Column Types for the Chromatographic Analysis of Oleochemicals

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ABSTRACT

Chromatography has developed into one of the principle methods of analysis of oleochemicals. Gas chromatography has been used extensively for the analysis of long-chain fatty acids as well as for the analysis of triglycerides and plant sterols. In recent years, high pressure liquid chromatography (HPLC) has been used for the analysis of triglycerides as well as for other related materials. Specialized gas chromatography columns have been developed for the separation of long-chain fatty acids such as the methyl esters. These columns have generally used high polarity stationary phases which separate fatty acids by degree of unsaturation. A specialized use of these high polarity stationary phases is separation of *cis-trans* isomers as well as *cis-cis* and *trans-trans* isomers. In this paper, packed and capillary columns are compared for the separation of the *cis-trans* isomers of fatty acid methyl esters prepared from a hydrogenated vegetable oil. For HPLC separations, the presence of a double bond is approximately equivalent chromatographically to shortening the alkyl chain by two carbons. The long-chain polyenic acids or ethyl esters thus elute near but are resolved from the short-chain saturated fatty acids or esters. HPLC is the method of choice for relatively complex, high molecular weight, or labile esters, such as those of retinyl or cholesterol. Glyceryl esters are particularly well resolved by HPLC in terms of both total chain length and degree of unsaturation. This technique is also useful for lipid class separations and for the analysis of modified fatty acid products, such as prostaglandins and related materials. In general, these analyses are conducted with octadecyl bonded phase column packings.

INTRODUCTION

Chromatography has developed into one of the principle methods of analysis of oleochemicals. Gas chromatography (GC) has been used extensively for the analysis of long-chain fatty acids as well as for triglycerides and plant sterols. In recent years, high pressure liquid chromatography (HPLC) has been used for the analysis of triglycerides and other related materials. Major advances in both equipment design and column technology make it possible for the analyst to do not only more difficult analysis but also more rapid analysis.

COLUMNS FOR GAS CHROMATOGRAPHY

Specialized gas chromatography columns have been developed for separation of long-chain fatty acids as the methyl esters. These columns generally use high polarity stationary phases which separate fatty acids by the degree of unsaturation. A specialized use of these highly polar stationary phases is the separation of *cis-trans* isomers as well as *cis-cis* and *trans-trans* isomers. The work is traced through the development of both packed and capillary columns.

The two major factors contributing to separation of the various fatty acids are selectivity of the stationary phase (i.e., that which governs the order of elution of the compounds from the column) and efficiency of the column or the narrowness of the GC peak.

In packed column work, the stationary phase was the major variable in column development. Largely because of practical considerations, it was difficult to operate a column which produced more than 6,000 theoretical plates. Although a larger number of plates could be produced, back pressure considerations discouraged the use of columns with a large number of plates. It became apparent that to separate the various fatty acid mixtures, a variety of different column types would be necessary. A given column might be excellent for one type of sample but might be inadequate for another.

It was found that the polyester stationary phase was the most versatile type of phase for fatty acid separation. Since the polyester stationary phase separates by degree of unsaturation, the order of elution for the various C_{18} fatty acids is stearate ($C_{18:0}$), oleate ($C_{18:1}$), linoleate ($C_{18:2}$), and linolenate ($C_{18:3}$). With increased polarity of the phase, the relative spacing between the elution of the components changed. For a moderately polar phase, such as butanediol succinate (BDS) or Carbowax 20M (polyethylene glycol), there is little or no chain overlap between the even carbon number fatty acids. Thus $C_{16:4\omega1}$ is eluted before $C_{18:0}$, and $C_{18:4}$ is eluted before C_{20}. As the polarity of the phase increases, the chain overlap increases; or, looking at it another way, as the polarity of phase increases, the elution of polyunsaturated compounds is considerably retarded. For example, with diethylene glycol succinate (DEGS), the $C_{18:3}$ is eluted considerably after $C_{20:0}$.

The separating characteristics of the column could also be modified by the percentage of coating or the loading on the support. For a given polar phase, the effective polarity would diminish as the amount of coating was reduced. In addition, the relative separation also depends on the column temperature. So, in the development of a given separation, the type of stationary phase, the percentage coating used and the column temperature could be used to obtain a given separation.

