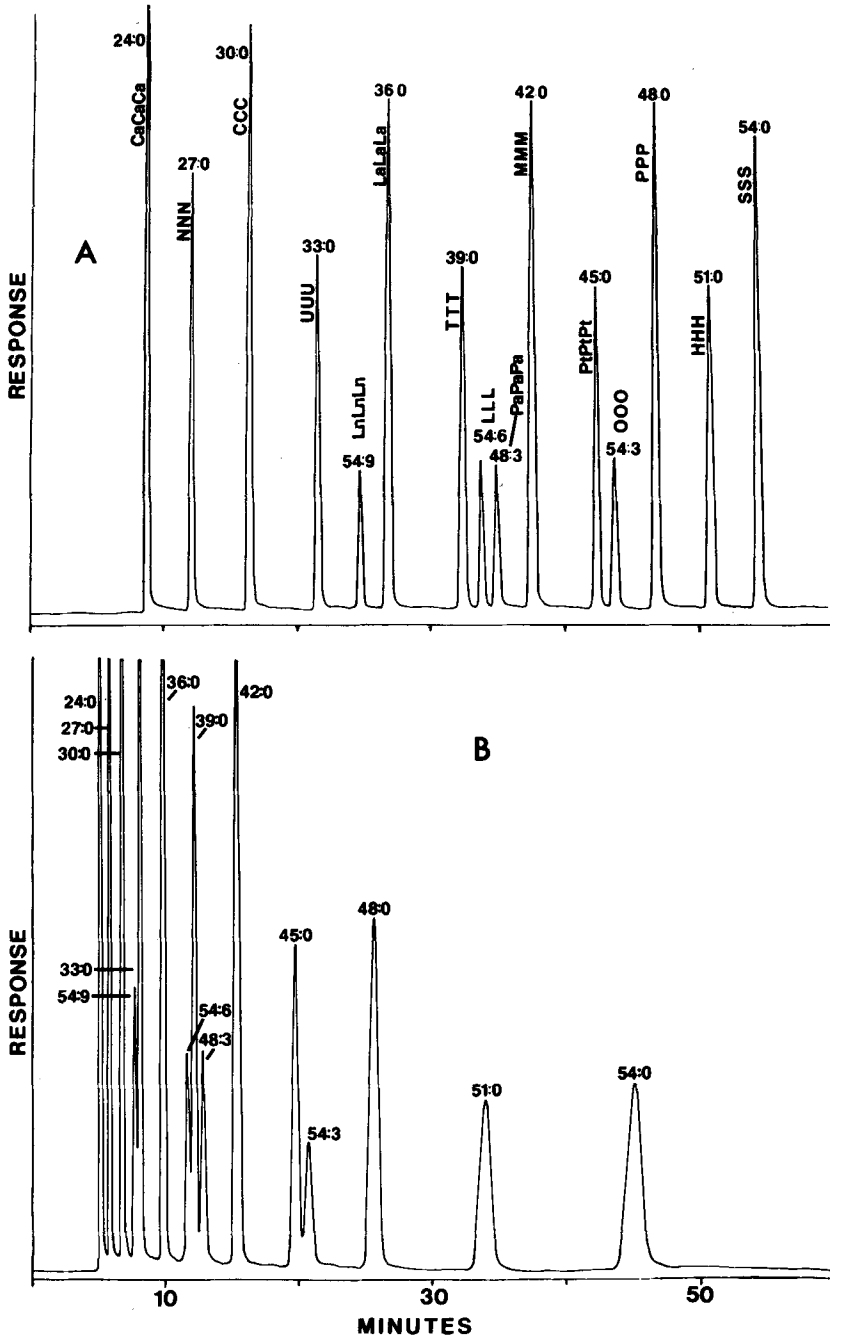

FIG. 1. Separation of triglyceride mixture HPLC #G-1; Column–250 X 4.6 mm, 5 μm, Zorbax C_{18} ODS with 15% carbon content (Column I); A) mobile phase–60 min linear gradient from 15 to 55% methylene chloride in acetonitrile; flow rate–0.8 ml per min; sample size–40 μg; detector–flame ionization. B) Isocratic elution with 45% methylene chloride in acetonitrile. Peaks: number before colon = number of carbon atoms in acyl chains, number after colon = number of double bonds in acyl chains. CaCaCa = tricaprylin, NNN = trinonanoin, CCC = tricaprin, UUU = triundecanoin, LnLnLn = trilinolenin, LaLaLa = trilaurin, TTT = tritridecanoin, LLL = trilinolein, PaPaPa = tripalmitolein, MMM = trimyristin, PtPtPt = tripentadecanoin, OOO = triolein, PPP = tripalmitin, HHH = triheptadecanoin, SSS = tristearin.

groups of critical pairs, 54:9/36:0; 54:6/48:3/42:0 and 54:3/48:0, making it a good overall mixture for testing the efficiency of ODS columns.