In capillary column work, one has to deal with both major variables: selectivity of the stationary phase, and column efficiency. On first consideration, the stationary phase becomes less critical because with a given stationary phase, even one producing very small separation factors for various pairs of components, the separation is easily achieved simply by having a large number of plates generated by the column. One can readily increase the capillary column length—15 to 30 to 60 to 100 m—and generate 40,000, 80,000, 160,000 and 250,000 effective plates.

The greater separating power of the capillary column then gives the user several advantages:

- —A single general purpose capillary column can be used to separate a variety of sample types, each of which would have required a different specialized packed column.
- —A capillary column of sufficient length can separate exceedingly complex mixtures which simply would not be separated on the most specialized packed column.
- —A capillary column can frequently show totally unexpected separation of what had been thought to be a single component.

cis-trans Isomer Separation

Partial hydrogenation of unsaturated oils produces an array of geometrical and positional isomers. Analysis of these geometrical isomers—particularly the monoene *cis-trans* pair, oleate/elaidate—has been a challenge to the analytical chemist. Although packed GC columns were readily adapted to the analysis of common vegetable oils, the *cis-trans* separation was not easily achieved.

The common vegetable oil fatty acids are readily sepa-

rated if the triglyceride fatty acids are first converted to the methyl esters. These can then be separated using a polyester stationary phase which separates the esters by carbon number as well as by the degree of unsaturation. The oleate/elaidate pair is eluted as a single peak using a polyester phase packed column.

During the 1960s, a number of methods were devised for separation of elaidate/oleate. These methods involved special derivatization techniques. Stein (1) converted the oleate and elaidate to the corresponding vinyl bromide compounds by bromonation and subsequent dehydrobromonation. The vinyl bromide esters can be separated on a polyester stationary phase. Christie and Holman (2) prepared cyclopropane derivatives which could be separated on both polar and nonpolar columns. These and other schemes, although novel, were generally laborious and not widely used. Conacher (3) has extensively reviewed this topic.

Little progress was made in packed column separation of the methyl esters till the 1970s with the introduction of various cyanosilicone stationary phases. It was shown that in 1973 the Silar 10 C and in 1974 the SP-2340 cyanosilicone phases were able to separate both geometrical and positional isomers to a far greater extent than the conventional polyester stationary phases.

In 1976, Ottenstein et al. (4) studied in detail the factors which contributed to the oleate/elaidate separation for packed GC columns. Their study showed the following:

- —The oleate/elaidate separation factor increases with increasing nitrile content of the stationary phase.
- —The oleate/elaidate separation factor increases with increasing concentration of stationary phase with most satisfactory separation occurring at 15% loading of phase. At higher concentration of phase (higher than 15%), the increase in the separation factor was offset by the decrease in column efficiency.
- —The type of support contributes to the separation with Chromosorb P giving better separation than Chromosorb W. The greater capacity of Chromosorb P allows for a higher loading of stationary phase which contributes to the improved separation.
- —The nature of the support surface contributes to the separation. An acid-washed silanized support (AW-DMCS) gives better separation than does an acid-washed (AW) support. Although the AW support gave better column efficiency, the AW-DMCS support showed a better separation factor for the oleate/elaidate pair when the support is coated with the same amount of stationary phase.

Combining these four factors, a packing consisting of 15% OV-275 on 100/120 Chromosorb P AW-DMCS gave the best overall separation of oleate/elaidate. A 20 ft × $^1/_8$ in. stainless-steel column is able to separate oleate/elaidate in under 30 min. This is shown in Figure 1.

The 15% OV-275 column was demonstrated for the separation of the *cis* and *trans* fatty acids of a number of commercial margarines (5). Good agreement was found between the total $C_{18:1}$ content obtained with a DEGS column and the oleate/elaidate content found with the OV-275 column. The samples were fractionated by argentation-TLC and the various fractions tested on the OV-275 column. These tests confirmed that the "oleate" peak contained only $C_{18:1}$ *cis* positional isomers and the "elaidate" peak contained only $C_{18:1}$ *trans* positional isomers. Reasonable agreement (±1-2%) was also found between the GC analysis for the elaidate content and the IR analysis for *trans* unsaturation.

Perkins et al. (6) also studied the comparison of the GC determination of methyl oleate and the IR method for

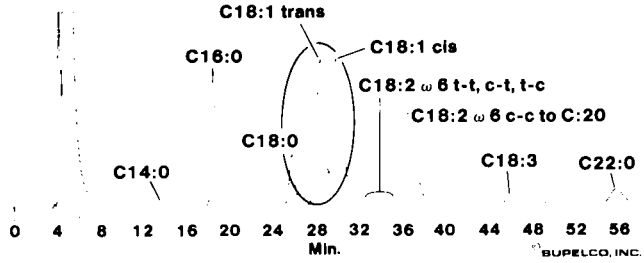

FIG. 1. Hydrogenated vegetable oil. Column description: 20 ft × $^1/_8$ in. stainless steel column with 15% OV-275 on 100/120 Chromosorb P AW DMCS; column temperature: 220 C; carrier gas flow: 10 mL/min; sample size: 1 μL (0.5 mg/mL) hydrogenated vegetable oil; detector: FID.

trans determination. Again, reasonable agreement between the two methods was found, with the GC method giving slightly lower results. Since 1979, the American Oil Chemists' Society has been conducting collaborative studies comparing the "elaidate" content obtained by the GC method using the OV-275 column to the *trans* content obtained by the IR method. Preliminary results confirm Perkins' data and indicate good agreement between the GC and IR methods. A final report is expected in the near future. Smith et al. (7) studied the GC/IR relationship of *trans* fatty acids in butter and margarine. Heckers and Melcher (8) have used GC to determine the *trans* content of various fats.

Capillary Separation

Development of capillary columns for *cis-trans* separations occurred concurrently with that of the OV-275 packed column. In the USA, the use of capillary columns was very much limited by limitations of equipment and a limited familiarity of capillary techniques by most workers. Columns available in the USA were made of stainless steel and were not highly efficient.

Separation of *cis-trans* isomers of fatty acid methyl esters on a capillary column was first accomplished by Lipsky and coworkers (9) in 1959 through the use of a nonpolar stationary phase, Apiezon L. Litchfield and coworkers (10-12) expanded on this development of columns for *cis-trans* separation in the mid-1960s with a variety of stationary phases including Apiezon L, the diethylene glycol succinate polymer (DEGS) and various cyanosilicones. In 1974, Ackman and Hooker (13) studied the *cis-trans* separation with BDS, Silar 5 CP and Apiezon L.

Use of capillary chromatography for *cis-trans* isomer separation was limited during the 1960s and 1970s because of the limitations of stainless-steel columns. These columns were neither very efficient nor inert. They were generally made in the worker's laboratory and were available commercially only in very limited quantities. It was not until the late 1970s when glass capillary columns became commercially available that cyanosilicone columns for *cis-trans* separation came into more common use. The availability and use of capillary columns further expanded with the advent of fused silica capillary columns which allowed easier installation of the columns by the user.

The ability of a cyanosilicone column to separate the *cis-trans* isomers is illustrated in Figure 2 using a 15-m SP-2330 cyanosilicone fused silica capillary column. The relatively short length and lower polarity were chosen to obtain the minimum separation of the positional isomers of the two geometric isomers, thus crowding the positional isomers into a readily integrated size peak. Using a longer length (60 m) and a more polar phase (SP-2340), the separation of the hydrogenated oil is shown in Figure 3. Although the greater length and higher polarity are starting to

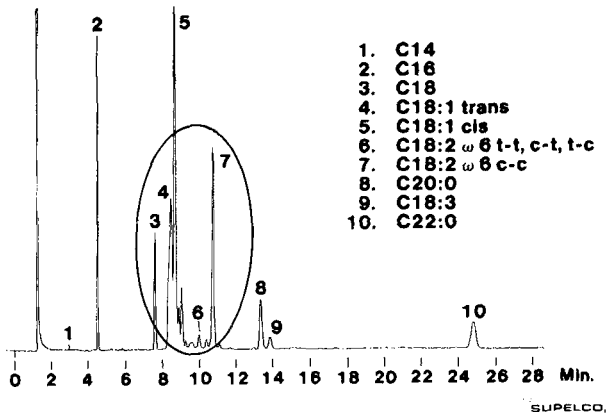

FIG. 2. Hydrogenated vegetable oil. Column description: 15 m × 0.25 mm id SP-2340 fused silica capillary column; column temperature: 155 C; carrier gas: helium, 20 cm/sec; sample size: 1 μL (100:1 split ratio) (0.5 mg/cc) hydrogenated vegetable oil; detector: FID.