For comparison, Figure 1B shows the best separation of the components of mixture HPLC #G-1 that could be obtained isocratically with methylene chloride and acetonitrile. The initial peaks are sharp but loss of resolution, namely between 54:9-33:0, 54:6-39:0-48:3, and 45:0-54:3, and band spreading occur with the increase in retention time.

The log-linear plots of the retention time-equivalent carbon number (ECN) of the simple saturated triglycerides of the separation of the components of HPLC #G-1 by both the gradient (curve A) and isocratic (curve B) solvent systems are shown in Figure 2. The isocratic system generally gives a linear relationship (3-5) in a log-linear plot. Gradient solvent systems are like GC with temperature programming and cannot be expected to give a linear relationship in a log-linear plot. The curve shown in Figure 2 is highly reproducible and can be used just as well as a linear plot. Using this relationship, the constants for the calculation of TCN values for triglycerides containing 1, 2 and 3 double-bond acyl groups [oleate (0.68), linoleate (0.73) and linolenate (0.39)] were determined. The application of these constants to the determination of TCN values of the triglyceride species identified in olive oil, reference mixture F (Fig. 3) and other standards, is shown in Table 1. Although the TCN value is an empirical determination and is influenced by acyl group composition, the experimental and calculated values agree sufficiently

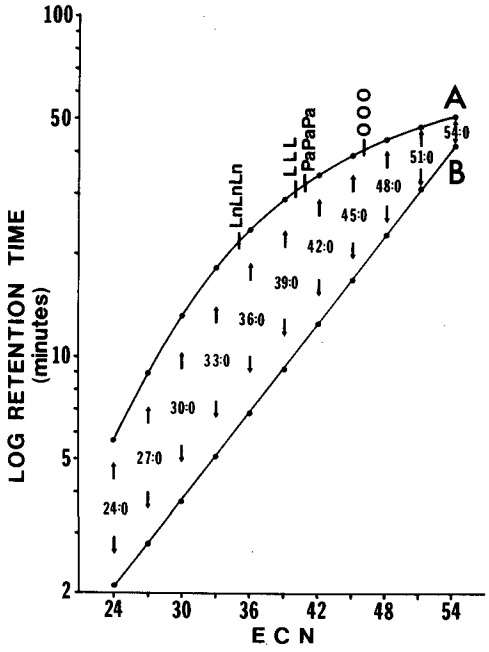

FIG. 2. Relationship of the log-linear plot of the ECN of the simple saturated triglycerides and retention time; Curve A = gradient elution, Curve B = isocratic elution. Identifications: number before colon = number of carbon atoms in acyl chains, number after colon = number of double bonds in acyl chains.

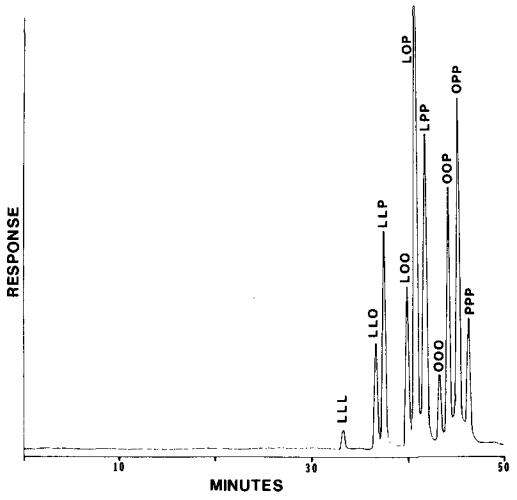

FIG. 3. Chromatogram of interesterification mixture F under the same conditions as in Figure 1A. P = palmitate, O = oleate and L = linoleate (the order of the designations does not indicate the separation of positional isomers).