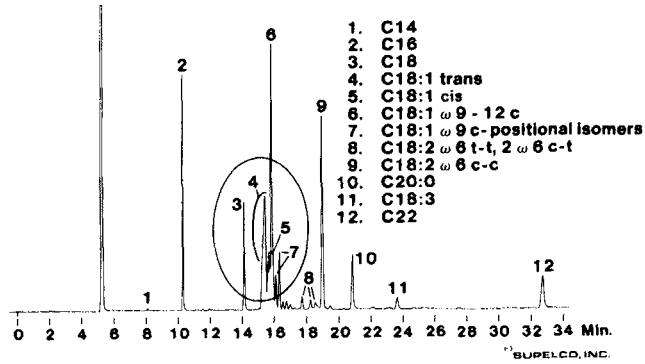

FIG. 3. Hydrogenated vegetable oil. Column description: 60 m × 0.25 mm id SP-2340 fused silica capillary column; column temperature: 175 C; carrier gas: helium, 20 cm/sec; sample size: 1 μL (100:1 split ratio) (0.5 mg/cc) hydrogenated vegetable oil; detector: FID.

separate the *trans* isomers into positional isomers, the column cannot obtain an adequate separation of the positional isomers.

Slover and Lanza (14) demonstrated separation of hydrogenated oils as methyl esters using a 100-m SP-2340 capillary column. With this column length, they were able to obtain separation of the $C_{18:1}$ *cis-trans* positional isomers and the *cis, cis*-methylene interrupted diene fatty acids. The separation obtained with a 100-m column is shown in Figure 4, with the $C_{18:1}$ *trans* isomer group encircled. The $C_{18:1}$ positional isomers separated by the column are thought to be those shown in Table I.

To obtain the separation shown in Figure 4, we found it necessary to prepare a column which differs from the standard SP-2340 capillary column in order to obtain consistent column-to-column separation of the *trans* positional isomers. The column is sold with the designation SP-2560.

TABLE I

$C_{18:1}$ *cis-trans* Positional Isomers

	trans			*cis*	
(1)	18:1	12*t*	(1)	18:1	12*c*
(2)	18:1	9*t*	(2)	18:1	9*c*
(3)	18:1	?*t*	(3)	18:1	?*c*
(4)	18:1	7*t*	(4)	18:1	7*c*
(5)	18:1	?*t*			

The column and conditions are the same as Figure 4.

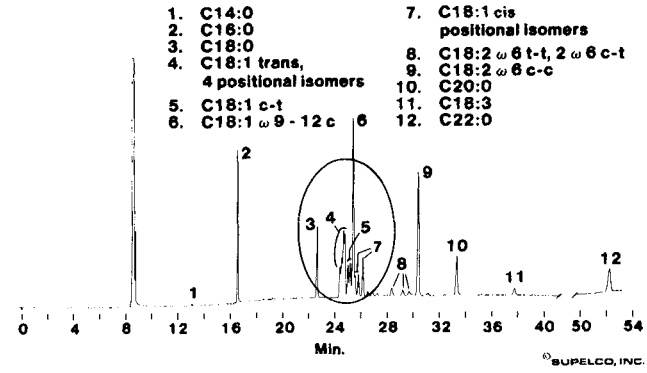

FIG. 4. Hydrogenated vegetable oil. Column description: 100 m × 0.25 mm id SP-2560 fused silica capillary column; column temperature: 175 C; carrier gas: helium, 20 cm/sec; sample size: 1 μL (100:1 split ratio) (0.5 mg/cc) hydrogenated vegetable oil; detector: FID.