TABLE 1

TCN Values of Triglyceride Species

Molecular species	Calculated*	Found
LLO	41.8	41.8
LnOO	42.2	42.1
LLP	42.5	42.3
LnOP	42.9	42.7
LOO	43.9	43.9
OOPa	44.2	44.3
LOP	44.6	44.5
OPaP	44.8	44.9
LPP	45.3	45.2
OOO	46.0	45.9
OOP	46.6	46.6
OPP	47.3	47.3
PPP	48.0	48.1
OOS	48.6	48.5
OPS	49.3	49.3
OSS	51.3	51.3

*TCN constants: Ln = 0.39, L = 0.73, O = 0.68, Pa = 0.45. Ln = linolenate, L = linoleate, O = oleate, Pa = palmitoleate, P = palmitate, S = stearate (the order of designation does not indicate positional isomers).

to be useful in the identification of triglyceride species.

In order to determine further the efficiency of the gradient system for the separation of triglyceride species, it was tested with the interesterification mixtures described in *Methods.* In reference mixture HPLC #G-1 (Fig. 1A), the species varied by differences of 3 carbon atoms or 3 double bonds. Figure 4 shows the separation of triglyceride species differing by 1 double bond (Fig. 4A) or 1 methylene group (Fig. 4B), mixtures B and E, respectively. The triglycerides differing by 1 double bond were separated better than those differing by 1 methylene group, but the components of both mixtures were separated in sharp peaks with little band spreading and, essentially, baseline efficiency.

The analysis of the 3 groups of 4 component critical pair mixtures, C, D and A, with ECN of 36, 42 and 48, respectively, is shown in Figure 5. These mixtures were added together for the analysis shown in Figure 5 and demonstrate the efficiency of the gradient system for the separation of 4 component critical pair mixtures.

Mixture G, whose 4 components differ by only one *trans* double bond, can be readily detected but are not completely separated as shown in Figure 6A. Figure 6B shows that separating some triglyceride species that differ in their acyl groups only by the position of the double bond (HPLC #G-3) is possible. These mixtures provide an indication of the limit of the efficiency of the column for the analysis of natural and partially hydrogenated fats.

Analysis of Olive Oil

The application of the gradient method to olive oil with column I and a combination of columns I and II is shown in Figure 7. The chromatogram obtained with column II (not shown) gave a better separation of the more polar species than column I, although the overall separation with column I shown in Figure 7 was better. When the 2 columns were connected together, the individual characteristics of each column were retained and the separation of both more polar and less polar components was superior, as shown in Figure 7B. The triglyceride species composition of olive oil is well known, hence, identification of the com-

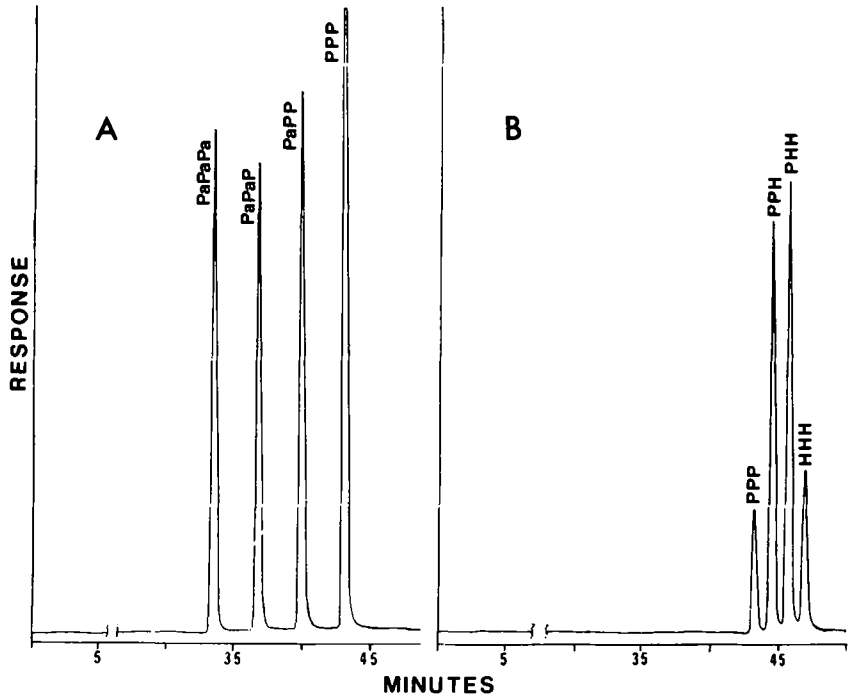

FIG. 4. Separation of interesterified test mixtures B and E under the same conditions as in Figure 1A. A = test mixture B, differences by one double bond; B = test mixture E, differences by one methylene group. (Pa = palmitoleate, P = palmitate and H = heptadecanoate; the order of the designations does not indicate the separation of positional isomers.)

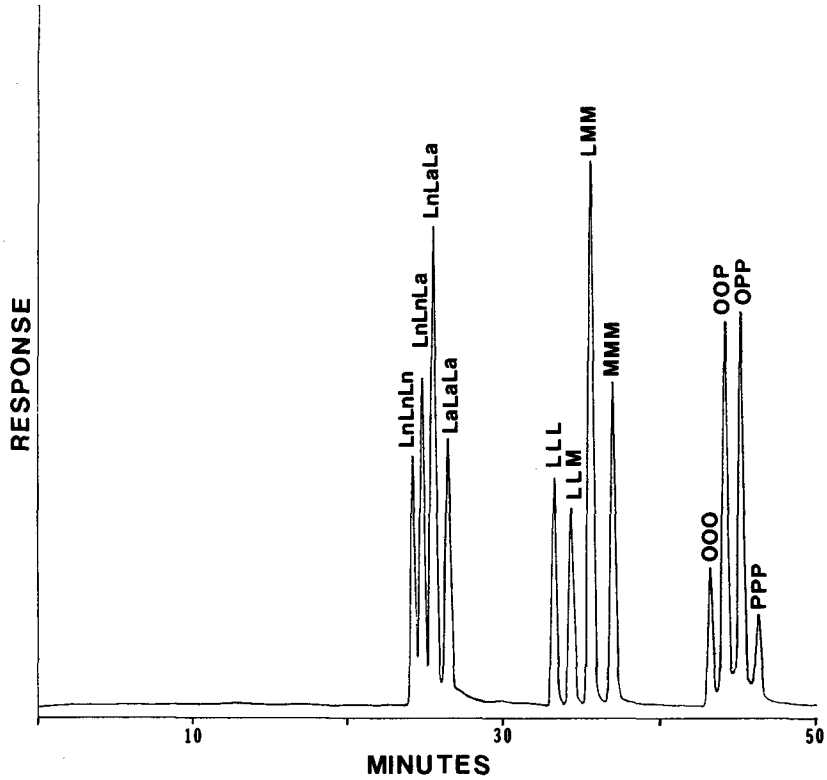

FIG. 5. Separation of a mixture of 3 groups of 4 component critical pairs with ECN's of 36, 42 and 48, triglyceride reference mixtures C, D and A, respectively. Conditions are the same as in Figure 1A. Ln = linolenate, La = laurate, L = linoleate, M = myristate, O = oleate and P = palmitate (the order of the designations does not indicate the separation of positional isomers).

ponents in Figure 7 was made by using references from the literature (4,5,7) that relate to the retention time of pure triglycerides, by comparing peak retention times to the components of mixture F, and by comparing calculated and experimental TCN values.

Figure 8 shows the analysis of olive oil with the third column using an isocratic solvent system at 2 different flow rates (1.2 and 0.6 ml/min). The best resolution was obtained with the slower flow rate, but the speed of the elution was increased proportionately by doubling the flow rate with this column. In either case, the minor components were not separated as well as with the longer columns. A gradient system was not worked out for this column, but might have increased both speed and resolution.

DISCUSSION

A major feature of the FID is that it permits the use of gradient elution, increasing the versatility of the chromatography. By using a gradient solvent system, triglyceride species can be separated on the basis of differences of one methylene group or one double bond. Two and 4 component critical pair mixtures were separated over the molecular weight range of the triglycerides of most common vegetable oils. Two columns connected together provide greater resolution than a single column but require approximately twice as long for an analysis, as shown in Figure 7. However, not only does the longer column provide greater resolution, it permits the use of larger loads, increasing the sensitivity for the detection and analysis of minor components, as shown in Figure 7. The long lead time and relatively short emergence time of the 2-column system demonstrated on olive oil should also facilitate the analyses of triglycerides containing highly polyunsaturated fatty acids that occur in some animal fats and fish oils.