COLUMNS FOR LIPID CHROMATOGRAPHY—HPLC

Lipids consist of a series of classes—triglycerides, phosphatidylcholines, sphingomyelins and sterols, to name a few. Each class is heterogeneous and usually contains members of one or more homologous series. Fatty acids and sphingosine bases are members of homologous series which generate heterogeneity within lipid classes.

Most HPLC is based on the use of so-called normal phase columns (useful for class separations), reversed-phase columns (useful for homolog separations), and polar columns (used in either the normal or reversed-phase mode). Since reversed-phase HPLC columns are generally easier to work with, most of the applications presented there use such columns. Ion exchange and size exclusion columns are also available but are not discussed here.

Detection of eluted lipids in HPLC is a definite problem. Refractive index detectors are relatively universally applicable but are relatively insensitive and preclude the use of gradient elution. Low wavelength UV detection (i.e., 200-210 nm) is more sensitive and permits the use of gradients but precludes use of certain common lipid solvents such as chloroform or acetone which are opaque in the UV region of interest. With low wavelength UV detection, the response will also be somewhat dependent on fatty acid composition. For these reasons, the mobile phases used in lipid analysis by HPLC may seem rather strange to workers familiar with thin layer chromatography (TLC) or open column separations.

Certain simple lipids, including triglycerides, sterol esters, wax esters and fat-soluble vitamins, may be separated by gas chromatography, frequently after derivatization. These same separations may also be made by HPLC, but the real interest in this technique relates to separation of complex lipids or to separations which are relatively difficult by GC. Several examples are presented to illustrate the types of HPLC columns and mobile phases used in lipid separations.

Triglycerides

Common triglycerides contain a series of even chain length saturated fatty acids and several corresponding series of unsaturated fatty acids. Such triglycerides may be separated by GC at high temperatures (350 C) by total fatty acid chain length (i.e., tripalmitin and tristearin have total fatty acid chain lengths of 48 carbons and 54 carbons, respectively). Unsaturation has relatively little effect on such separations. In older liquid chromatographic methods, including TLC, it has been found that introduction of one double bond is approximately chromatographically equiva-

lent to shortening the alkyl chain by two carbon number atoms (i.e., both tripalmitin and triolein have equivalent carbon numbers of 48). Addition of silver ions to the chromatographic media (argentation chromatography) produces separations which are dependent on the number of double bonds but relatively independent of fatty acid chain lengths.

Reversed-phase HPLC on the Supelcosil LC-18 column has shown that triglycerides can now be separated in terms of both fatty acid chain length and the degree of unsaturation (15,16). This is illustrated with soybean oil but is also applicable to other natural oils. Soybean oil triglycerides contain palmitic, stearic, oleic, linoleic and linolenic acids; the main triglyceride types are listed in Table II. There are only two species in terms of total fatty acid chain lengths—52 and 54—and five species in terms of equivalent carbon numbers—42, 44, 46, 48 and 50. Figure 5 shows that in HPLC the triglycerides with equivalent carbon numbers are grouped together chromatographically but are resolved according to the number of double bonds. Oleyldilinolein (54:5) and palmitodilinolein (52:4) both at an equivalent carbon number of 44 are detected as adjacent but separate peaks. Similarly, the pair LO_2 (54:4) and LOP (52:3) with equivalent carbon numbers of 46, appear as two peaks by HPLC. This system fails to give separations of triglycerides having the same total carbon chain length and the same number of double bonds such as triolein, O_3 (54:3) and oleoyl stearoyl linolein, OSL (54:3). The first peak in this chromatogram was not characterized but presumably represents a dilinolenoilolein (54:7) with an equivalent carbon number of 40.

For this type of separation, a totally nonaqueous mobile phase is used—acetone/acetonitrile (63.6:36.4, v/v). Since acetone is opaque in the UV region, a refractive index detection is used. Use of the refractive index detector precludes use of a gradient to spread out the separation. This mobile phase produces relatively low back pressures, and several columns may be used in series to enhance resolution.

A typical industrial use of such a separation is illustrated with a commercial cocoa butter in Figure 6. Triolein and trielaidin are readily separated in this system, but the mixture of intermediate triglyceride species and positional isomers present in margarine samples presents a far more difficult problem.

TABLE II

Soybean Oil Triglycerides

Composition		Carbon number	Equivalent carbon number
L_3	(54:6)	54	42
L_2O	(54:5)	54	44
L_2	(52:4)	52	44
LO_2	(54:4)	54	46
LOP	(52:3)	52	46
LSO	(54:3)	54	48
LSP	(52:2)	52	50

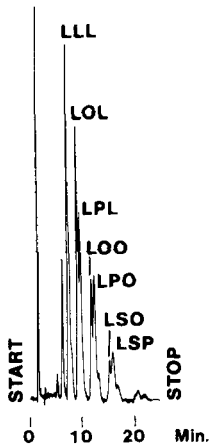

FIG. 5. HPLC of soybean oil triglycerides (16). Column: Supelcosil LC-18, two 15 cm × 4.6 mm in series, 5 micron packing; mobile phase: acetone/acetonitrile (63.6:36); flow rate: 1 mL/min; temperature: ambient; detection: refractive index; sample volume: 10 μL; sample concentration: 100 mg/mL. The peak before L_3 is probably L_2Le (54:7).

Fatty Acid Methyl Esters (FAME)

Since it is possible to separate triglycerides by fatty acid chain length and degree of unsaturation, such a system is also applicable to fatty acid methyl esters, stearyl esters and such other miscellaneous items as retinyl esters. The Supelcosil LC-18 column is used for this application with an acetone/acetonitrile/tetrahydrofuran (50:42:76, v/v/v) mobile phase. A refractive index detector is used for this application. A model mixture of 18:3, 18:2, 14:0, 18:1, 16:0 and 18:0 (4-7021) is readily separated in ca. 15 min (Fig. 7).

This HPLC system works well with relatively simple fatty acid mixtures and offers certain advantages in terms of the clear-cut resolution of the set of compounds—18:3, 20:0 and 20:1. However, with complex mixtures of animal

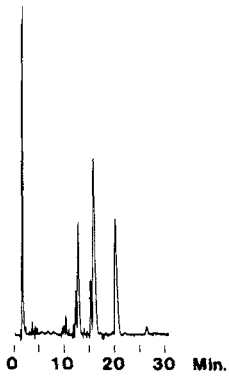

FIG. 6. HPLC of cocoa butter triglycerides. Column: Supelcosil LC-18 25 cm × 4.6 mm, 3 micron packing; mobile phase: acetone/acetonitrile (64:36); temperature: ambient; flow rate: 2 mL/min; pressure: 2690 psig; chart speed: 0.2 cm/min; sample volume: 5 μL; sample concentration: 200 mg/mL. The peak sequence was O_3; P_2O; SO_2; PSO; S_2O.

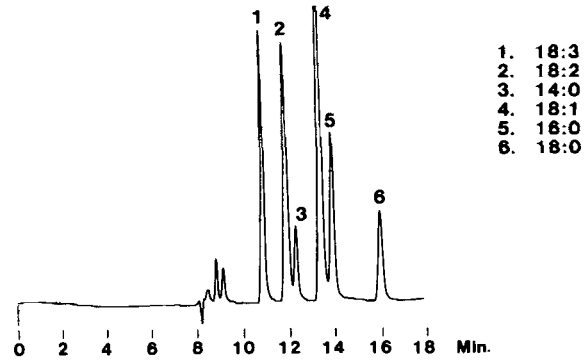

FIG. 7. HPLC of fatty acid methyl esters. Column: Supelcosil LC-18, three 25 cm × 4.6 mm columns in series, 5 micron packing; mobile phase: acetone/acetonitrile/tetrahydrofuran (50:42:7.6); flow rate: 1 mL/min; pressure: 1500 psig; temperature: ambient; chart speed: 0.5 cm/min; detection: refractive index; range: 2X; sample size: 10 μL; sample concentration: 50 mg/mL.

tissue origin, the separation of sets such as 22:5, 20:4, 18:3, and 18:2, 16:1, 14:0 is somewhat more difficult by HPLC than by GC. Oleate and elaidate are readily resolved by HPLC, but the complex mixture of positional and geometric isomers present in samples prepared from commercial margarines presents a difficult problem. Horrocks (17) recently described the separation of fatty acid methyl esters on an ODS column using acetonitrile as the mobile phase. This system permits use of low wavelength UV detection.