The analysis of olive oil by both the single and double column systems gave results similar

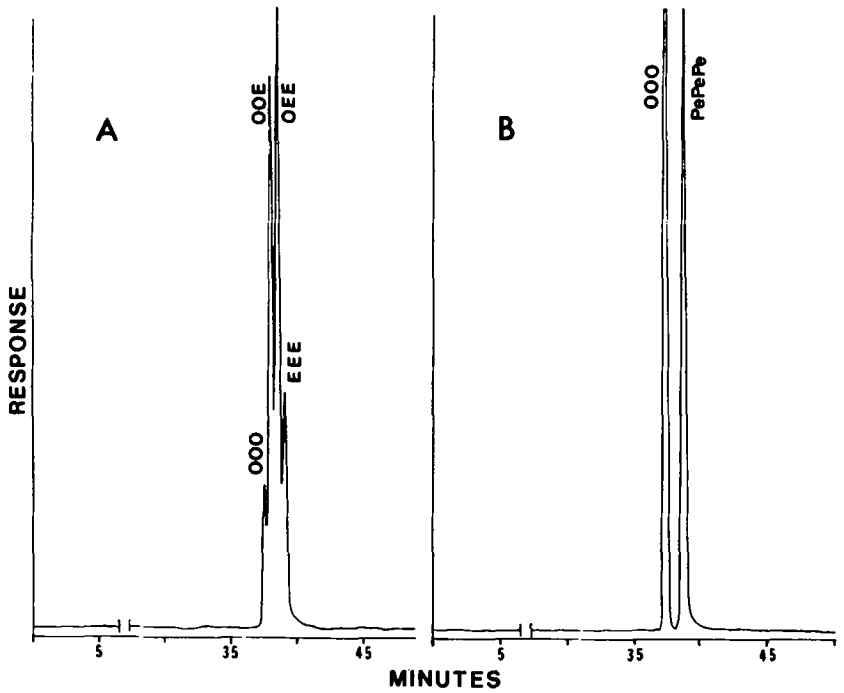

FIG. 6. Separation of interesterified test mixture G and triglyceride reference mixture HPLC #G-3, A and B, respectively. The conditions are the same as in Figure 1A. O = oleate, E = elaidate and Pe = petroselinate (the order of the designations in A does not indicate the separation of positional isomers).

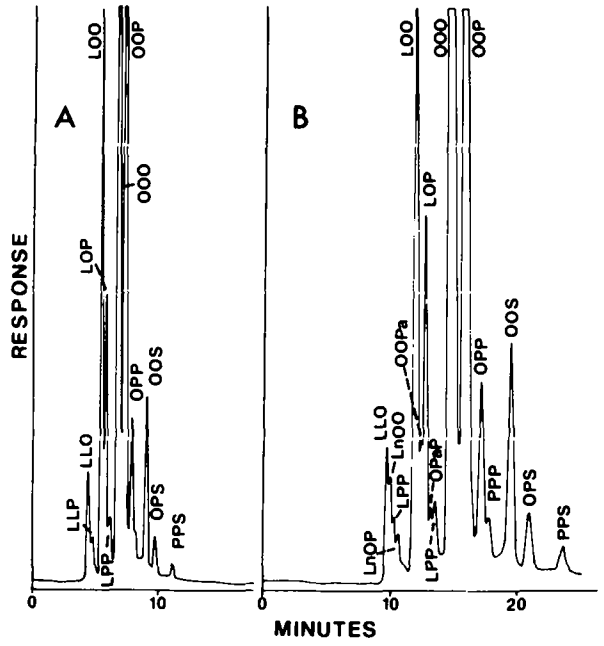

FIG. 7. Triglyceride species analyses of olive oil. A, the conditions are as in Figure 1A except that a gradient from 30 to 60% was used; B, Zorbax columns I and II were connected and a 120 min gradient used. Species identification as indicated where P = palmitate, S = stearate, O = oleate, L = linoleate, Ln = linolenate, Pa = palmitoleate and U = unidentified. The order of the designations does not indicate the separation of isomers.