Free Fatty Acids

Free fatty acids are separable by GC by inclusion of phosphoric acid in the packing. For HPLC, the phosphoric acid or other equivalent strong acid is included in the mobile phase. We have used a variation of the system of Bailie et al. (18) for this purpose. A somewhat similar system has also been described by Horrocks (17). On a Supelcosil LC-18 column, a model mixture of free fatty acids is separated with a mobile phase containing tetrahydrofuran, acetonitrile, water and phosphoric acid, 6:64:30:0.1 at pH 2 (Fig. 8). As noted for triglycerides and fatty acid methyl esters, oleic and elaidic acids, palmitoleic and palmiteloidic acids, and linoleic and linoelaidic are well separated; but margarine fatty acids present a difficult problem. UV detection at 220 nm was used to prepare this chromatogram. Oxidation of polyunsaturated fatty acids leads to oxygenated species with a pair of conjugated double bonds. The product from linoleic acid has an extraction value of ca. 28,000 at 232 nm. Trace quantities of such oxidation products in 98-99% pure polyunsaturated fatty acid standards will produce relatively huge peaks when UV detection at this wavelength is used. HPLC provides an excellent separation of such oxidation products and may thus be very useful in this area. When there is no interest in oxidation products, either refractive index detection or a wavelength nearer 200 nm would be preferred for fatty acid detection.

Fat-Soluble Vitamins

There are numerous publications illustrating separations of various forms of vitamins A, D and E. A typical separation of α, β, γ and δ tocopherols is shown (Fig. 9) on a Supelcosil LC-Si column eluted with hexane/methyl-*t*-butyl ether (90:10). This procedure is quite sensitive and produces excellent separation of the four tocopherols. We have not looked at the tocotrienols in this system.

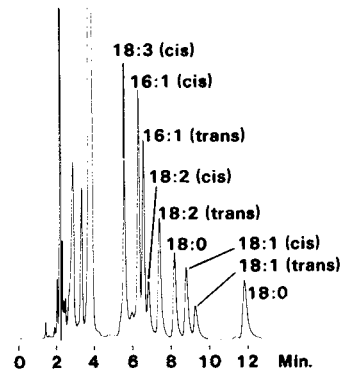

FIG. 8. HPLC of free fatty acids. Column: Supelcosil LC-18, 25 cm × 4.6 mm, 5 micron packing; mobile phase: tetrahydrofuran/acetonitrile/0.1% phosphoric acid, pH 2.2 (21.6:50.4:28.0); flow rate: 1.5 mL/min; pressure: 1070 psig; temperature: 35 C; chart speed: 1 cm/min; detection: UV at 220 nm, 0.02 AUFS; sample volume: 10 μL; sample concentration: 1-2 mg/mL per component.

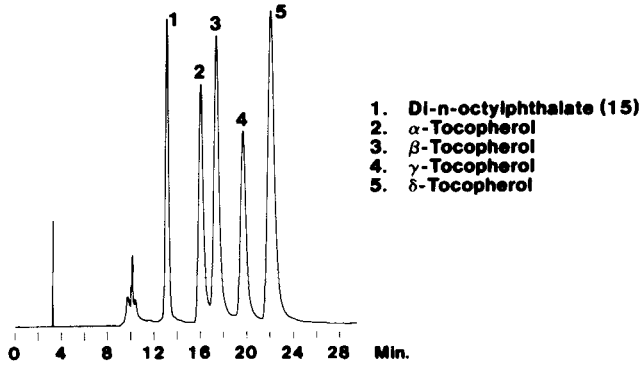

FIG. 9. HPLC of tocopherols. Column: Supelcosil LC-Si, 25 cm × 4.6 mm, 5 micron packing; mobile phase: hexane/methyl-*t*-butyl ether (90:10); flow rate: 1 mL/min; pressure: 500 psig; temperature: ambient; chart speed: 0.1 cm/min; detection: UV at 295 nm, 0.05 AUFS; sample size: 10 μL; sample concentration: 1 mg/mL.

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