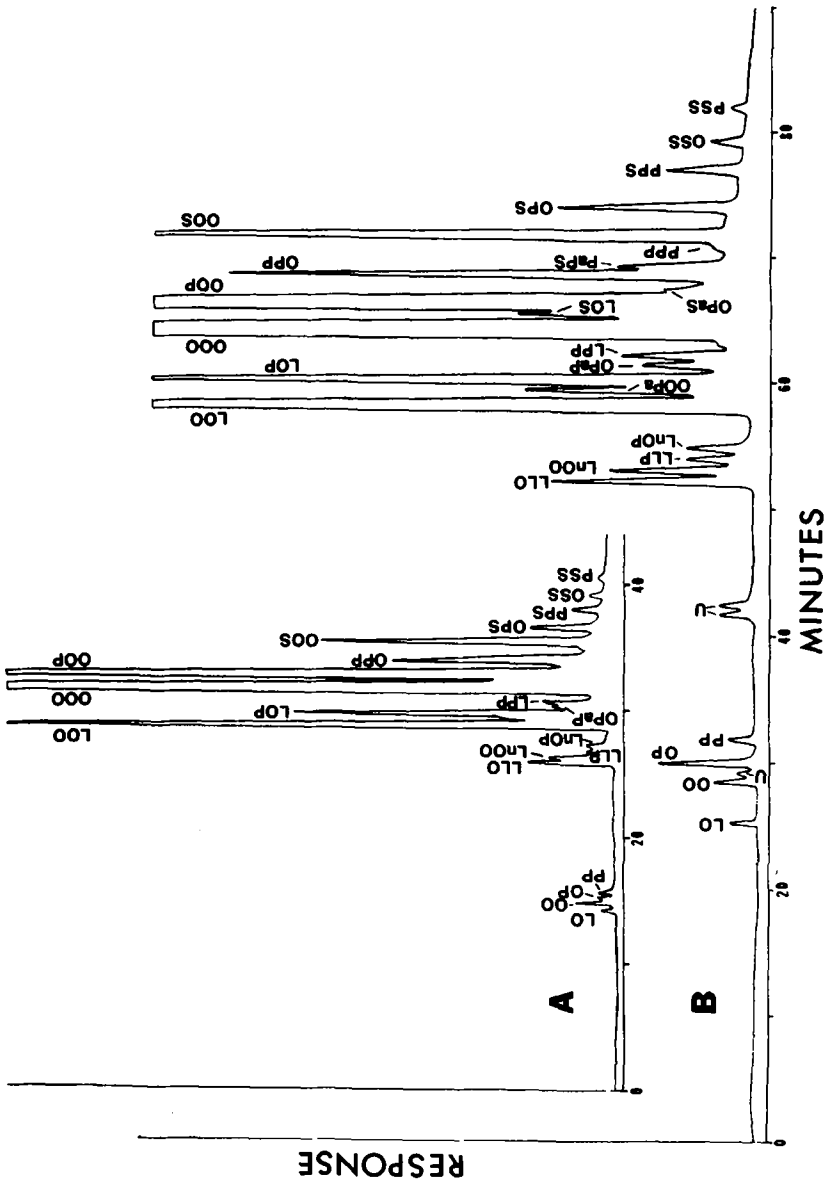

FIG. 8. The triglyceride species analysis of olive oil with Zorbax column III. Isocratic elution with 45% methylene chloride in acetonitrile; sample size 100 μg and FID with a 1:1 effluent split. A = flow rate of 1.2 ml/min and B = 0.6 ml/min.

to those reported by Dong and DiCesare (7) and El-Hamdy and Perkins (5) except in the analysis of the minor components, particularly linolenate species, which were readily detected. These components were well separated into sharp, distinct peaks by the 2-column systems, as shown in Figure 7. The resolution and identification of the linolenate species of olive oil is very important as the level of this fatty acid is used as a parameter for the detection of adulteration of this oil. Obtaining similar separations of these and other minor components with isocratic systems would be very difficult because fast elution times are virtually mandatory in these systems to avoid excessive peak broadening with a consequent loss in sensitivity, as well as resolution. Progressive peak broadening, which occurs with isocratic systems, has been demonstrated by Snyder et al. (15), on a theoretical basis as well as by Dong and DiCesare (7) with olive oil.

The most important factor in the analysis of natural oils is still the need for greater resolution. The FID provides advances for more efficient systems which are required for the complete physical separation of triglyceride species. Such an accomplishment does not seem to be outside of the realm of possibility in view of the versatility that can be obtained with gradient solvent systems and the continued improvement in ODS columns. Moreover, the triglyceride mixtures described here provide a good test for advances in this area. Although the identification of peaks by their TCN values is satisfactory for most oils, it is somewhat laborious without a computer and not always certain, especially in the case of minor components and those species not well separated. To obtain unequivocal identification by GLC of methyl esters of isolated fractions is laborious and not always possible because of the difficulty of collecting closely separated peaks. A more positive technique of identification is the combination of HPLC with chemical ionization/mass spectrometry as recently reported by Kuksis et al. (16) and as being developed in our laboratory (17). However, this technique is not quantitative, does not distinguish positional and geometric acyl groups and is generally more complex and expensive. Hence, the FID coupled with a high resolution column and a good library of reference compounds is the method of choice.

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